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Transcription Profile of Candidate Genes for the Acquisition of Competence During Oocyte Growth in Cattle

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Contents

The aim of this study was to investigate the expression profile of candidate genes involved in competence during oocyte growth. The candidate genes (*BMP15, OOSP1, H1FOO, H2A, H3A, H4, SLBP, DNMT1, DNMT3B, HAT1, HDAC2 and SUV39H1*) were selected because of their possible involvement in determining oocyte developmental competence. Pre-antral and antral follicles were isolated from the ovaries of Zebu (*Bos indicus*) cows, measured and classified into the following categories according to their diameter: (i) oocytes from primordial follicles: diameter <20 μm, (ii) oocytes from primary follicles: 25–35 μm, (iii) oocytes from small secondary follicles: 40–60 μm, (iv) oocytes from large secondary follicles: 65–85 μm, (v) oocytes from small antral follicles: 100–120 μm, and (vi) oocytes from large antral follicles: >128 μm. Total RNA was extracted from four pools of 25 oocytes for each category of follicles, and the genes were quantified by qPCR. Target gene expression was normalized using the gene *PPIA*. The results suggest that stocks of the studied transcript genes accumulate before the final phase of folliculogenesis. The *HDAC2* gene was the only gene in which a differential expression was observed at stage associated with competence acquisition.

Introduction

Many studies have shown that oocyte developmental competence is determined, in part, by the composition and quantity of maternal transcripts stored during oocyte growth and the final phases of folliculogenesis (Lonergan et al. 2003; Gandolfi et al. 2005; Sirard et al. 2006). Taking into consideration that this stored mRNA is essential for determining competence (Allard et al. 2005; Arnold et al. 2008), an alternative approach to studying competence determination is to characterize how and during which stages of oogenesis and folliculogenesis these stocks are formed. Characterizing this process would be the first step to understanding the molecular basis involved in the formation of a good-quality oocyte.

Even though the different expression levels of some genes are already associated with oocyte developmental competence in cattle (Donnison and Pfeffer 2004; Fair et al. 2004; Dode et al. 2006; Mourot et al. 2006; Racedo et al. 2008; Caixeta et al. 2009), the mechanisms involved and the molecular characteristics of competent oocytes are still unknown. Therefore, the various genes that participate during the critical events that take place during gametogenesis are possible candidate genes that may be involved in competence determination.

Oocyte-secreted protein 1 (*OOSP1*) and bone morphogenetic protein 15 (*BMP15*) are paracrine factors secreted by the oocyte. Although the function of *OOSP1* has not yet been defined (Tremblay et al. 2006), the fact that it is specifically secreted by the oocyte suggests that, similar to *BMP15*, it may play an important role in growth and follicular development (Gilchrist et al. 2004; Hussein et al. 2005).

Histone proteins, which are structural proteins that associate with chromatin to form nucleosomes, also function in controlling gene expression (McGraw et al. 2006), embryonic genome activation, DNA methylation and X-chromosome inactivation (Fair et al. 2004). Recently, it was reported that an oocyte-specific histone named H1 histone family, member O, oocyte-specific (*H1FOO*) is involved in controlling gene expression during oogenesis and initial embryogenesis (Tanaka et al. 2005; McGraw et al. 2006). The gene coding for the stem-loop binding protein (*SLBP*), which is related to histone proteins, plays an important role in the accumulation, stabilization and translation of histone mRNA during oocyte maturation and fertilization (Allard et al. 2002, 2005; Arnold et al. 2008).

An increase in the gene expression level of histone *H2A* (Dode et al. 2006; Mourot et al. 2006; Caixeta et al. 2009), *H3* (Fair et al. 2004) and *SLBP* (Donnison and Pfeffer 2004) was observed in oocytes with greater developmental competence. During oogenesis, in addition to forming the stockpiles of mRNA, the epigenetic pattern in the oocyte genome, which was completely erased, has to be restored in preparation for fertilization. DNA methyl (cytosine-5-) transferases (*DMNTs*) are the enzymes responsible for establishing (*DNMT3a* and *3b*) and maintaining (*DNMT1*) methylation patterns. The expression levels of these enzymes correlate with the diameter of the oocyte in mice (Luccifero et al. 2007), which indicates that a specific size must be reached for the completion of epigenetic modification in the oocyte (Hiura et al. 2006; Luccifero et al. 2007).

Post-translational modifications to histone tails and DNA methylation are essential mechanisms in gene regulation and play an important role in controlling the reprogramming that occurs during gametogenesis and embryogenesis (Prather et al. 2009). The level of histone acetylation is controlled by histone acetyltransferase (*HAT*) and histone deacetylase (*HDAC*) enzymes, and the level of histone methylation is controlled by histone methyl transferases (*HMT*s) enzyme. Among the various *HMT*s, the supressor of variegation 3–9 homolog 1 (*SUV39H1*) specifically methylates histone H3 at position lysine 9 (*H3K9*), and this modification plays an important role in forming heterochromatin, which then

leads to the repression of gene expression (Lachner and Jenuwein 2002; Racedo et al. 2009).

