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ORIGINAL RESEARCH

# Methylation status in the intragenic differentially methylated region of the *IGF2* locus in *Bos taurus indicus* oocytes with different developmental competencies

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**ABSTRACT:** Oocyte quality is one of the most important aspects of *in vitro* embryo development. Extensive epigenetic programming must occur during oocyte growth and maturation. A specific DNA methylation pattern of the imprinted genes must be established on differentially methylated regions (DMR). The insulin-like growth factor 2 (*IGF2*) gene is an important growth factor, and it is imprinted in several mammalian species. The aim of this study was to evaluate the methylation pattern on the DMR of the last exon of *IGF2* in immature and mature bovine oocytes with different developmental competencies. Mature oocytes from large follicles were less methylated (28.93%) than immature oocytes from large follicles (77.38% $P = 0.002$), and there was also a tendency towards lower methylation in mature oocytes from large follicles (28.93%) compared with mature oocytes from small follicles (52.58% $P = 0.07$). Immature oocytes from small and large follicles showed 53.85% (7/13) and 91.66% (11/12) hypermethylated sequences, respectively, whereas mature oocytes from small and large follicles showed 61.11% (11/18) and 40% (4/10), respectively. The hypomethylation pattern in mature oocytes from large follicles may be related to the higher competence of these oocytes. Our results suggest that the methylation pattern in this DMR may be a useful parameter to investigate as a molecular marker for oocyte competence in cattle and as a model for studies in other species.

**Key words:** imprinting / methylation / oocyte competence / bovine oocytes / insulin-growth factor 2

## Introduction

Epigenetics refers to changes in gene function that do not depend on changes in the primary sequence of DNA (Singal and Ginder, 1999). It can be reversible (Rush and Plass, 2002) and heritable in the genome during cell division (Feinberg, 2004). Its regulation is characterized by several modifications, such as DNA methylation and post-translational changes in histone proteins, which include methylation, acetylation, phosphorylation, glycosylation, ubiquitination, sumoylation, ADP ribosylation and nitrilation (Rush and Plass, 2002; Lebedev and Sazhenova, 2008). Additionally, these modifications may be associated with antisense transcripts and non-coding RNA, including microRNA (Spahm and Barlow, 2003), that control gene expression.

DNA methylation occurs in regions of the genome that are generally rich in CpG dinucleotides (Li, 2002; Jaenisch and Bird, 2003). Cytosine methylation (5 mC) normally occurs on a cytosine that is 5′ of a guanine (5′CpG3′; Singal and Ginder, 1999), although it may occur in non-CpG sites (Imamura *et al.*, 2005).

In mammals, correct DNA methylation reprogramming is essential to gametogenesis and normal preimplantation embryonic development, involving demethylation and *de novo* methylation processes (Newell-Price *et al.*, 2000).

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At the beginning of the 1980s, experiments showed that genetic material could be differentially expressed, depending on parental origin (Reik and Walter, 2001). This phenomenon was named genomic imprinting, meaning that the mono-allelic expression of some genes was dependent on the parental origin of the chromosome (Bartolomei et al., 1991; DeChiara et al., 1991; Feil et al., 1994; Manipalviratn et al., 2009). Allele-specific methylation is involved in imprinted gene regulation, specifically, in the differentially methylated region (DMR) of DNA, and is established during primordial germ cell differentiation in gametogenesis (Murrell et al., 2001; Reik and Walter, 2001).

Genome-wide demethylation in mouse primordial germ cells occurs between E10.5 and E13.5. At this point, these cells enter the gonad, and the majority of DMRs is demethylated, which is coincident with genome arrest in meiosis I. In the female germ line, the *de novo* methylation process starts after birth during the oocyte growth phase. Because this is a long period in the oocyte's lifespan, it is likely that the methylation of different sequences in the genome occurs at different time-points (Reik et al., 2001).

Removal, stabilization and maintenance of the imprinting pattern are dynamic processes that must be correctly reprogrammed in each reproductive cycle to ensure the formation of gametes that are able to support normal development after fertilization. It has been well established that only competent oocytes are able to undergo complete maturation *in vitro* to develop into the blastocyst stage and culminate in a full-term pregnancy. However, it is not known whether the correct establishment and maintenance of the imprinted genes' methylation patterns are associated with the acquisition of competence, and subsequently, oocyte quality.

In *in vitro* embryo development, morphological evaluation based on the number and compaction of cumulus cells and cytoplasmic homogeneity (de Wit et al., 2000; Lonergan et al., 2003) has been routinely used as a selection criterion for oocyte quality. However, such evaluation alone is insufficient to identify competent oocytes (Lonergan et al., 2003; Krisher, 2004).

