Identification of microRNAs in the follicular and luteal tissues of Holstein-Friesian cows

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Abstract

MicroRNAs (miRNAs) regulate many physiological pathways, including development, cell differentiation, immune response as well as diseases by post-transcriptional processes. However, information is limited regarding the biological roles of miRNAs in the development of the follicular and luteal tissues in cows. The main aim of the present study was to identify miRNAs that are expressed in the follicular and luteal tissues of cows. We used a comparative RNA sequencing method to identify miRNA candidates that might play key roles in the follicular and luteal phases of Holstein cows. Nine miRNAs were expressed at a high ratio: 8 in the follicular tissue and one in the luteal tissue. Bioinformatics analysis predicted 2,479 target genes. RNA sequencing identified several miRNA candidates, including bta-miR-196a, bta-miR-490, bta-miR-1247-5p, bta-miR-34c, and bta-miR-222. These were associated with pathways like TGF-beta signaling, gonadotropin-releasing hormone receptor, prolactin signaling, and progesterone-mediated oocyte maturation associated with the reproductive

system. These findings will be beneficial in future comprehensive studies on the miRNAs involved in regulating the development of follicular and luteal tissues in cows.

Keywords: miRNA, Preovulatory follicle, Corpora lutea, Cows, Reproductive system.

1. Introduction

Several physiological events are occurring in the ovary. The events that take place as the follicles grow, develop, and transform into the corpora lutea are controlled at the molecular level. These events continuously repeat themselves in female reproductive system [1, 2]. The details of the transcriptional processes in follicular and luteal phases of an ovary are well understood; however, information regarding post-transcriptional regulation is limited.

Folliculogenesis process take place into the cortex of the ovary. During this process, four regulatory events occur, including recruitment, preantral follicle development, selection, and atresia [3]. First follicle is primordial follicles. After this follicle, primary follicle, secondary follicle, and tertiary follicle are formed, respectively. At later, mature graafian follicle or preovulatory follicle is formed which contains follicular fluid or liquor folliculi. Lastly, after the ovulation is occured, corpora lutea is formed [3]. The progesterone level increases in the luteal phase. At this stage, progesterone is secreted by the corpora lutea [4]. Previous studies have indicated that some proteins and intracellular transmitters, such as bone morphogenetic protein (BMP), activin, mothers against decapentaplegic homolog (SMADs), and Wnt regulate many of the cyclical activities in the follicular and luteal phases [5, 6].

The growth of follicle in the cow ovary is regulated by FSH. The first stage of ovarian growth is the follicular stage, during which the largest follicle continues to grow and transforms into the corpora lutea after ovulation, whereas the other small follicles go through atresia [3]. These ovary development stages in cows are controlled by thousands of genes, and transcriptome analysis has identified many of the genes related to follicular selection,

maturation, and the passage from the follicular to the luteal phase. However, the molecular mechanisms that regulate the expression of these defined genes are still not completely defined [7, 8].

One possible mode of regulation may involve microRNAs (miRNAs), which are small noncoding RNAs, 22 to 25 nucleotides in length, that regulate gene expression by degrading target mRNA or blocking 3 UTR region of target mRNA [9, 10, 11]. MiRNAs play regulatory roles in cell growth and differentiation [12]. Various studies have provided that miRNAs are also involved in steroidogenesis, proliferation, differentiation, recruitment, selection, dominance, atresia, and cumulus enlargement [13, 14, 15, 16]. Previous studies have identified numerous miRNAs in ovarian tissues, follicular and luteal cells, but few of these studies were conducted on cows [15, 16]. Confirmation that miRNAs have a role in follicle development and the luteal phase in cows will open up new perspective for more detailed research on the molecular mechanisms of follicular development [17, 18, 19, 20].

The main aim of the present study was to identify miRNAs that are expressed in the follicular and luteal tissues of cows. A secondary aim was to determine whether miRNAs have a role in the molecular mechanisms guiding the follicular and luteal phases by identifying the gene targets of these miRNAs.

2. Materials and Methods

2.1.Sample collection

The experimental Holstein cattle were obtained from the Oral meat production company (Erzurum, Turkey). The selection criteria of cows are: around 5 years old, multiparous, and body condition score of 3.5 to ≤ 4.0. In total, 20 Holstein cows (n = 20) were selected for collection of the ovary tissues.

After slaughter, all ovarian tissues from each cow were collected. Macroscopic examination of ovarian tissues was performed for follicular phase and luteal phase. After rinsing with 70% ethanol, ovarian pairs containing preovulatory follicle that is the largest of the follicles (POF) (n = 12) and corpora lutea (CL) (n = 8) were selected for dissection. ELISA test was performed on the liquid samples. Preovulatory follicles with the highest estradiol level were selected (n = 6). Corpora luteas with the highest progesterone level were selected (n = 6). Tissue samples taken from selected follicle and corpora lutea was examined (Table 1). These samples were subjected to RNAseq procedures. After that samples were stored at – 80 °C.