Transcripts for all of these genes are present in fully grown oocytes at the GV stage (McGraw et al. 2003, 2006; Racedo et al. 2009). However, how and during which stage of oogenesis their stocks are established has not been described in cattle. Additionally, the studies evaluating the expression of some of these genes in bovine have been limited to the use of immature oocytes obtained from antral follicles and, in some rare cases, large secondary follicles (Sanchez et al. 2009). No reports using oocytes isolated from primordial and primary follicles were found. Therefore, the aim of this study was to characterize, in the bovine, the expression pattern of oocyte-specific genes and genes related to histone proteins and epigenetic changes, during oocyte growth from the primordial follicle to the final antral stage.

Material and Methods

Isolation of pre-antral follicles

Ovaries from Nellore cows (*Bos indicus*) were collected at a local slaughterhouse and transported to the laboratory in saline solution (0.9% NaCl) supplemented with antibiotics (100 IU ml penicillin G and 100 μg ml streptomycin sulphate; Sigma, St Louis MO, USA) at 37°C.

In the laboratory, cortical slices were cut from the ovarian surface using a surgical blade (no. 22) and then washed with phosphate-buffered saline (PBS). For isolating pre-antral follicles, slices were cut using the tissue chopper (The Mickle Laboratory Engineering Co. Ltd, Gomshall, Surrey, England) with four different cutting intervals (200, 250, 300, 350 μm) followed by filtration and successive pipetting (Lucci et al. 2002). The supernatant was discarded, and the pellet was collected and searched for pre-antral follicles or treated for oocyte isolation. Given the difficulty in obtaining large secondary follicles with the previously described procedure, a different protocol was used for isolating these follicles. The ovarian cortex was sliced in the tissue chopper in two directions (longitudinal and transverse) into approximately 1-mm sections. The sections were transferred to a Petri dish containing PBS, and the follicles were manually isolated using a microdissection technique with needles (no. 29) under a stereomicroscope (Telfer 1996; Sharma et al. 2009).

Characterization of the diameter of oocytes obtained from pre-antral follicles

The isolated pre-antral follicles were classified by their morphological appearance according to Kacinskis et al. (2005), into four developmental stages: primordial, primary, small secondary and large secondary (Fig. 1). After classification, the follicles in each category were separately measured using the Motic Images Plus 2.0 software (Motic China Group Co. Ltd., Xiamen, China). Then, each follicle was individually transferred into 50-μl TCM 199 (Sigma, St. Louis MO, USA) supplemented with 0.5 mg/ml of collagenase type II (Sigma, St. Louis MO, USA) and successively pipetted until the oocyte was completely free of the cumulus cells. Once isolated, the individual oocytes were measured, excluding the zona pellucida, using the Motic Images program. For each category of follicles, 50 oocytes were individually measured, and the mean size was calculated. Based on the mean size, the oocytes were classified according to their diameter into the following categories: (i) oocytes obtained from primordial follicles: diameter <20 μm, (ii) oocytes obtained from primary follicles: 25–35 μm, (iii) oocytes obtained from small secondary follicles: 40–60 μm, (iv) oocytes obtained from large secondary follicles: 65–85 μm.

Harvesting oocytes from pre-antral and antral follicles

For gene expression analysis, oocytes were recovered from the various categories of follicles according to a modified protocol described by Monti et al. (2006). After pre-antral follicle isolation, 1 ml of the pellet was collected from the suspension containing the pre-antral follicles and transferred to another tube containing TCM 199 supplemented with 0.5 mg/ml of collagenase type II. The solution was incubated at 37°C in a water bath for 20 min. After incubation, a solution of BSA (1%) was added to the tube and centrifuged at 190 g for 2 min. The supernatant was discarded, leaving 500 μl of the pellet, which was resuspended in EDTA (2.4 mM) and 460 μl of TCM-199 with Hank's salts. The final solution (1 ml) was incubated at 37°C in a water bath for 3 min and then subjected to repeated pipetting (40 times) using a graduated pipette. The final solution was taken to an inverted microscope for oocytes search. The oocytes were washed several times until they were free of follicle cells and impurities. Only oocytes completely released from the follicles and having a normal appearance (no structural damage to the plasma membrane,