Studies in our laboratory using oocytes from *Bos taurus indicus* females (Caixeta et al., 2009) have corroborated results obtained by others (Lonergan et al., 1994; Racedo et al., 2008) regarding the relationship between follicle size and oocyte developmental competence. Therefore, this follicle size model is a useful tool for the comparison of oocytes with different levels of competence.

The insulin-like growth factor 2 (*IGF2*) gene regulates cell growth and differentiation in many species (Chao and D'Amore, 2008). It is located on chromosome 29 (29qter) in the bovine genome (Goodall and Schmutz, 2003). It is a potent mitogen that is involved in placental (Constancia et al., 2002; Chao and D'amore, 2008) and fetal development (DeChiara et al., 1990; Curchoe et al., 2005). *IGF2* is predominantly expressed by the paternal allele while the maternal allele is silenced, and several DMRs have been identified in this gene (Feil et al., 1994).

A different methylation pattern was identified between spermatozoa and oocytes within a region of the last exon of the bovine and porcine *IGF2* gene, suggesting the presence of an intragenic DMR, which has been shown to be more highly methylated in spermatozoa than in oocytes (Gebert et al., 2006; Han et al., 2008). This methylated DMR is involved in the initiation of *IGF2* transcription, contributing to a high rate of gene transcription (Murrell et al., 2001).

Regarding the control of *IGF2* expression, a DMR that contains 'CTCF-binding' sites is located in the *IGF2/H19* locus upstream of the *H19* promoter, and CTCF-binding proteins regulate transcription of both genes. *IGF2* is expressed in the paternal chromosome with a methylated DMR and *H19* is expressed in the maternal chromosome with an unmethylated DMR (Chao and D'Amore, 2008). Another model of *IGF2/H19* locus regulation, called the chromatin loop, has been proposed (Murrell et al., 2004) to explain the interactions among DMRs in this locus.

The aim of this study was to evaluate the methylation pattern in the DMR of the last exon of the *IGF2* gene in immature and mature bovine oocytes with different developmental competencies.

# Materials and Methods

## Oocyte recovery and *in vitro* maturation

Ovaries from Nellore (*B. taurus indicus*) cows were collected immediately after slaughter and transported to the laboratory in a saline solution (0.9% NaCl) supplemented with penicillin G (100 IU/ml) and streptomycin sulphate (100 μg/ml) at 35–37°C.

Follicles were dissected from the ovarian cortex at room temperature using scissors, scalpels and tweezers. During dissection, follicles were kept in phosphate-buffered saline (PBS) with 10% fetal calf serum (FCS; Gibco®) at 36°C. Follicles were measured using a stereomicroscope with a graduated eyepiece (OSM-4; Olympus, Tokyo, Japan) and were then classified morphologically as small or large follicles according to their diameter, as described by Caixeta et al. (2009). Follicles with a diameter of 1–3 mm were classified as small and represented less competent oocytes, and those with a diameter ≥8.0 mm were considered large and represented more competent oocytes (Caixeta et al., 2009). Cumulus–oocyte complexes (COCs) were released by rupturing the follicles. Only COCs with a homogeneous granulated cytoplasm and at least five layers of compact cumulus cells were considered viable and used in the present study. For each follicle size group, immature COCs were either denuded by repeated pipetting in PBS and frozen at −80°C until DNA extraction or submitted to *in vitro* maturation.

COCs selected from each category of follicles were washed and transferred to 50 μl (≤10 oocytes) of maturation medium, covered with silicone oil and incubated for 22–24 h at 39°C in 5% $CO_2$. The maturation medium consisted of tissue culture medium-199 (Invitrogen®, CA, USA) supplemented with 10% FCS (Gibco®), 12 IU/ml LH (Sigma®), 0.1 IU/ml FSH (Sigma®), 0.1 mg/ml L-glutamine (Sigma®) and an antibiotic (amicacyn, 0.075 mg/ml). We used the same batch of FCS (Gibco®; Lot. no. 210.030K) in all replicates.

Following the maturation period, COCs were incubated with 0.2% hyaluronidase for 10 min and then denuded by repeated pipetting. Only oocytes that had extruded their first polar body were considered mature and used in the experiments. Immature and mature oocytes were obtained from both small and large follicles. Two pools of 60 oocytes for each experimental group (immature small, mature small, immature large and mature large follicles), except for one pool of mature small that had 44 oocytes, were frozen at −80°C until DNA extraction.