2.2.ELISA Test

To determine follicular health, we measured the concentrations of estradiol (E2) and progesterone [21] using 17β - Estradiol high sensitivity ELISA kit (Enzo Life Sciences, UK) and using Progesterone Competitive ELISA Kit (Thermo Fisher Scientific, USA) following the manufacturer's instructions (range, 15.6 - 1,000 pg/ml E2 and 50 pg/mL – 3,200 pg/mL progesterone) in the preovulatory follicle and corpora lutea.

2.3.Total RNA Isolation and library construction

Total RNA was isolated from ovary tissues with Qiazol® Plus RNA Purification Kit (Qiagen, Germany) according to the manufacturer's instructions. Total RNA concentration was measured with NanoDrop (Epoch Microplate Spectrophotometer). RNA samples were stored at - 80 °C until analysis. Sequencing libraries were created by using the NEBNext® Multiplex Small RNA Library Prep Set for Illumina® Kit (NEB, USA) in line with the manufacturer's instructions. 2 µg RNA was used for library construction using PEG-8000 (Sigma, USA) and T4 RNA ligase (NEB, USA). RNA sequence was performed with the Illumina Genome Analyzer (GeneXPro, Frankfurt, Germany).

2.4.Data analysis

FASTX-toolkit was used to obtain clean reads data in FASTQ files from the libraries. The quality of the sequencing data was evaluated with FastQC software. The RNA sequence length distribution analyses was performed with the FASTQ Information. 18 and 45 nt in length of small RNAs were determined from Rfam database (Rfam 10.0) using Rfamscan). The conserved miRNAs were identified from the miRBase (miRBase19.0) using miRDeep2 [22, 23, 24, 25].

2.5.Identification and target prediction

The expression analysis was carried out using the conserved miRNAs which were obtained from the Fol and Lut libraries [26, 27]. The R package "edgeR" program was used to evaluate the differentially expressed miRNAs. The heatmap analysis of miRNAs was performed with with Clustvis [28]. Lastly, the target genes of miRNAs were identified with miRanda and RNAhybrid software [29].

2.6. miRNA-Protein interactions

miRNA-protein interaction analysis was conducted for selected miRNAs (bta-miR-196a, bta-miR-490, bta-miR-222, bta-miR-34c) and target proteins (PRL, PRLR, IGF1, PAPPA, ESR1, IRS1, IGFBP1, SMAD4 to identify interactions using the STRING database.

2.7.qPCR

A total of 5 differently-expressed miRNAs were selected, and specific primers were designed for them (Table 2). Then, the cDNA was generated from the RNA samples that was isolated from ovarium samples by using QuantiTect Rev. Transcription Kit (Qiagen, Germany). The QuantiTect SYBR Green PCR Kit (Qiagen, Germany) was used in RT-PCR by using the Rotor-Gene Q 5plex HRM System (Qiagen, Germany). The CT/CQ values were identified, and the

expression levels was determined with the $2^{-[CTmiRNA-CT5SRNA]}$ method. 5S snRNA was used as internal control.

2.8.Statistical analysis

IBM SPSS 20 was used to perform the statistical analyses. A one-way analysis of variance was used to detect statistically differences in selected miRNAs expressions in the Fol and Lut groups. TPM was used to identify the miRNA expressions in the following formula “equation”, librarysize represents the total counts of all miRNAs identified and miR_readscounts stands for the read number for a specific miRNA [24].

$$TPM = \frac{miR\_readscounts \ * 1000000}{librarysize}$$

3. Results

3.1. ELISA

As expected, the levels of estradiol was higher in preovulatory follicles compare to corpora lutea (Figure 1). On the other hand, the level of progesterone also was relatively high in the corpora lutea compare to preovulatory follicles (Figure 1).

3.2. Establishing the Small RNA Library

We examined the differential expression of miRNAs in the follicular and luteal tissues by establishing Fol and Lut small RNA libraries, respectively. Illumina sequencing technology generated 5, 430, 473 raw reads for the follicular tissue and 6, 009, 702 raw reads for the luteal tissue. We eliminated the low quality reads, PolyA series, series that were smaller than 18 nucleotides, large series, and repeating series to leave a clean read of 4,738, 630 (7.26 %) for the follicular tissue and 5, 203,800 (86.59 %) for the luteal tissue; these data were used for further analysis (Table 3).

143 The length distributions for the small RNAs were given in Figure 2. The horizontal
144 coordinates show the lengths of the RNAs and the vertical coordinates showed the distribution
145 percentages. Most of the small RNAs showed a length distribution that ranged from 20 to 24
146 nucleotides. The distribution percentages of a typical 22-nucleotide miRNA with a Dicer origin
147 was 46.3 in the follicular tissue and 48.5 in the luteal tissue. Filtering the clean read data in the
148 Rfam database for noncoding RNAs, such as tRNA, rRNA, snoRNA, and snRNA, revealed that
149 35.60 % of the miRNA was located in the follicular tissues and 33.40 % in the luteal tissues
150 (Figure 3A, 3B). This result confirmed that the sequencing process used in this study had been
151 successful since it is parallel to the qRT-PCR result.

152

153 **3.3. MiRNAs expressed in the Follicular and Luteal tissues**

154 The reads were also filtered and aligned with the cow genome, and the conserved miRNAs
155 and their expression levels were specified by blasting the mapped miRNA series as per
156 miRBase. In total, 457 miRNAs were expressed in both the follicular and luteal tissues. In
157 addition, 23 miRNAs were expressed specifically in the follicular tissue, and 14 miRNAs were
158 expressed specifically in the luteal tissue (Figure 3C).