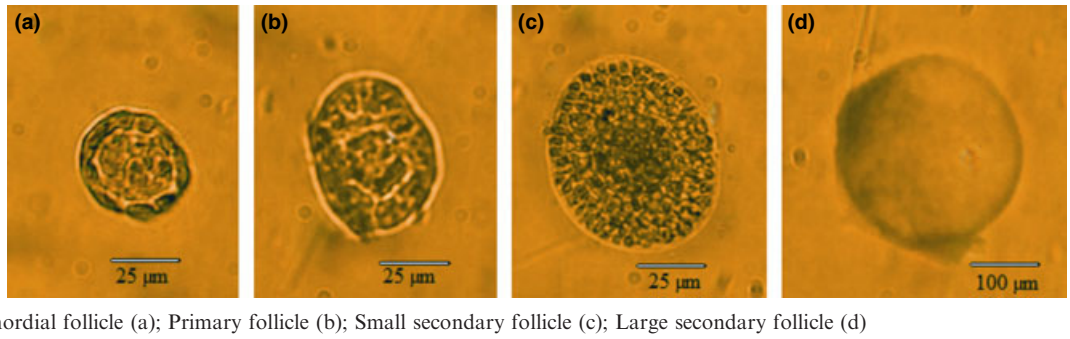

Fig. 1. Primordial follicle (a); Primary follicle (b); Small secondary follicle (c); Large secondary follicle (d)

pellucida zone and homogeneous cytoplasm) were used. The oocytes recovered were measured and separated by size into the various categories. To identify oocytes from antral follicles (Fig. 2) with different levels of competence, the diameter classification described by Caixeta et al. (2009) was used. Oocytes with a diameter of 100–120 μm were classified as oocytes less competent and derived from small antral follicles (1–3 mm), and oocytes with a diameter greater than 128 μm were classified as oocytes more competent and derived from large antral follicles (>6 mm). Oocytes from each group were then transferred to microtubes, 4 μl of RNA later was added, and the samples were stored at −20°C until RNA extraction.

RNA extraction and reverse transcription (RT)

Total RNA was isolated from four pools consisting of 25 oocytes for each category using the RNeasy Plus® kit (Qiagen, Mississauga, Ontario, CA, USA) and following the manufacturer's instructions. Total RNA was subjected to a reverse transcription reaction containing 1 μl of SuperScript III (200 U/μl; Invitrogen, Carlsbad, CA, USA), 1 μl oligo-dT primer (500 μg/ml), dNTPs (2.0 mM), 1 μl DTT (0.1 mM), 1 μl ribonuclease inhibitor recombinant RNaseOUT ® (40 U/μl; Invitrogen) and 1 × first strand buffer. Reactions were performed at 65°C for 5 min, 50°C for 60 min and, finally, 70°C for 15 min to inactivate the enzyme.

Real-Time PCR (qPCR)

Real-time PCR analysis was run in a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the Power SYBR Green PCR Master Mix (Applied Biosystems). Reactions were performed in a total volume of 25 μl using cDNA equivalent to 0.5 oocytes, and PCR cycling conditions were 95°C for 5 min following by 50 cycles of denaturing at 95°C for 15 s and then annealing at the temperatures given in Table 1 for 1 min. The primer sequences, fragment size and annealing temperature for each gene are listed in Table 1. Reactions were optimized to provide maximum amplification efficiency for each gene (>90%) that was calculated using relative standard curves in 7500 software 2.0.3 (Applied Biosystem). Each sample was analysed in duplicate, and the specificity of each PCR product was determined by melting curve analysis and amplicon size determination in agarose gels. Non-template controls were not amplified or showed 10 Cq higher than the average of Cq of the genes. The expression level of the target genes was normalized to the expression level of the constitutive gene peptidylprolyl isomerase A (cyclophilin A/*PPIA*), which was expressed at similar levels (Cq values) in the all oocyte samples and was stable under the conditions used. The relative expression of each gene was calculated using the ΔΔCt method with efficiency correction (Pfaffl 2001).

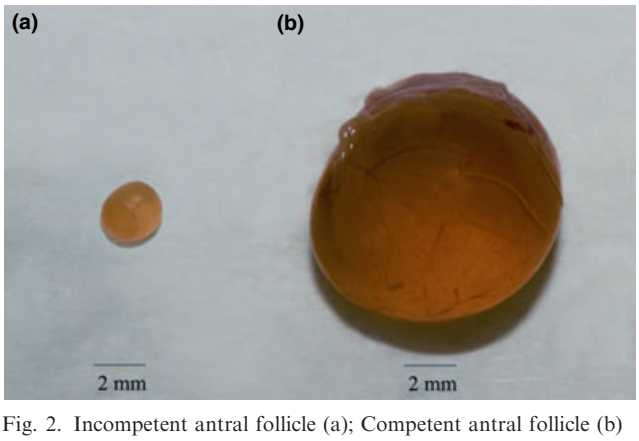

Fig. 2. Incompetent antral follicle (a); Competent antral follicle (b)

Statistical analysis

To compare the groups by size of oocytes, follicles and gene expression, we used the nonparametric Kruskal–Wallis test. All statistical analyses were performed using the program Prophet version 5.0 (BBN Technologies System, NIH, Bethesda, MD, USA, 1996).

Results

Because the oocytes from the different pre-antral follicles categories were collected according to their diameter size, and not follicle morphology, a preliminary experiment was performed. The aim of this experiment was to characterize the size of oocytes isolated from the different pre-antral follicle categories in Nellore females.

