Expression of genes involved in BMP and estrogen signaling and AMPK production can be important factors affecting total number of antral follicles in ewes

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Abstract

Follicular growth and ovulation of healthy oocytes is a complicated process which is regulated by several endocrine and paracrine factors as well as cross-talk between the oocyte and its surrounding somatic cells. This study compared the expression profile of some candidate genes involved in BMP signaling as well as estrogen and AMPK production in cumulus-oocyte complex (COC) of small and large antral follicles and their associated somatic cell layers in ovaries from ewes with high- and low-antral follicle count (AFC). Expression of GDF9 was increased by increasing the size of antral follicles, while BMP15 expression was decreased by follicular size. It should be noteworthy that transcription of both GDF9 and BMP15 was also detected in the adjacent cellular layers under the follicles. There was a very strong positive correlation between BMP15 and BMPR2 in ovary tissues. Expression of GDF9 was highly correlated with BMP15, BMPR1B, and BMPR2 in large antral follicles. Expression of BMP7 in small antral follicles and BMPR2 in ovary tissues was significantly increased in the high-AFC group. Expression of ESR1 and ESR2 involved in estrogen production as well as PRKAA1 which involved in AMPK production were significantly greater in large antral follicles of high-AFC. There was a very high correlation between Cyp19 and ESR1 in large antral follicles and ovary tissues. Expression of Cyp19 and PRKAA1 were positively correlated with GDF9, BMP15, and BMP7 in large follicles. In conclusion, this study suggests that apart from the BMP signaling, genes involved in AMPK and estrogen production can be pivotal players in ewe's follicular development process. In addition, a strong cross-talk can exist among AMPK, BMP signaling, and estrogen synthesis systems in ewe ovary.

Keywords: AFC, BMPs, Cross-talk, Cyp19, PRKAA1, Sheep

Introduction

Litter size is an important economic trait in both traditional and industrial sheep production systems. It has been well-documented that antral follicle count (AFC) is a reliable index for predicting the number of morphologically healthy oocytes and follicles in mammalian ovaries [1-3]. While there is a high variability in AFC between individuals, AFC is relatively constant within an individual animal related to follicular development and ovulation rate [1, 4].

The importance of growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) belonging to transforming growth factor β (TGF β) superfamily were identified as pivotal growth factors for follicular development and ovulation in mammalian species [5-7]. Several mutations in these genes have been detected and related to ewe ovulation rate and multiple-birth [8-12]. Moreover, the importance of BMP7 has also been highlighted in the growth and development of the reproductive system as well as litter size attributes [13]. In parallel with changes in BMP ligands within the oocyte, the importance of BMP receptors has also been evident for follicular development and antral follicle count [14]. It has been evident that GDF9, BMP7, and BMP15 can bind to both types of BMPRII and BMPRII, although depending on the mammalian species, they may have a higher tendency to one of these ligands [15]. Discovery of a single mutation in BMPRII in Booroola sheep was the first report on segregation of a major gene which considerably increases ewe prolificacy [16-18]. However, it has been shown that a large proportion of high prolific ewes did not carry the known significant mutations in GDF9, BMP15, and BMPRII [10, 19-21]. Therefore, studies are still needed to assess the importance of other genes which might involve in high antral follicle count (AFC), ovulation rate, and multiple-birth in ewes.

Expression of cytochrome P450 family 19 subfamily A member 1 (Cyp19A1), aromatase, is also essential for follicular growth and development as well as ovulation [22, 23]. Aromatase directs estrogen synthesis, and estrogen acts through estrogen receptor (ESR), i.e., ESR1 and ESR2 (see [24] for review). It has been shown that ESR1 is critical for follicular growth and development and successful ovulation whereas the estrogen ESR2 is unable to compensate the lack of ESR1 expression in mouse [25]. Moreover, nutritionally stimulated ewes by flushing have a higher ovulation and twinning-birth rate [26, 27]. Short-term intravenous injection of glucosamine substantially increased the total number of follicles in synchronized ewes [22]. The exact mechanism by which flushing induces higher prolificacy has not been fully determined. It has been proposed that flushing indirectly results in transiently higher FSH production [27].