159 MiRNAs expressed in the follicular and luteal tissues were quantified using TPM, which
160 indicated a total of 494 miRNAs. If TPM $|\log2FC| \geq 1$ and the FDR p-value < 0.05, the
161 expression of the specified miRNAs was considered statistically significant. Differential
162 expression was presented as heatmaps (Figure 4). In this study, we identified 9 miRNAs that
163 were expressed in the follicular and luteal tissues at a high ratio. Among these miRNAs, 8 were
164 expressed in the follicular tissue and one was expressed in luteal tissue (Table 4).

165 We determined that, among these specific miRNAs, the bta-miR-196a family regulated the
166 expression of the transforming growth factor, beta receptor III (TGFBR3), mitogen-activated

protein kinase kinase kinase 1, E3 ubiquitin protein ligase (MAP3K1) genes, whereas bta-miR-490 regulated the pregnancy-associated plasma protein A, pappalysin 1 (Table 5).

Bta-miR-34c'nin DMRT-like family A1 (DMRTA1), DMRT-like family B with proline rich C-terminal 1, F-box and WD repeat domain containing 8 (FBXW8), F-box protein 5 (FBXO5), SMAD family member 4 (SMAD4), and Wnt family member 2B (WNT2B) genes (Table 5).

Bta-miR-1247-5p regulated the Wnt family member 2B (WNT2B) and Wnt family member 4 (WNT4) genes, and bta-miR-222 regulated estrogen receptor 1 (ESR1), doublesex and mab-3 related transcription factor 3 (DMRT3), and insulin-like growth factor 1 (IGF1), which were related with the reproductive system (Table 5).

The expression values of 5 miRNA candidates were measured by qRT-PCR. The qRT-PCR results agreed with the data obtained in the small RNA sequencing ($P < 0.01$, $P < 0.001$), thereby confirming the reliability of the small RNA sequencing results (Figure 5A, 5B).

3.4. miRNA-mRNA network analysis

MiRNA-protein interactions were analyzed to determine the relationship of target genes to the prolactin signaling, ovarian steroidogenesis, oocyte maturation, and oocyte meiosis pathways. We identified the candidate miRNAs such as bta-miR-196a, bta-miR-490, bta-miR-1247-5p, bta-miR-34c, and bta-miR-222 appeared to act as potential regulators (Figure 6).

4. Discussion

One of the known regulators of ovarian events is estrogen. Estrogen regulates follicular development by stimulating the proliferation of granulosa cells in the ovary [30, 31, 32, 33]. Steroidogenesis is increased by the augmentation of FSH activity increases and lead to greater progesterone and estradiol synthesis. Progesterone plays an important role in regulating the reproductive cycle by releasing it from the corpora lutea [34]. The secretion of progesterone from the corpora lutea is affected by several hormones.

We determined miRNA candidates that show differential expression in the follicular and luteal ovaries of Holstein cows by small RNA sequencing. Our results revealed that bta-miR-196a, 196a-1, 196a-2, bta-miR-490, bta-miR-34c, bta-miR-1247, and bta-miR-1247-5p were expressed in the follicular tissue at a high level and that bta-miR-222 was expressed in luteal tissue at a high level.

Our data also indicated that bta-miR-196a is expressed in the follicular tissue with a high ratio. -miR-196a targeted genes having roles in the development of cells of the follicular tissue, such as the cow newborn ovary homeobox gene (NOBOX), transforming growth factor, beta receptor III (TGFBR3), mitogen-activated protein kinase kinase kinase 1, E3 ubiquitin protein ligase (MAP3K1), and prolactin receptor (PRLR) (Table 3) and that it has a role in cow reproduction as a biological process. A study conducted by Tripurani et al. [35] also showed that bta-miR-196a targeted the cow newborn ovary homeobox gene (NOBOX) in the early embryogenesis stage and regulated its expression. NOBOX mRNA and protein are expressed at a high level in the oocytes of cows during the folliculogenesis stage [36]. Some microRNAs were strongly downregulated in newborn ovaries that absent NOBOX [37]. Therefore, bta-miR-196a may be used as a biomarker for the early embryonic development failure in cattle. TGFBR3 has a role in the multiplication and differentiation of follicular cells in the ovary [38]. A previous study has observed that TGFBR3 is reduced in a variety of cancers such as ovary carcinomas [39]. Thus, bta-miR-196a would be beneficial for ovarian cancer prognosis in cattle.

MAP3K1 interacts with Wnt signaling pathway to perform roles in various physiological events, such as follicular development, embryogenesis, cell reproduction, and differentiation [40, 41, 42]. Present study may be revealed that bta-miR-196a induces bovine follicles cell apoptosis by inhibiting the MAP3K1 gene. PRLR has a role in physiological paths, such as reproduction, growth, and development [43]. Recent studies have focused on the potential use of the PRLR gene as a biomarker for reproductive performance [44, 45]. Our review of the gene targets of bta-miR-196a indicated that these miRNAs regulate the follicular development process in cows.