The size of the follicles and oocytes from each category is presented in Table 2. There were significant differences ($p < 0.01$) in the sizes of follicles and oocytes for all of the categories studied. The analyses of the relative amount of mRNA for the individual genes studied are depicted on Fig. 3. The relative abundance of the *OOSP1, BMP-15, H1FOO* and *SLBP* transcripts increased with the size of the oocyte, with the level of transcripts being greater in oocytes obtained from antral follicles than from pre-antral follicles. The oocytes obtained from the large secondary follicles had *BMP-15* and *H1FOO* gene expression levels that were intermediate to the expression levels found in oocytes from the pre-antral and antral follicle categories. Transcripts for the *OOSP1* gene only start to be detected in small secondary follicles (Fig. 3).

For the *H3A* and *H2A* genes, the relative abundance of their transcripts did not differ ($p > 0.05$) between the oocytes from various stages of growth, including the primary to final antral follicle stages. The *H3A* gene was the only gene in our study whose expression could be detected in oocytes derived from primordial follicles. The amount of *H4* gene mRNA was similar between many of the groups, and a difference was only detected between oocytes from the primary and small antral follicles (Fig. 3).

The expression level of *DNMT3B* was different between oocyte groups from the pre-antral and antral follicles, and the expression of *DNMT1* was similar between oocytes from the large secondary follicles and antral follicles (Fig. 3).

The transcripts for the enzyme *SUV39H1* can first be detected in oocytes from the small secondary follicle stage, and the expression level in this group is

Table 1. Gene names, sequence of primer, amplicon size in base pairs (bp), annealing temperature and accession number

Gene name	Gene symbol	Primer sequence (5′–3′)	Size (bp)	Temperature (°C)	GeneBank accession number
Peptidylprolyl isomerase A (cyclophilin A)	*PPIA*	F: GCCATGGAGCGCTTTGG R: CCACAGTCAGCAATGGTGATCT	65	60	BC105173
Histone H4	*H4*	F: TGCGCGACAATATCCAGGGTATCA R: AACCACGTCCATAGCGGTTACAGT	197	60	NM_173880.2
Histone H2A type 2-A-like	*H2A*	F: GAGGAGCTGAACAAGCTGTTG R: TTGTGGTGGCTCTCAGTCTTC	104	60	XM_003582302.1
H1 Histone family, member O, oocyte-specific	*H1FOO*	F:AGTCGAAGGTCAAAGAAAGAGGGAGC R: TGAACTCTGACTTCCAGGCTGTGT	80	60	NM_001035372.1
Histone H3.3-like	*H3A*	F: GTACAAAGCAGACTGCCCGCAAAT R: ACCAGGCCTGTAACGATGAGGTTT	128	60	XM_003583514.1
Stem-loop binding protein	*SLBP*	F: CAGTCTTGCCACAACTTCAATC R: ATGGAGCCGATTATGAGAACAC	208	53	AY630919.1
DNA (cytosine-5-) methyl transferase 1	*DNMT1*	F: TTGGCTTTAGCACCTCATTTGCCG R: TCCTGCATCACGCTGAATAGTGGT	82	57	AY173048.1
DNA (cytosine-5-)-methyl transferase 3 beta	*DNMT3B*	F: AGCAACCAGAGAATAAGACGCGGA R: AATTTGTCTTGAGGCGCTTGGGTG	90	57	NM_181813.2
Bone morphogenetic protein 15	*BMP15*	F: GTCAGCAGCCAAGAGGTAGTG R: CCCGAGGACATACTCCCTTAC	360	59	NM_001031752.1
Histone acetyltransferase 1	*HAT1*	F: AATTGAGAGACTTTGTGCTTGTGA R: TTCAATGACACGTCGATAATCTTC	392	60	NM_001034347.1
Supressor of variegation 3-9 homolog 1	*SUV39H1*	F: GGACTGAATCCTGCCGCAAATACCT R: GGCCATGAATCCCAACTGCAGAAAG	307	62	NM_001046264.1
Histone deacetylase 2	*HDAC2*	F: TTATTTGAAAATTTACGCATGTT R: TTGCTCCTTTCTTATGATCAGTC	229	56	NM_001075146.1
Oocyte-secreted protein 1	*OOSP1*	F: GCCAAGATTAAACCCACACTATTT R: ATAATGAGCATCTGGTGAAACGTA	182	60	NM_001038687.1

Table 2. Pre-antral follicle and oocyte diameters obtained from ovaries from Nellore (*Bos indicus*) females (mean ± SE)

Follicular classes	N	Follicle diameter (μm) (range)	Oocyte diameter (μm) (range)
Primordial	50	38.8 ± 0.64^{a} (29.5–53.0)	22.34 ± 0.45^{a} (16.2–30.0)
Primary	50	54.5 ± 0.89^{b} (43.7 – 72.2)	27.86 ± 0.37^{b} (23.7–33.3)
Small secondary	50	88.9 ± 2.55^{c} (53.1–125.1)	44.17 ± 1.37^{c} (28.8 – 63.3)
Large secondary	50	192.84 ± 6.71^{d} (129.8–334)	74.4 ± 1.28^{d} (61.1–93.8)

[a,b,c,d]Different letters indicate different values ($p < 0.01$).

statistically similar to the expression level in oocytes from antral follicles. Interestingly, the expression level of the transcript in oocytes obtained from large secondary follicles is similar only to the level in the small secondary follicle group (Fig. 3).