## DNA isolation

Genomic DNA was isolated from each category of oocytes as described by Melo et al. (2005), with minor modifications. Briefly, Pronase E (10 mg/ml; Sigma®) was added to tubes containing pooled oocytes to a total volume of 30 μl, and the tubes were then incubated at 37°C for

45 min. Following incubation, the samples were covered with a drop of mineral oil and the enzyme was inactivated at 85°C for 15 min. To extract the DNA, the samples were frozen in liquid nitrogen and immediately heated to 95°C for 1 min. This step was repeated five times. Finally, the samples were stored at −20°C until sodium bisulphite treatment.

## Sodium bisulphite treatment

DNA treatment with sodium bisulphite was performed using the EZ DNA methylation kit (Zymo Research) according to the manufacturer's protocol, except that the conversion temperature was changed to 55°C. The samples were diluted in 10 μl of distilled water and maintained at −80°C until PCR amplification.

## PCR amplification, cloning and bisulphite sequencing

Nested PCR using bisulphite-treated DNA was performed using the primers listed in Table I. PCR was performed in a total volume of 20 μl using 1× Taq buffer, 2.0 mM $MgCl_2$, 100 μM of each dNTP, 0.5 μM of each primer, 1 U Platinum Taq polymerase (Invitrogen) and 3 μl template DNA for the first round and 1 μl template DNA for the second round. Both rounds of nested PCR were performed with an initial denaturing step at 94°C for 3 min followed by 45 cycles at 94°C for 40 s, 45°C (first round) or 40°C (second round) for 1 min and 72°C for 1 min, with a final extension at 72°C for 15 min. The PCR products were recovered from an agarose gel and purified using the GenClean III kit (MP Biomedicals, LLC) according to the manufacturer's protocol. Then, the purified products were cloned into the pGEMT-Easy vector (Promega) and transformed into *Escherichia coli* (XL-1 Blue). The resulting individual clones were sequenced using a dideoxy fluorescence terminator system (ABI 3130xl). Only sequences originating from clones with ≥95% cytosine conversion were used. At least 10 clones from each treatment were sequenced from two independent sets of DNA modification, amplification, cloning and sequencing. Methylation patterns of non-CpG cytosines were used to identify clones from different DNA templates. The sequences were analysed using CHROMAS software and DNAMAN 4.0 software and GenBank accession no. X53553 as a reference.

## Statistical analysis

Differences in the percentage of viable COCs and the maturation rate between follicle groups were analysed using the $\chi^2$ test. To compare the methylation pattern among groups of oocytes, we calculated the total percentage of methylated CpGs in each group. We also compared the number of hypermethylated (≥50% of CpG sites on a given methylated strand) and hypomethylated (<50% of CpG sites on a given methylated strand) clones in each group, as cited by Imamura *et al.* (2005). The experiment was repeated twice. The methylation pattern was compared using the *t*-test or the Mann–Whitney test for data showing normality or not, respectively. All data were compared using the Prophet Program, version 5.0 (BBN Systems and Technologies, 1996), and are shown as the mean ± SEM.

# Results

A total of 2249 follicles of 1–3 mm and 1118 follicles of ≥8.0 mm were dissected. The results showed that follicles ≥8.0 mm presented a higher percentage of viable COCs (40.5%) than those follicles that measured from 1 to 3 mm (22%; $P < 0.05$).

Immature COCs were selected from the 1–3-mm ($n = 375$) and ≥8.0-mm ($n = 333$) follicles and submitted to *in vitro* maturation. The presence of the first polar body was used to characterize the success of the nuclear maturation process. COCs from ≥8.0-mm follicles presented a higher maturation rate (60.6%) than those from the 1–3-mm follicles (52.5%; $P < 0.01$). As a control of the system, 22 protocols for *in vitro* embryo production were performed. A total of 3127 oocytes were used for these protocols with cleavage and D7 blastocyst rates of 83% ($n = 2588$) and 40.2% ($n = 1256$), respectively.

The methylation pattern of the intragenic *IGF2* DMR in the oocytes is shown in Fig. 1A–E. In all of the clones we analysed, 28 CpG sites were identified. On the basis of the methylation pattern of each of the clones, we found that at least 10 alleles were different for each oocyte group.

No significant differences in DNA methylation were found between immature oocytes from small follicles (51.10 ± 10.75%; Fig. 1A) and those from large follicles (77.38 ± 6.76%; Fig. 1B; $P = 0.152$).

DNA methylation was higher in immature oocytes from large follicles than in those that were mature (77.38 ± 6.76 and 28.93 ± 10.26%, respectively; $P = 0.00201$; Fig. 1B and D). When comparing only mature oocytes, the DNA of oocytes from small follicles displayed a tendency toward greater methylation than those from large follicles (52.58 ± 8.17 and 28.93 ± 10.26%, respectively; $P = 0.0717$; Fig. 1C and D).