Our gene target determination, GO enrichment, and KEGG analysis data for bta-miR-490 indicated that this miRNA targeted pregnancy-associated plasma protein A and pappalysin 1(PAPP-A) (Table 3) and that it has a role in the reproductive system as a biological process. PAPPA has functions in cell proliferation and ovulation [46]. Overall, PAPP-A, a granulosa cells-derived protease in the ovary and a biomarker of follicle selection and corpora lutea formation [47]. A study conducted with goats revealed that after embryo implantation, miR-490 expression was high in ovaries and this miRNA played a role in embryonic development [48]. However, no similar information exists for a role for miR-490 in the follicular development in cow ovaries. The results obtained in the present study confirm that bta-miR-490 plays a role in the follicular development of cow ovaries. We showed that bta-miR-34 was expressed in the follicular tissue with a high ratio. Our gene target determination, GO enrichment, and KEGG analysis conducted for this miRNA also indicated that bta-miR-34c targeted the DMRT like family A1 (DMRTA1), DMRT like family B with proline rich C-terminal 1, F-box and WD repeat domain containing 8 (FBXW8), F-box protein 5 (FBXO5), SMAD family member 4 (SMAD4), and Wnt family member 2B (WNT2B) genes (Table 3) and that it had a role in reproduction as a biological process. DMRTA1 has a role in postnatal differentiation of germ cells [49], FBXW8 has a role in cell reproduction and differentiation

[50], SMAD4 has a role in suppressing progesterone synthesis in the ovaries [51], and WNT2B has a role in follicular development, embryogenesis, cell reproduction, and differentiation. Previous studies showed that bta-miR-34c played a role in embryonic development and ovarian maturation in cows [52, 53]. Review of the genes targeted by bta-miR-34c also confirmed that these miRNAs play a role in the developmental stage of ovarian follicles in cows.

We observed that bta-miR-1247-5p targeted the Wnt family member 2B (WNT2B) and Wnt family member 4 (WNT4) genes (Table 5) and that it has a role in reproduction as a biological process. WNT2B and WNT4 have roles in follicular development, embryogenesis, cell reproduction, and differentiation. A study realized by Huang et al. showed bta-miR-1247-5 expression in the ovaries of Holstein cows [54], but the authors provided no detailed information about this miRNA. Our study revealed that these miRNAs play a role in the development of follicular ovaries in the cows.

In addition, we confirmed that miR222 regulates *ESR1* in our previous study [55]. In agreement with previous study, our data also indicated that bta-miR-222 plays a role in the development of the luteal tissue in cows.

5. Conclusion

To the best of our knowledge, this study identifies the miRNAs that are differentially expressed in the follicular and luteal stages and determines the probably molecular paths that these miRNAs regulate in Holstein cow. The pathway analysis conducted for 5 miRNAs expressed at high levels in the follicular and luteal tissues indicated that these miRNAs are associated with the TGF-beta signaling, gonadotropin-releasing hormone receptor, Wnt signaling, prolactin signaling, ovarian steroidogenesis, progesterone-mediated oocyte maturation, and oocyte meiosis pathways. Therefore, we can assert that bta-miR-196a, bta-miR-

490, bta-miR-1247-5p, and bta-miR-34c play roles in the different stages of follicular tissue development in cows and that bta-miR-222 has a role in luteal tissue development.

Acknowledgements

The Atatürk University Local Board of Ethics Committee for Animal Experiments has approved the study protocol of this research (HADYEK decision no: 2018/30). We thanks to Eastern Anatolia Advanced Technology Application and Research Center (DAYTAM) of Atatürk University for supporting. Funds for this work were provided by Ataturk University Scientific Research Projects SRP-2016/84.

Declaration of conflicting interests

The authors declare that they have no conflict of interest.

Funding

This work was supported by grants from the fundamental research funds for Ataturk University Scientific Research Projects SRP-2016/84.

Author Contribution

S.Ö conceived and designed the study, performed molecular experiments and wrote the paper. S.Ç performed the histopathology and immunohistochemistry and data analysis. S.Ö and S.Ç performed the experiments and interpreted the data. All authors read and approved the final manuscript

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442 **Figure Legends**

443 **Figure 1:** The levels of estradiol and progesterone in the POF (n=6) and CL (n=6). POF:
444 Preovulatory Follicle, CL: Corpora lutea. Values represent the mean ± SD of 3 indipendent
445 samples; error bars indicate standard deviation. Statistical significance (* $P < 0.05$, ** $P < 0.01$
446 and *** $P < 0.001$) was analyzed using One Way Anova.

447 **Figure 2:** Frequency distribution of sequence lengths of sequencing results in all samples. A)
448 Follicular library (Fol), n = 6 and B) Luteal library (Lut), n = 6.

449 **Figure 3:** Pie charts of small RNA percentages**.** A) Percentage of various ncRNA reads in total
450 distinct reads of Follicular library (Fol) n = 6. B) Percentage of various ncRNA reads in total
451 distinct reads of Luteal library (Lut) n = 6. C) Venn diagram of conserved miRNAs in both Fol
452 and Lut libraries.