The expression level of the *HDAC2* gene in oocytes from primary follicles was similar to the level in oocytes from the other categories of follicles. A difference in expression was observed within the antral follicles category, in which there is a decrease in the expression level in large antral group. The relative abundance of the *HAT1* transcripts was similar among the primary, large secondary, small and large antral follicle groups. The oocytes from the small secondary follicles had a lower expression level than the oocytes from the large secondary and small antral follicle groups (Fig. 3).

Finally, to characterize the general pattern of expression during oocyte growth, a global analysis including all the genes used in this study in each treatment group was conducted (Fig. 4). The result shows that the pattern of expression increases with oocyte growth, which makes it possible to identify three distinct groups. These three groups are the initial pre-antral group (primordial, primary and small secondary), which has a low expression level, the large secondary group, which has an intermediate expression level, and the antral follicle group, which has the highest expression level compared with the other groups.

Discussion

In the present study, we analysed the expression profile of candidate genes responsible for determining developmental competence during oocyte growth using oocytes from the primordial to the antral follicle stages. This molecular characterization is important because it provides relevant information concerning the major changes that occur during oogenesis and are related to establishing competence during development.

To characterize the size of oocytes from each category of pre-antral follicles, a preliminary experiment was conducted. This experiment was necessary to ensure the accuracy of the samples. Previous studies (Braw-Tal and Yossefi 1997; Lucci et al. 2002; Kacinskis et al. 2005) have only reported measurements in histological sections and not for isolated oocytes. Histological evaluation involves sample manipulations, such as fixation and section preparation, that could result in changes in the oocyte shape, which could affect the size measurements. As expected, the oocytes increased in size as the follicle size increased. The mean diameter for both follicles and oocytes for each group was similar to the diameters reported in previous studies (Braw-Tal and Yossefi 1997; Lucci et al. 2002; Kacinskis et al. 2005). The only difference we found between our results and previously published results was that we found lower