Meanwhile, 5' adenosine monophosphate-activated protein kinase (AMPK) is a very decisive regulatory factor or “fuel gauge” for energy production and expenditure [28]. AMPK involved in the synthesis of fatty acid, cholesterol, and protein as well as glucose transport, fatty acid oxidation, and glycolysis (see [29] for review). It has been shown that AMPK is also involved in ketone body production in cows[30], FSH production in hens[31], progesterone and GnRH production in rats [32, 33], and estradiol and progesterone production in cows [34]. It consists of $\alpha 1$ and $\alpha 2$ as catalytic subunits and $\beta 1$, $\beta 2$, $\gamma 1$, $\gamma 2$, and $\gamma 3$ as non-catalytic regulatory subunits [28, 35, 36]. It has been shown that any mutation in AMPK $\gamma 1$ isoform, protein kinase AMP-activated catalytic subunit alpha 1 (PRKAA1), which causes a small modification in the adenyl-binding site of PRKAA1 can severely affect AMP and ATP binding to AMPK [36]. Because AMPK involves in circadian oscillation as well as the physiological and seasonal behavior of

mammalians [29], evaluation of its expression can be helpful for the understanding of reproduction performance in ewes, as a prominent seasonal breeder livestock.

In addition to these factors, intracellular communication between oocyte and its companion somatic cells has been revealed as the key factor for follicular development, ovulation, successful fertilization, and embryogenesis[37]. Therefore, gene expression patterns in the companion cells around follicles can be related to follicular health criteria. Thereof, we devised a retrospective study on high- and low-AFC ewe ovaries to compare the expression profile of some candidate genes involved in BMP signaling as well as estrogen and AMPK production. The expression profile was evaluated in COCs and the neighboring somatic cell layers beneath the follicles.

Materials and methods

Ovary and COC collection

Ovaries were collected from adult ewes of Iranian Afshari sheep breed by daily visits of an industrial abattoir, Fasaran, Isfahan, Iran, during the early breeding season in November 2014. Each pair of ovaries which contained any corpus luteum was discarded. Therefore, the studied ovaries were all in the follicular phase in the estrous cycle [38]. During the collection period, only five pairs of ovaries belonging to five ewes with very high number of antral follicle count (AFC) were found and were collected and designated the high-AFC group. This low number of animals with high AFC is consistent with the low incidence of multiple ovulations and lambing in this breed of sheep. Ten pairs of ovaries from ten ewes with the lowest AFC were collected and designated as the low-AFC group (Table 1) [39]. Immediately following the ovary

collection, cumulus oocyte-complex (COCs) from the selected ovaries were aspirated from each small (3-4 mm) or large follicles (> 5 mm) and washed twice in phosphate buffer saline (PBS) supplemented with 0.5 % BSA (Sigma-Aldrich, Germany). To provide enough cDNA for gene expression assay, each two COCs from the same ovary were pooled and transferred into a tube with the minimum media. The whole cumulus-oocyte complex was used for RNA extraction and gene expression assay. Moreover, the superficial layer which was located under a random sample of aspirated follicles on ovary tissues was sliced and considered as ovary tissue. We cut only one thin layer on the cortical section of the ovary which was located in the closest vicinity to the follicles which may interact with the COC and support follicular development.

Quantitative PCR analysis

The detail of cDNA synthesis procedure was described previously [12]. Briefly, total RNA was extracted using the RNX PLUS kit (SinaClon, Iran). The quality and quantity of extracted RNA were checked by 1% agarose gel electrophoresis and spectrophotometer, respectively. Three micrograms of total RNA was treated with 3 I. U. of DNase-I (#AM2222, Invitrogen™, Germany) for 15 min at 37°C followed by heat inactivation at 75°C for 5 min. Then, 1 µg of RNA was quickly reverse-transcribed into cDNA using M-MLV enzyme (#28025013, Invitrogen™, Germany) and random hexamer primers as follows: incubated initially at 70°C for 5 minutes, chilled on ice, and mixed with RT ingredients including 5X reaction buffer, dNTP mix (200 µM), RNase inhibitor, and RT enzyme (1 µl). The cDNA synthesis was carried out using incubation at 25°C for 5 minutes, 42°C for 60 minutes, 70°C for 5 minutes, and finally was stored at -80 °C for further analysis. One microliter of cDNA was used for real-time PCR (Corbett, RG-6000, Germany) using KAPA SYBR FAST qPCR Master mix (KapaBiosystems,