453 **Figure 4:** Hierarchical clustering of all differentially expressed sRNAs. The three rows
454 represent the overall TPM cluster analysis result, clustered by log10(TPM+1) value. Heatmap
455 of miRNA expression (TPM) across follicular and luteal ovaries for differentially expressed

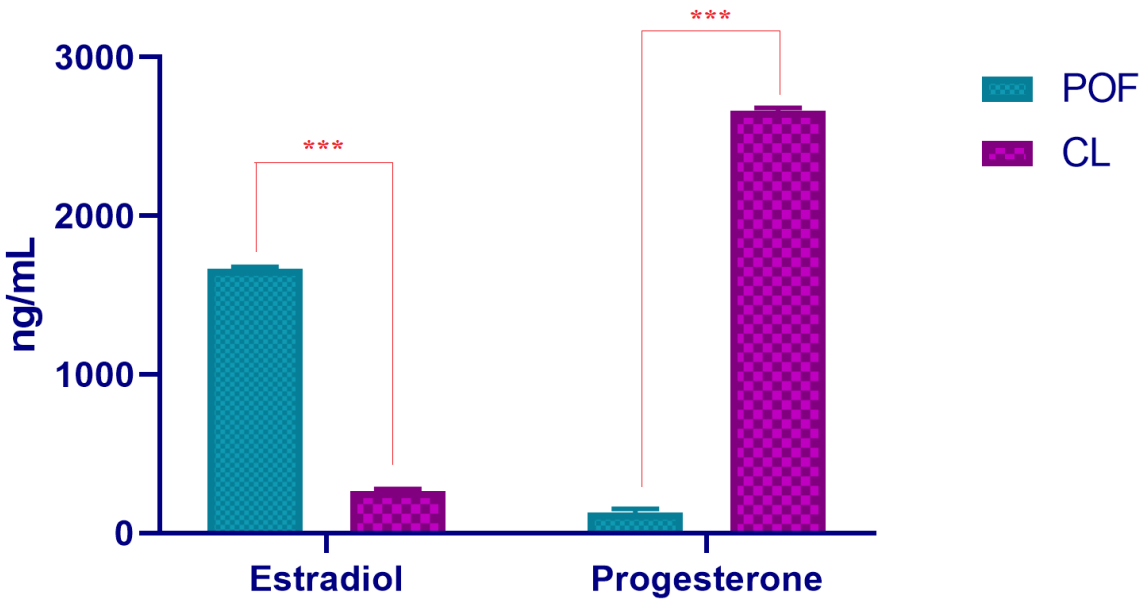
miRNA. Log2FoldChange, pvalue, and padj were observed. Dendrograms of clustering analysis for samples and miRNAs were displayed on the top and left respectively.

Figure 5: Comparison of relative expression and TPM values of five selected differentially expressed miRNAs. A) The relative expression level of selected miRNAs (five microRNAs) were measured by quantitative real-time PCR, data were expressed as the means±SD. ** , *** $P < 0.01$, or $P < 0.001$, respectively. B) The TPM of selected miRNAs analyzed in RNA-seq.

Figure 6: The relationship between the identified microRNAs and the target mRNAs. Interactive relationship between differentially expressed miRNAs and their target genes in Gonadotropin-releasing hormone receptor, Prolactin signaling, Ovarian steroidogenesis, Progesterone-mediated oocyte maturation and Oocyte meiosis pathway.

Figures

Figure 1



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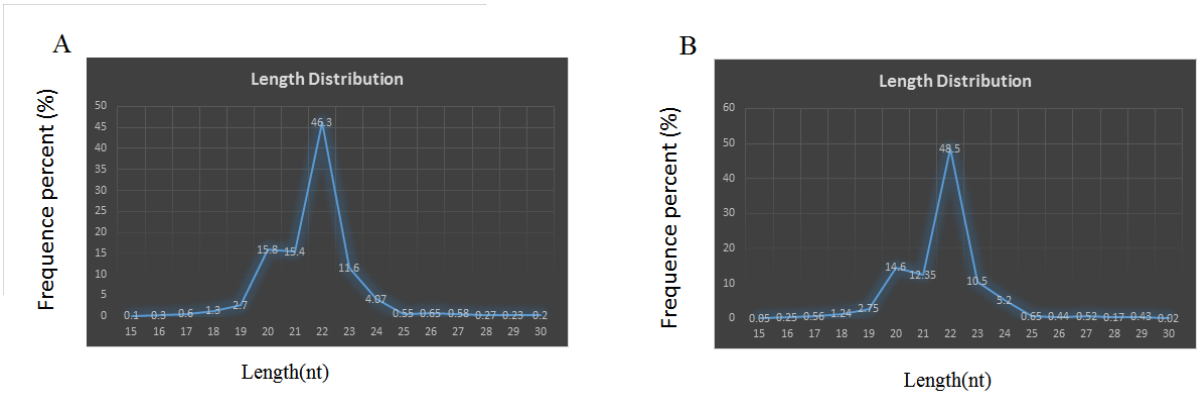
484 **Figure 2**