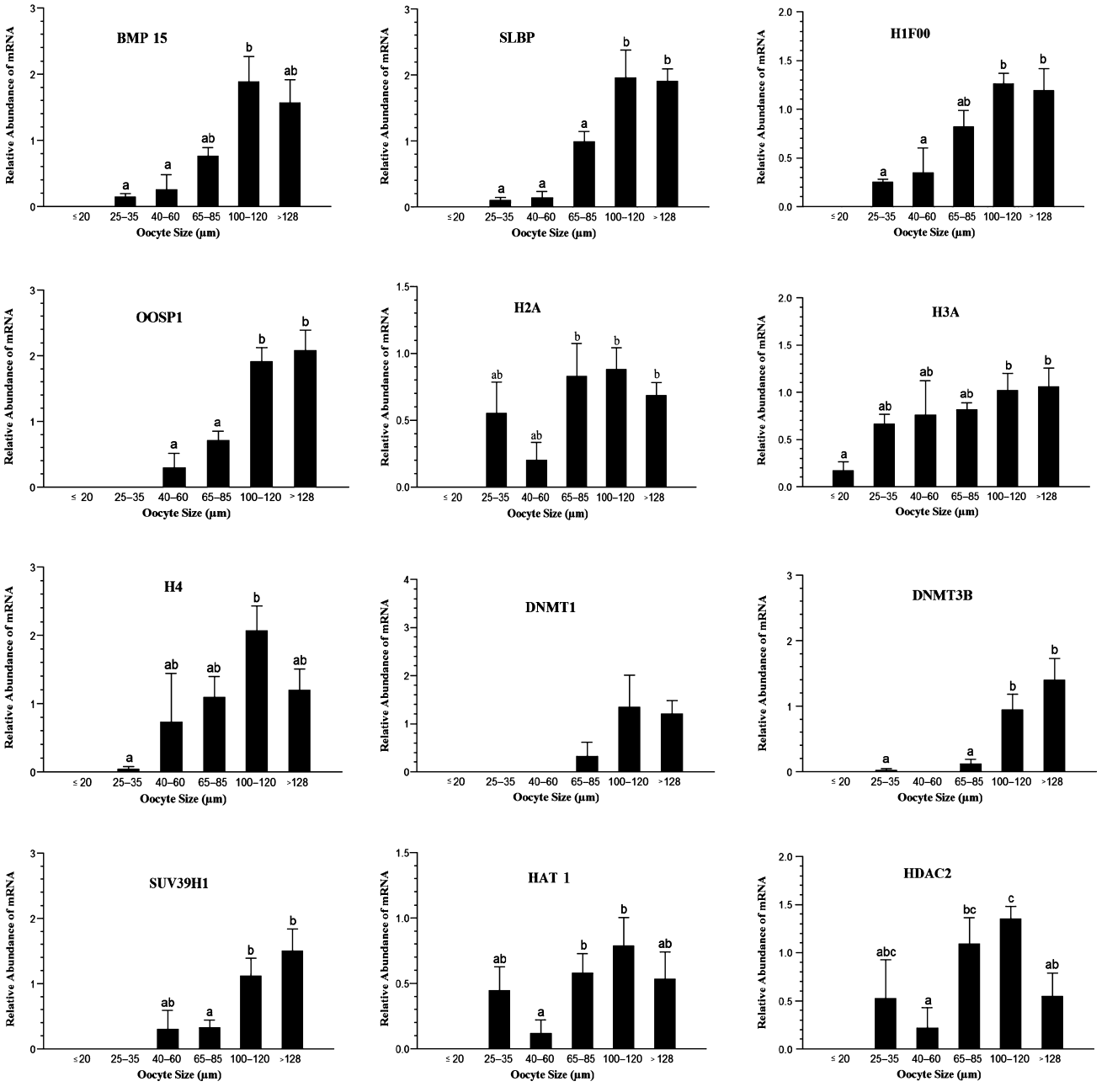

Fig. 3. Level of transcripts for the genes *BMP15, OOSP1, H1FOO, SLBP H2A*, *H3A*, *H4, DNMT1, DNMT3B, SUV39H1, HAT1* and *HDAC2* that were analysed by RT-PCR in immature oocytes obtained from primordial follicles (≤ 20 µm), primary follicles (25–35 µm), small secondary follicles (40–60 µm), large secondary follicles (65–85 µm), small antral follicles (100–120 µm) and large antral follicles (>128 µm) from *Bos indicus* females. Each group was analysed using four pools of different replicas. The data (mean ± SD) were normalized to the PPIA gene. [a,b]Different letters in the bars indicate different values ($p < 0.05$)

variation in the diameter within each oocyte and follicle category, which was probably a result of measuring isolated follicles versus histological sections.

The earliest detection of *OOSP1* gene expression occurred in oocytes from secondary follicles, and its expression level increased significantly in oocytes from antral follicles. In murine oocytes, the expression of *OOSP1* was detected much earlier, at the primary follicle stage, than in the present study and increased as the oocyte developed (Yan et al. 2001). Another oocyte-specific gene evaluated was *BMP15*, and similar to murine oocyte development (Sanchez et al. 2009), it was detected in oocytes from the primary follicle stage to the large antral stage. Analysing the transcript levels of these two genes in only oocytes from antral follicles, we observed that the expression of either *OOSP1* or *BMP15* was already established in the small antral follicles. These results are consistent with studies by Caixeta et al. (2009) and Mourot et al. (2006), who reported that the expression of these genes is similar in oocytes from antral follicles although they show different levels of competence. Similarly, Ghanem et al. (2007) evaluating transcript abundance of bovine oocytes of different developmental competence found

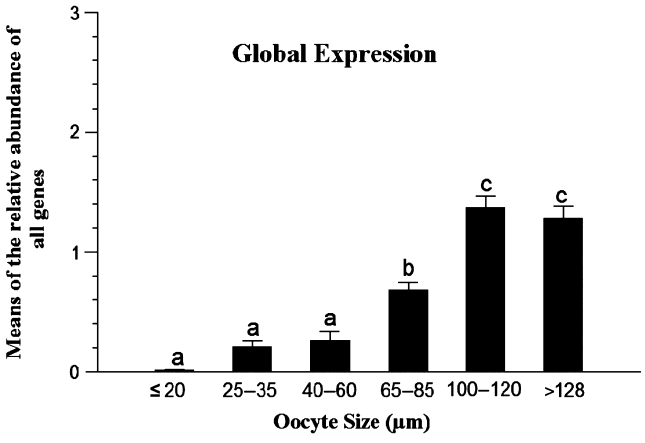

Fig. 4. Means of the relative abundance of all genes (*H1FOO, H2A, H3A, H4, SLBP, OOSP1, DNMT1, DNMT3B, HAT1, HDAC2, SUV39H1* and *BMP-15*) grouped by oocyte size

no difference in *BMP15* gene expression. Taken together, these results suggest that stocks of *OOSP1* and *BMP15* accumulate before oocytes begin to acquire competence in cattle.

In contrast to somatic cells, which have little or no capacity to store histone proteins and the level of histone mRNA is especially dependent on DNA replication, the oocyte can store histone mRNA and proteins during oogenesis (Marzluff and Duronio 2002; Sanchez and Marzluff 2004). Additionally, studies have reported that a relationship exists between the amount of histones and oocytes competence in bovine.