UK). The relative expression of GDF9, BMP15, BMP7, BMPR1B, BMPR2, Cyp19 (aromatase), Estrogen receptor-1 (ESR1), ESR2, and PRKAA1 as candidate genes was evaluated compared to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the housekeeping gene [40]. Primer sequences for the above-mentioned genes are presented in Table 2. The qPCR for all genes were carried out using the following program: initial denaturation at 95°C for 3 min and 40 cycles of denaturation at 95°C for 30 s and annealing at 60°C for 40 s. Primer sets were verified for amplification of only the desired genes through qPCR assay by adding a melting curve program (denaturation at 95°C for 60 s and then a gradual increase in the temperature from 65 to 95°C) after the qPCR program. The melting curve and gel electrophoresis for each primer pair were performed to verify amplification of the desired band. The real-time data was analyzed using the Rotor-Gene 6000 Series 1.7 Software. The cycle of threshold (Ct) derived from the target gene was subtracted from the Ct of the housekeeping gene, and the relative expression of each gene was calculated using the 2-dCt method. The number of replicates was five and ten for high- and low-AFC group, respectively. Each sample (pooled COC pairs) was analyzed by real-time PCR in triplicate and the Ct average for this triplicate was considered as one repetition.

Statistical analysis

Except for the large antral follicles in the low-AFC ovaries in which only one COC was detected, each two-COC was pooled and considered as one replicate. Moreover, a very thin layer was excised from the beneath of random samples of small and large follicles. Three samples were randomly collected from the beneath of three follicles and considered as three repetitions from one ovary. Mean comparisons between high- and low-AFC groups were carried out using the independent t-test. To compare the gene expression profile in transcription sources (small and

large antral follicles and ovary tissue), means of combined classes of AFC and transcription source were compared using LSD test. Moreover, to evaluate possible interactions for each gene, Pearson-correlation coefficients were determined between expression levels of each two genes using the whole data collected from small and large antral follicles as well as ovary tissues using Proc CORR in SAS (version 9.1) software. A p-value ≤ 0.05 was considered as statistically significant level, while $0.05 < \text{P-value} < 0.10$ was considered as the presence of a trend between two groups.

Results

Expression of BMP signaling system

As shown in Figure 1a, GDF9 predominated in ovarian tissues, whereas BMP15 predominated in small follicles. The presence of a trend was observed for GDF9 expression in ovarian tissues of the high-AFC group compared to low-AFC group (P-value = 0.10), although the difference was significant when combined data of AFC and expression source was analyzed (P-value <0.05). Moreover, relative expression of BMP7 was higher in ovarian tissues of high-AFC than low-AFC (P-value = 0.03). Considering expression of BMP receptors in Figure 1b indicates that BMPR2 predominated in large follicles, whereas in small follicles it was greater in high-AFC than low-AFC (P-value = 0.04).

There were very strong positive correlations between GDF9 and BMP15 in small and large follicles as well as ovarian tissues (Tables 3 and 4). Also, a positive correlation was observed between BMP7 and BMP15 in large antral follicles (Table 3). Estimated correlations between

growth factors and their receptors indicated that BMP15 was negatively associated with BMPR1B in small follicles. However, both GDF9 and BMP15 associated positively with BMPR1B and BMPR2 in large antral follicles (Table 3). Expression analysis in ovary tissues suggested the presence of strong correlations between BMPR2 and both BMP15 and GDF9 (Table 4). Additionally, there was a significant relation between BMPR1B and BMPR2 in large antral follicles (Table 3).

Expression of estrogen signaling system

Expression of Cyp19 predominated in small follicles in which it was lower in high-AFC than in low-AFC (Figure 2). On the other side, ESR1 expression prevailed in large follicles. A higher level of transcription was detected in high-AFC than low-AFC in large follicles as well as the neighbor-follicle cell layers (P-value = 0.008, 0.002, and 0.0005, respectively). Expression of ESR2 predominated in ovarian tissues, in which it was greater in high-AFC than low-AFC as well as in large follicles ((P-value = 0.05 and 0.02, respectively). Association analysis of Cyp19 with estrogen receptors showed a very high correlation between Cyp19 and ESR1 in large antral follicles and ovary tissues. Moreover, there was a positive correlation between estrogens receptors, ESR1 vs. ESR2, in ovarian tissues (Table 4).