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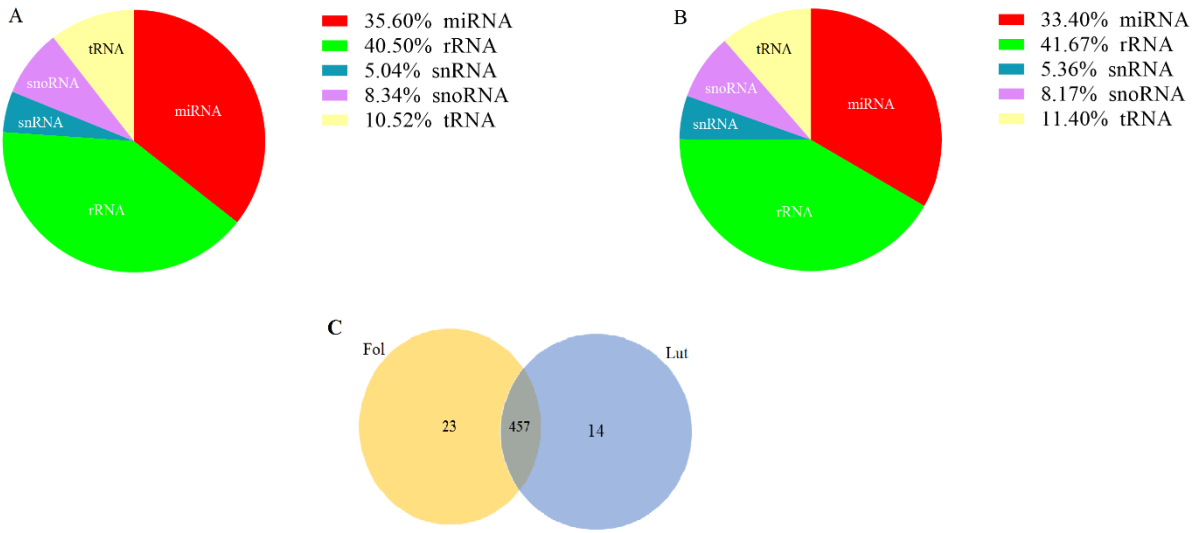
488 **Figure 3**


A
tRNA
snoRNA
snRNA
miRNA
rRNA
35.60% miRNA
40.50% rRNA
5.04% snRNA
8.34% snoRNA
10.52% tRNA
B
tRNA
snoRNA
snRNA
miRNA
rRNA
33.40% miRNA
41.67% rRNA
5.36% snRNA
8.17% snoRNA
11.40% tRNA
C
Fol
Lut
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457
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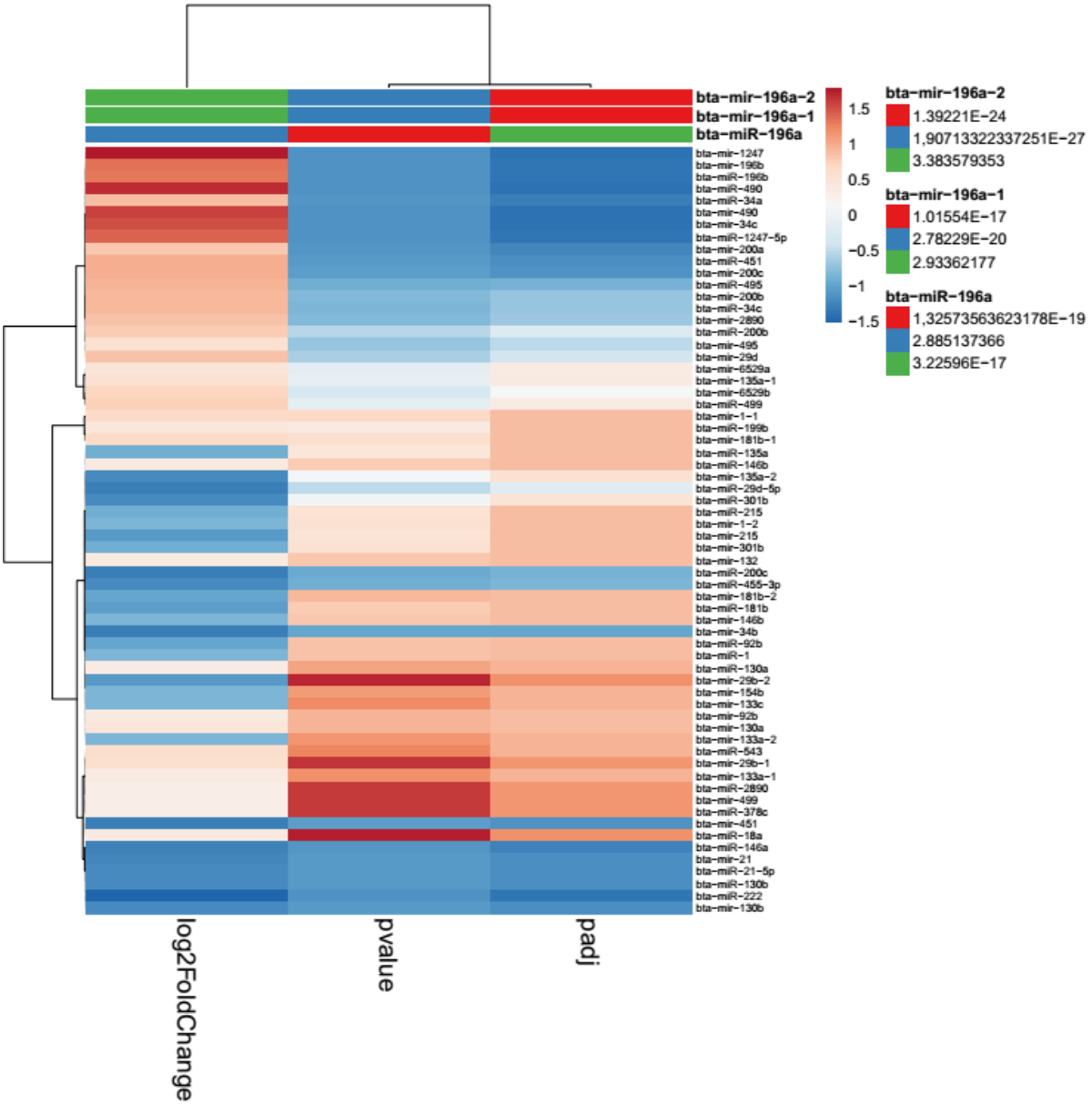
Figure 4