We determined the expression level of *H1FOO* gene, an oocyte-specific histone (McGraw et al. 2006) and *SBLP* gene, which plays a role in histone translation. Of all the histone genes evaluated, *H1FOO* demonstrated a more gradual increase in expression during oocyte growth. The relative abundance of the transcript, however, remained the same between oocytes from the small and large antral follicles. In murine oocytes, transcripts of *H1FOO* were detected in oocytes from primordial follicles, and the level of transcripts increased in primary and secondary follicles. The presence of the protein was also reported in human oocytes throughout oogenesis, including the primordial follicle stage (Mizusawa et al. 2010). Yet, we did not detect any expression of *H1FOO* in oocytes from primordial follicles. Unfortunately, it is not possible to determine a reason for this discrepancy at this because there have been very few studies of this type evaluating bovine oocytes.

The *H3A* gene was the only gene in which expression could be detected in oocytes derived from primordial follicles because the stock of *H3A* transcript is established by the primary follicle stage. Taking into consideration only the antral stages of development, these results are similar to the study by Caixeta et al. (2009), who reported that the amount of transcript was already established in the oocytes from small antral follicles. However, these findings differ from the results reported by Fair et al. (2004), in which high levels of *H3A* expression are related to competence. Future studies should determine why the detection and accumulation of the *H3A* transcript occur earlier than for any other histone transcript during oogenesis.

The level of mRNA for the *H4* and *H2A* genes stabilized very early in oogenesis, in the early secondary stage and primary follicle stage, respectively. Some studies found differences in *H2A* expression levels when evaluating competent and incompetent oocytes (Mourot et al. 2006; Caixeta et al. 2009). We, however, did not detect any differences in the expression level of this gene in oocytes from small and large antral follicles. Interestingly, the data published on *H2A* gene expression are very conflicting. Some studies report a difference in gene expression (Mourot et al. 2006; Caixeta et al. 2009), while other studies did not observe these differences. Some studies even used this gene as a constitutive gene to normalize data (Lonergan et al. 2003; Vigneault et al. 2007). Therefore, because a consensus on the relationship between the *H2A* expression level and acquisition of developmental competence (Donnison and Pfeffer 2004; Dode et al. 2006; Caixeta et al. 2009) has not been reached, more studies should be conducted to confirm its expression pattern.

Different from the expression profiles of the histone proteins, the transcript level for *SLBP* was higher in oocytes from antral follicles than those from pre-antral follicles. Taking into consideration that *SLBP* plays an important role in the accumulation and translation of histones (Allard et al. 2002), it was expected that its expression profile would follow the expression profiles of the histone proteins. This expected pattern, however, was not observed in the present study. It is possible that in the early stages of folliculogenesis, in which stocks of histones are formed, a higher activity level of *SLBP* is necessary. Then, when translation is occurring at a higher rate, it prevents the accumulation of an *SLBP* mRNA stock. Another factor to consider is that the mechanism for storing each histone may be different. This hypothesis is based on our results and a study using transgenic mice. In this study, transgenic mice were manipulated so that the amount of *SLBP* in oocytes changed, and this change affected the accumulation of histone *H3* and *H4* mRNA, but not histone *H2A* and *H2B* mRNA (Arnold et al. 2008).

Epigenetic phenomena such as DNA methylation and histone modifications play a crucial role in the reprogramming of the gametes during gametogenesis. These events are catalysed by various enzymes, such as *DNMTs, HMTs, HATs and HDACs*. Therefore, the expression of these enzymes may play an important role during oocyte growth and in determining competence. Our results show that transcripts for *DNMT3B*, which is responsible for *de novo* DNA methylation, were detected in oocytes from primary follicles, higher levels of transcript accumulated during the transition from the pre-antral to antral stage. Similar results were obtained in mice by Luccifero et al. (2007), who evaluated the expression of *DNMT3B* in oocytes from primordial follicles and observed an increase in its expression level in oocytes from antral follicles. The *DNMT1* expression pattern was similar to *DNMT3B*, however, no *DNMT1* transcript was detected in oocytes from primary follicles. Lodde et al. (2009) evaluated oocytes from pre-antral to antral follicles in cattle and observed the presence of *DNMT1* transcript in oocytes from all of these stages. Similarly, the presence of *DNMT*s – *DNMT3A, DNMT3B* and *DNMT3L* – is expressed in

parallel to the acquisition of maternal DNA methylation imprints during the period of oocyte growth when oocytes from 95 μm to >20 μm of diameter were studied (O'Doherty et al. 2012).

It is well known that in the germ cell line, the methylation pattern is erased at the beginning of development and is reestablished during gametogenesis. Therefore, the low level of transcripts observed for the *DNMT*s genes in the pre-antral stage may be due to the high rate of translation that is necessary for methylation to occur. And only in the antral stage, when the methylation pattern is reestablished, can a greater accumulation of these transcripts occur. Another hypothesis is that the methylation pattern in oocytes is established later in oogenesis then we originally believed and is only completed in oocytes in large antral follicles. This timeline for establishing the methylation pattern would explain why the expression of the *DNMT*s genes occurs later in oocyte development. This hypothesis is supported by the results from our laboratory that showed that the methylation pattern of the intragenic DMR of the *IGF2* gene in the bovine oocytes is only completed at the end of oogenesis, just before the initiation of oocyte maturation in oocytes obtained from $\geq$8.0 mm diameter follicles (Fagundes et al. 2011).