Interrelation between expression of growth factors and estrogen signaling systems

Positive correlations were observed between estrogen production key enzyme, Cyp19, and growth factors of GDF9, BMP15, and BMP7 in large follicles while Cyp19 variation was positively associated only with BMP7 in ovarian tissues (Tables 3 and 4). Moreover, Cyp19

correlation with growth factor receptors indicated that its expression positively associated with BMPR2 in large follicles and ovarian tissues.

Correlation coefficients between growth factors and estrogen receptors showed that both of GDF9 and BMP15 positively correlated with ESR2 in small follicles as well as ovarian tissues. Moreover, positive correlations were detected between GDF9 and ESR1 in large follicles as well as BMP7 and ESR1 in ovarian tissues. Correlations between growth factor receptors and estrogens receptors revealed that BMPR2 was positively associated with ESR1 and ESR2 in large follicles and ovarian tissues, respectively (Tables 3 and 4).

Expression of PRKAA1, as an energy sensor component

Expression of PRKAA1 was greater in high-AFC than in low-AFC in large follicles (P-value = 0.05) (Figure 3). Re the association analysis between PRKAA1 and growth factors, positive correlations were observed between PRKAA1 and BMP ligands, GDF9, BMP15, and BMP7, in large follicles. Moreover, PRKAA1 expression was positively correlated with BMPR2 in large antral follicles. There was a positive correlation between PRKAA1 and Cyp19 in large follicles and ovarian tissue and also between PRKAA and ESR1 in large follicles.

Discussion

In this study, the gene expression pattern in the cumulus-oocyte complex was assessed in the small and large antral follicles derived from ovaries with the high and low number of follicles. The expression profile of TGFb ligands and receptors in oocytes, cumulus, granulosa, and theca

cells have been analyzed previously [14]. There is a very high interaction between oocyte and granulosa cells [37, 41] [42]. Proliferation and differentiation of granulosa cells are directly affected by paracrine factors, particularly members of the TGFb superfamily[43]. Additionally, there is a high difference in the expression of TGFb members between granulosa and oocytes [14, 41]. Since the follicular development and ovulation is a complicated consequence of cascade events involving several paracrine factors, we studied the expression pattern in the combined cumulus/granulosa cells and oocyte (COCs), as well as one thin cellular layer of their adjacent cell layers, including granulosa, theca, and cumulus cells, beneath the follicles on the surface of ovary tissue. The main agenda of this project was to assess expression differences of some BMP ligands and receptors, aromatase and its receptors as well as PRKAA1 as the main component of AMPK in COCs and surrounding cells of high vs. low-AFC ewes.

Results of this study showed that BMP ligands, GDF9, BMP15, and BMP7, as well as both BMPRII and BMPRI, were expressed in COCs and the cellular layer of ovary tissues laid beneath of follicles in high- and low-AFC ewe. The relative gene expressions of GDF9 and BMP15 in small and large antral follicles were not significantly different between high- and low-AFC ovaries. Moreover, GDF9 transcription increased by increasing the size of antral follicles, while a reverse trend was observed for BMP15. The highest expression of GDF9 and BMP15 were observed in ovary tissue and small antral follicles, respectively. The results of this study clearly showed that GDF9 and BMP15 mRNA was present in the ovarian cell layer located in the close vicinity of the antral follicles. To our knowledge, this is the first report on in vivo evaluation of transcription profile in ovarian cell layers surrounding antral follicles. Although it has been suggested that expression of GDF9 and BMP15 occurs only in oocytes [6, 44], further

studies showed that the expression site of these BMP ligands is not limited to oocyte cells [40, 45, 46]. In the current study, we evaluated the differential gene expression pattern in the cortical slices which were located in the close vicinity of follicles to consider possible paracrine cross-communication approach between oocytes and the companion cell layers which can actively regulate follicular cell layers and oocyte development [37, 42]. Since these cell layers were sliced as the basal cell layers which may interact with the follicular cells, we did not work on specific cell types. Thus, the transcripts could be produced by these somatic cells per se, or by small oocytes which were located in the close vicinity of antral follicles and had not initiated follicular formation step which is [37, 42, 46]. The exact cell types which were the main sources for GDF9 and BMP15 in the cellular layer surrounding the follicles have yet to be defined. However, the concluding remark of this part of the project was that GDF9 expression in the cellular layer around the follicles could be much greater and even more important than the COCs per se. However, further studies are needed to discover the possible cross-communication between these adjacent cells and COCs. Nonetheless, our results indicated that differential expression of GDF9 and BMP15 as the main candidate genes for higher sheep prolificacy were not the pivotal reasons for the higher number of antral follicles in ewes which were used in the current study. In contrast to these two above-mentioned BMP ligands, high levels of BMP7 expression was observed in ovary tissues of high-AFC compared to the low-AFC group. These results agreed with previous reports indicating the important role of BMP7 in stimulating follicular development in mammals [13].