bta-mir-196a-2
bta-mir-196a-1
bta-miR-196a
bta-mir-1247
bta-mir-196b
bta-miR-196b
bta-miR-490
bta-miR-34a
bta-mir-490
bta-mir-34c
bta-miR-1247-5p
bta-mir-200a
bta-miR-451
bta-mir-200c
bta-miR-495
bta-mir-200b
bta-miR-34c
bta-mir-2890
bta-miR-200b
bta-mir-495
bta-mir-29d
bta-mir-6529a
bta-mir-135a-1
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bta-miR-199b
bta-mir-181b-1
bta-miR-135a
bta-miR-146b
bta-mir-135a-2
bta-miR-29d-5p
bta-miR-301b
bta-miR-215
bta-mir-1-2
bta-mir-215
bta-mir-301b
bta-mir-132
bta-miR-200c
bta-miR-455-3p
bta-mir-181b-2
bta-miR-181b
bta-mir-146b
bta-mir-34b
bta-miR-92b
bta-miR-1
bta-miR-130a
bta-mir-29b-2
bta-mir-154b
bta-mir-133c
bta-mir-92b
bta-mir-130a
bta-mir-133a-2
bta-miR-543
bta-mir-29b-1
bta-mir-133a-1
bta-miR-2890
bta-mir-499
bta-miR-378c
bta-mir-451
bta-miR-18a
bta-miR-146a
bta-mir-21
bta-miR-21-5p
bta-miR-130b
bta-miR-222
bta-mir-130b
log2FoldChange
pvalue
padj
1.5
1
0.5
0
−0.5
−1
−1.5
bta-mir-196a-2
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1,90713322337251E-27
3.383579353
bta-mir-196a-1
1.01554E-17
2.78229E-20
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bta-miR-196a
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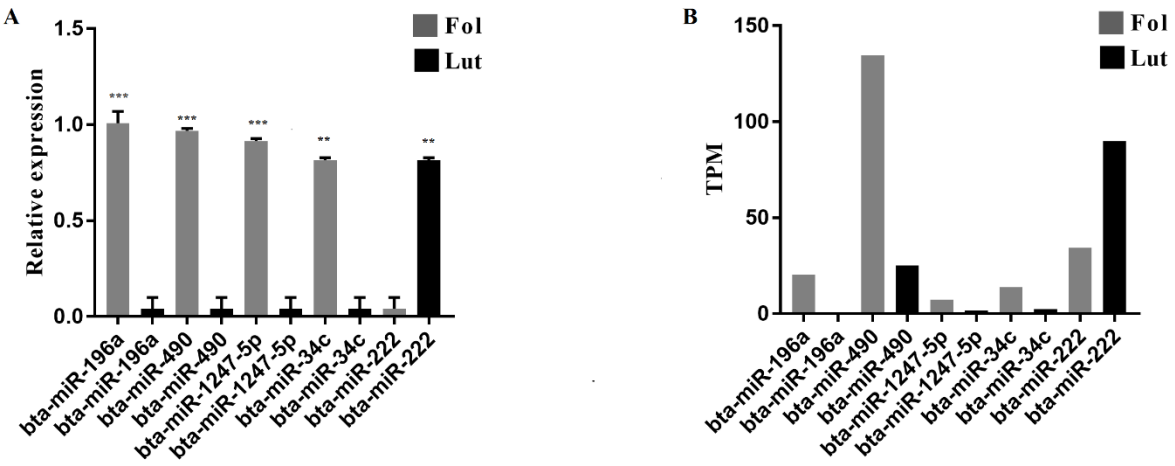
2.885137366
3.22596E-17