Regarding the genes involved in histone modifications, we detected mRNA for the *HAT1* gene in oocytes from primary follicles. The level of expression for this gene is maintained until the antral follicle stage, and its expression level varies among oocytes from the early secondary follicle and small antral follicle stages. The expression profile for *HDAC2* is similar to the expression profile for *HAT1*, except for the expression level at the large antral stage, in which we detect a decrease in *HDAC2* expression. Furthermore, Endo et al. (2008) suggested that *HAT1* and *HDAC2* proteins may also have similar expression patterns.

When a histone is acetylated, the DNA is usually more accessible to transcription factors (Segev et al. 2001). Therefore, it was expected that during oocyte growth, in which there is a high level of transcriptional activity, histones are being acetylated and would require *HAT1* activity. Acetylation is a reversible process, and histone deacetylase (HDAC) is responsible for this reversal and usually results in transcriptional repression (Ma and Schultz 2008). Therefore, the level of histone acetylation is controlled by a balance between the activity of HAT and HDAC, which explains the similar expression profile for these two enzymes. As the oocyte establishes competence and resumes meiosis, histones must be deacetylated to repress transcription. This repression is caused by an increase in HDAC activity. The oocytes from larger antral follicles are closer to ovulation and are more competent than those from smaller follicles. Therefore, a reduction in the quantity of the *HDAC2* transcript observed in this follicle category may be a result of an increase in the translation rate. This reduction would lead to an increase in enzyme activity, which is necessary to end transcription and to ensure the oocyte is prepared to resume meiosis. *HDAC2* is the only gene in this study that demonstrated a relationship between change of expression and competence. The relationship between status of acetylation and oocyte quality has also been suggested by others in human and mice oocytes (van den Berg et al. 2011; Ding et al. 2012; Huang et al. 2012).

The methylation of *H3*/K9 by histone methyltransferase *SUV39H1* is related to transcription repression. In this study, the expression of *SUV39H1* in bovine oocytes begins at the small secondary follicle stage, and a significant increase in transcript level was observed between oocytes from large secondary follicles and antral follicles. These results are inconsistent with the results reported by Racedo et al. (2009), in which the amount of mRNA was significantly higher in bovine oocytes from 2- to 8-mm follicles than from follicles smaller than 2 mm. In our study, the oocytes were specifically defined, from 1- to 3-mm follicles and follicles larger than 6 mm. Additionally, in the study by Racedo et al. (2009), the oocytes were obtained from aspirated and not isolated follicles, which may lead to errors because the size of follicle on the ovarian surface may not represent its actual size.

In an attempt to get a general overview of the gene expression profile during oocyte growth, an analysis using the expression of all of the genes in each group of oocytes was performed. This analysis showed that the total amount of transcripts increased as the oocyte developed. This analysis allowed us to distinguish three distinct developmental groups, an initial pre-antral group (primordial, primary and small secondary), an intermediate group, (large secondary follicles) and an antral group (small and large antral stages). This analysis also shows that mRNA stocks form before oocyte acquire competence. This model is supported by the results obtained for the majority of genes studied, the exception being the histone transcripts, which have a stable expression level by the pre-antral stage. Although only a few set of genes involved in the oocyte developmental competence have been evaluated in the present study, pattern of these genes could be used to select oocytes with better quality. In fact, in this case, in which oocytes with higher mRNA stocks are more competent than those with lower socks, the global expression could a more informative marker than the expression of a single gene. Attempts to use the global gene expression as a marker have been reported elsewhere (Mundim et al. 2009; Siqueira et al. 2011) however, more studies using higher number of genes are needed to establish the best way to use that information.

In conclusion, for the twelve genes analysed, all transcripts accumulate before competence is established, and the *HDAC2* gene was the only gene that showed a differential expression at the stage associated with competence acquisition. However, to obtain a complete gene expression profile and to better understand the mechanisms for regulating transcription and forming the mRNA stocks during oogenesis, more studies are needed that would evaluate the expression of proteins encoded by the genes in this study.

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Conflicts of interests

None of the authors have any conflict of interest to declare.

Author contributions

Isabela Rebouças Bessa performed all the experiments determining oocyte size, collected all the oocytes for gene expression analysis, drafting the article.

Rosana Camargo Nishimura performed all genes expression analysis. Maurício Machaim Franco substantial contributed to the experiment design, statistical analysis and interpretation of the data, critical discussion and critical review of the article.

Margot Alves Nunes Dode conceived the study, design the experiments, interpretation of the data, secured funding for research, drafting and critical review of the manuscript.

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