Results of gene expression pattern of BMP receptors showed that relative expression of BMP2 was moderately higher in large follicles compared to small follicles and ovary tissues, while

BMPR1B did not associate with the follicle number. On the other side, a very strong positive correlation was detected between the expression of GDF9 and BMP15 in both large antral follicles and the cell layers beneath follicles on ovary tissues. It has been shown that GDF9 can bind to BMPR1 and BMPR2 [41, 47]. Results of the current study indicated that GDF9 expression was associated with BMPR2 but not with BMPR1B in large antral follicles and the ovary tissue. This agreed with the previous finding indicating the crucial importance of BMPR2 for GDF9 action mechanism [41]. It has been shown that BMP15 and BMP7 can bind to both types of BMP receptors [15]. Results from the current study indicated that BMP15 expression was highly correlated with the expression of both BMPR1B and BMPR2 in COCs derived from large antral follicles. In the cellular layer surrounding the follicles on ovary tissues, BMP15 expression was correlated with BMPR2, suggesting a potentially important role for BMPR2 in the theca and granulosa cells surrounding the follicles. Results of this study did not show an association of BMP7 expression with either BMPR1B or BMPR2. This finding suggests that in agreement with the previous finding in rats [48], BMP7 in ewe ovaries may preferentially bind to activin receptors.

Expression of Cyp19, the key gene responsible for estrogen biosynthesis, in granulosa cells is essential for follicular growth and coordination of the ovulatory process (15). It has been verified that Cyp19 and estrogen receptors express in both granulosa cells and oocytes throughout follicular growth in ewes [49-51]. Our results indicated that the relative expression of estrogen receptor-1 and -2 in large antral follicles as well as ovary tissues which were derived from high-AFC ovaries were considerably greater than the low-AFC group. Moreover, expression of Cyp19 was highly correlated with ESR1 in large antral follicles and ovary tissues. These results clearly

suggested the important role of estrogen in the number of antral follicles. Agreeing with this finding, higher levels of estradiol was detected in granulosa cells and follicular fluid of low-AFC compared to high-AFC ovaries in cows experiencing follicular phase [51]. It should be noteworthy that results of the current study showed that Cyp19 expression was positively correlated with GDF9, BMP15, BMP7, and BMPR2 expression in large follicles as well as BMP7 and BMPR2 in ovary tissues. It has been reported that growth factors and aromatase inhibitors have a synergistic effect on chicken sex-reversal, although the exact mechanism has yet to be discovered [52]. On the other side, there is a high relation between AFC and plasma levels of circulating anti-Müllerian hormone (AMH) [53], oocyte quality[54], ovarian response to FSH stimulation, and subsequently pregnancy rates in ewes [53]. It has been verified that AMH had a strong negative effect on the secretion of estradiol through down-regulation of Cyp19 [55-57]. The effect of AMH and aromatase inhibitors on blocking estrogen production in early fetuses and subsequently female-to-male sex-reversal has been evident in sheep and chicken [52, 55]. Association of AMH has been validated with BMPR1A in mouse [58] and BMPR1B in ewes which carried the FecB mutation [59]. Further studies showed that presence of the FecB mutation attenuated the granulosa cell response to the stimulatory effect of BMP4 on AMH production [60]. However, it needs to be explored if the observed associations in the current study between estrogen synthesis signals and a set of growth factors are associated with AMH expression.

It has been well-documented that flushing of ewes increases the number of antral follicles [26]. It directly inhibits follicular oestradiol production which may lead to a compensatory secretion of FSH [61]. Although short-term intravenous injection of glucosamine did not affect P450-

aromatase expression [22], flushing of ewes *per se* decreased aromatase expression 12 h after flushing. They suggested that reduction of Cyp19 expression could reduce estradiol synthesis in follicles, transiently increase FSH, and finally increase the number of follicles with >3 mm diameter [61]. In the current study, we tracked PRKAA1 expression as an AMPK production indicator to see if its differential expression can increase the total number of antral follicles. Our results showed that PRKAA1 was expressed at higher levels in large antral follicles of high-AFC than that of low-AFC. More importantly, there were strong correlations between PRKAA1 and variation of estrogen synthesis signals of Cyp19 and ESR1, BMP ligands of GDF9, BMP15, and BMP7, and BMPRI1B in large antral follicles. This is the first report suggesting the relation of AMPK with estrogen as well as BMP ligands and receptors. In higher demands of ATP, cyclic AMP (cAMP) stimulates AMPK activity [62-64] to increase food intake and turn on catabolic pathways that generate ATP [64]. On the other side, cAMP is an important stimulatory factor for estrogen synthesis [65, 66].