522 **Figure 5**



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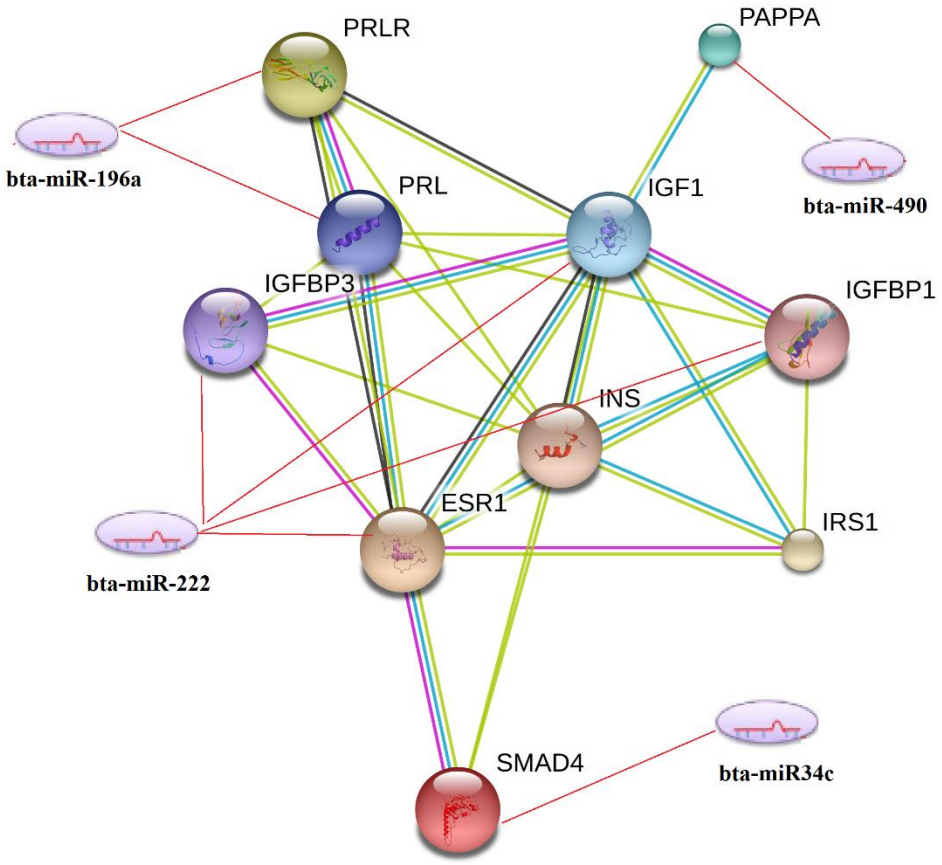
524 **Figure 6**



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Tables

Table 1: Selected and examined ovarium tissues

Number of cows	Number of ovarian tissues	Folicular phase	Luteal phase	Highest estradiol level	Highest progesterone level	Appropriate histology for preovulatory follicle	Appropriate histology for corpus luteum	IHC and RNAseq
20	20	12	8	6	6	6	6	6 POF 6 CL

Table 2: Summary of miRNA primers sequences for the RT-PCR

Name	Sequence [5′ → 3′]	Length [nt]	GC [%]
bta-miR-196a	TAGGTAGTTTCATGTTGTTGGG	22	40.9
bta-miR-490	CAACCTGGAGGACTCCATGCTG	22	59.1
bta-miR-1247-5p	ACCCGTCCCGTGCGTCCCCGGA	22	77.3
bta-miR-34c	AGGCAGTGTAGTTAGCTGATTG	22	45.5
bta-miR-222	AGCTACATCTGGCTACTGGGT	21	52.4

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Table 3: Results of raw reads before and afer quality control of the Follicular [Fol] and Luteal [Lut] libraries.

	Fol		Lut	
Read Type	**Reads Number**	**Ratio**	**Reads Number**	**Ratio**
Total reads number	5430473	100%	6009702	100%
Low quality	4887	0.09%	4206	0.07%
Adaptor 3 null	59735	1.1%	64904	1.08%
Insert null	73311	1.35%	67308	1.12%
5′ adaptor contaminants	19006	0.35%	52284	0.87%
Size< 18 nt	534901	9.85%	615393	10.24%
PolyA	1086	0.02%	1802	0.03%
High quality [size≥ 18 nt]	4738630	87.26%	5203800	86.59%

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Table 4: Differentially expressed known miRNAs identifed in the Fol and Lut libraries.

miRNA name	Log_2FC	FDR	Fol_TPM	Lut_TPM
bta-miR-196a-2	3.383579353	0	20.569	0.395
bta-miR-196a-1	2.93362177	0.0001	14.719	0.395
bta-miR-196a	2.885137366	0.0003	14.247	0.395
bta-miR-490	1.519984898	0.0145	134.735	25.199
bta-miR-1247	1.609613751	0.0013	7.265	1.68
bta-miR-34c	1.393009541	0.0145	13.964	2.471
bta-miR-196b	1.266352464	0.0178	1.604	0
bta-miR-222	-1.014635668	0.0001	28.439	90.008
bta-miR-1247-5p	1.303824475	0.0003	12.293	0.907

Table 5: Specific gene targets of differently expressed miRNAs

miRNA name	Fol or Lut	Genes
bta-miR-196a	Fol	[PRLR], [NOBOX] , [TGFBR3], [MAP3K1]
bta-miR-490	Fol	[PAPPA]
bta-miR-34c	Fol	[DMRTA1] [FBXW8] [FBXO5] [SMAD4] [WNT2B]
bta-miR-1247-5p	Fol	[WNT2B] [WNT4]
bta-miR-222	Lut	1[ESR1] [DMRT3] 1[IGF1]

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