Higher expression of the majority of studied genes in the adjacent cells beneath the follicles comparing to small and large antral follicles *per se* can support this idea that local micro environmental regulation on the ovarian surface epithelium can be more important than systemic factors [38]. However, further prospective studies using a controlled homogenous experimental design controlling for age, parity, and exact stage of the oestrus cycle are required to determine the exact mechanisms involved in the cross-talk between AMPK, estrogen synthesis, and growth factors in the ovary.

Conclusion

This study was carried out to explore the mRNA expression profile in COCs and the theca/granulosa cells beneath the follicles in ovaries with high- and low-AFC. Results indicated that expression of BMP7 and BMPR2 in COCs and ovary tissues are highly associated with the antral follicle number. The results revealed that expression of a set of genes involving in estrogen synthesis, including Cyp19, ESR1, and ESR2, as well as AMPK production, PRKAA1, is closely associated with the number of antral follicles in ewe ovaries. Also, PRKAA1 had significant correlation with Cyp19, ESR1, GDF9, BMP15, BMP7, and BMPR1B in large antral follicles. Overall, this study confirmed the importance of multiple genes in the ovulation rate in ewe and suggested that apart from the BMP signaling, AMPK and estrogen may act as pivotal players in this reproduction process. These data can be useful for further elucidation of the molecular mechanism by which nutritional flushing increases the number of antral follicles and ovulation rate.

Acknowledgement

Authors would like to appreciate Professor Alan McNeilly for his invaluable collaboration for getting the funding, conducting the project, and editing the manuscript. This project was supported by Iran National Science Foundation (INSF) through grant number 90002710.

Conflict of interest

Authors have no conflict of interest to declare.

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Figure Legends

Figure 1. Relative expression pattern of three BMP ligands (A) and two BMP receptors (B) in COCs derived from small and large antral follicle as well as the ovary tissue, one thin layer on the cortical section of the ovary which was located beneath the follicles in ewe ovaries. GAPDH was used as the house keeping gene. White and black bars represent high- and low-AFC ovaries, respectively. The difference between high- and low-AFC groups which was significant using independent t-test at P-value <0.05 is indicated by *. Also mean comparison among combined classes of AFC (high and low) and transcription source (small and large antral follicles and ovary tissue) were carried out using LSD test and means with common letter did not have a significant difference (P-value <0.05).

Figure 2. Relative expression pattern of major genes involving in estrogen synthesis in COCs derived from small and large antral follicle as well as the ovary tissue, one thin layer on the cortical section of the ovary which was located beneath the follicles in ewe ovaries. GAPDH was used as the house keeping gene. White and black bars represent high- and low-AFC ovaries, respectively. The difference between high- and low-AFC groups which was significant using independent t-test at P-value <0.05 and <0.01 are indicated by * and **, respectively. Also mean comparison among combined classes of AFC (high and low) and transcription source (small and large antral follicles and ovary tissue) were carried out using LSD test and means with common letter did not have a significant difference (P-value <0.05).

Figure 3. Relative expression pattern of PRKAA1 gene involving in AMPK production in COCs derived from small and large antral follicle as well as the ovary tissue, one thin layer on the cortical section of the ovary which was located beneath the follicles in ewe ovaries. GAPDH was

used as the house keeping gene. White and black bars represent high- and low-AFC ovaries, respectively. The difference between high- and low-AFC groups which was significant using independent t-test at P-value <0.05 is indicated by *. Also, mean comparison among combined classes of AFC (high and low) and transcription source (small and large antral follicles and ovary tissue) were carried out using LSD test and means with common letter did not have a significant difference (P-value <0.05).

Table 1. A summary of antral follicle count (AFC) in ewe ovaries.

Group	no. of ovaries	No. of small antral follicle (3-4 mm)	No. of large antral follicle (> 5 mm)	Total no. of antral follicles
High-AFC	5	14.3 ± 2.0	4.3 ± 0.5	18.5 ± 1.8
Low-AFC	10	5.5 ± 0.6	1.7 ± 0.2	7.3 ± 0.6

Table 2. Primer pairs for gene expression assay in ewe ovaries

Gene name	Gene ID	Primer sequence	Amplicon Length (bp)	Reference
GAPDH	refNM_001190390.1	F: GAGAAACCTGCCAAGTATGA R: CGAAGGTAGAAGAGTGAGTG	139	[31]
GDF9	gb KR063137.1	F: TAGAGGTTCTGTATGATGGG R: AGCCGAACAGTGTGTAGAG	151	[31]
BMP15	gb KT013294.1	F: CGCTGGCTAGTGTAGCAAGG R: GTGGGAATGAGTTAGGTGAAG	143	[31]
BMPR1B	gb AF312016.1	F: CCTGTGGTCACTTCTGGATGTC R: TTCCGTTCTGTGCAGCATTC	107	[31]
Cyp19	emb AJ012153.1	F: TACTTCCCCTGAGATCAAGTAACA R: CTTTAGCACTCGAGGCACTTG	161	[39]
BMP7	refNM_001308564.1	F: AACGTCGCAGAAAACAGCAG R: AGTAGTAGGCGGCATAGCCT	127	Current paper
BMPR2	refNM_001306123.1	F: AGAATGAGCGCAACCTGTCA R: TGTGCTGGACATCGAATGCT	149	Current paper
ESR1	emb Z49257.1	F: AGAGGGTGCCAGGATTTGTG R: TTCCCTGGTTCCTGTCCAGA	162	Current paper
ESR2	refNM_001009737.1	F: AATGGTGAAGTGTGGCTCCC R: TGCACCGTTCCTCTTGTTT	112	Current paper
PRKAA1	refXM_004017020.2	F: TGGAAGGAAATCTGATGTACCTGG R: CTGTGGACCACCATATGCCT	135	Current paper

Table 3. Correlation coefficients between gene expressions in large and small antral follicles (upper and lower triangles, respectively) in ewe ovaries. To estimate the correlation coefficients, all data from high- and low-AFC ovaries were pooled. P-value < 0.05, < 0.01, and < 0.001 are indicated by *, **, ***, respectively.

	GDF9	BMP15	BMP7	BMPR1B	BMPR2	Cyp19	ESR1	ESR2	PRKAA1
GDF9		0.76***	0.46	0.64**	0.99***	0.54*	0.53*	0.19	0.54*
BMP15	0.77***		0.73**	0.62**	0.76***	0.64*	0.32	0.01	0.56*
BMP7	-0.08	0.11		0.16	-0.33	0.91***	0.48	0.03	0.89***
BMPR1B	-0.47	-0.57*	0.11		0.64*	0.05	0.01	0.17	0.02
BMPR2	-0.25	0.24	0.45	-0.11		0.53*	0.54*	0.19	0.53*
Cyp19	-0.06	0.01	0.34	-0.12	-0.22		0.63**	0.17	0.83***
ESR1	0.01	0.22	0.03	-0.17	0.1	-0.03		0.29	0.56*
ESR2	0.66**	0.85***	-0.02	-0.38	0.43	-0.02	0.07		0.14
PRKAA1	0.11	-0.17	0.4	0.42	-0.29	0.05	0.39	-0.39	

Table 4. Correlation coefficients between gene expressions in ewe ovary tissue. To estimate the correlation coefficients, all data from high- and low-AFC ovaries were pooled. P-value < 0.05, < 0.01, and < 0.001 are indicated by *, **, ***, respectively.

	GDF9	BMP15	BMP7	BMPR1B	BMPR2	Cyp19	ESR1	ESR2
BMP15	0.74***							
BMP7	0.61	0.39						
BMPR1B	0.4	0.49	-0.15					
BMPR2	0.79***	0.99***	0.42	0.47				
Cyp19	0.44	0.49	0.60*	-0.19	0.51*			
ESR1	0.6	0.37	0.73***	-0.1	0.4	0.75***		
ESR2	0.90***	0.64**	0.45	0.33	0.70**	0.45	0.67**	
PRKAA1	0.26	0.37	0.47	-0.19	0.34	0.78***	0.38	0.1

Figure 1

Figure 2

Figure 3