We also compared hypermethylated and hypomethylated sequences (Imamura *et al.*, 2005) among the four groups of oocytes. Immature oocytes from small and large follicles had 53.85% (7/13) and 91.66% (11/12) hypermethylated sequences, respectively, and mature oocytes had 61.11% (11/18) and 40% (4/10), respectively.

**Table I Primers used for bisulphite sequencing.**

| Gene | Primers (5′–3′)[a] | Accession no. | Amplicon size |
|---|---|---|---|
| | | X53553.1 | |
| *IGF2* out | Foward:TGGGTAAGTTTTTTTAATATGATATT<br>Reverse:TTTAAAACCAATTAATTTTATACATT | F:243–268<br>R:672–697 | 455 bp |
| | | X53553.1 | |
| *IGF2* inner | Foward:TAATATGATATTTGGAAGTAGT<br>Reverse:ACATTTTTAAAAATATTATTCT | F:257–278<br>R:655–676 | 420 bp |

[a]Gebert *et al.* (2006).

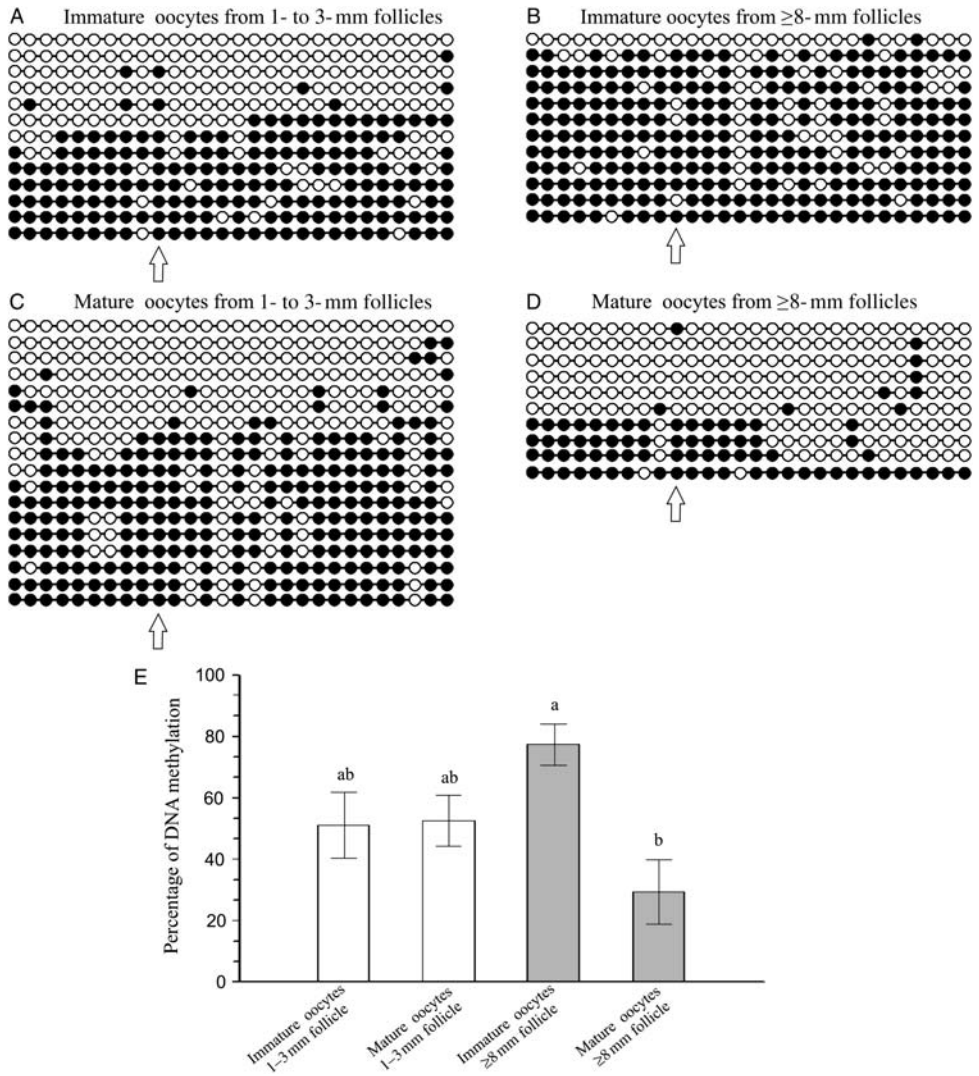


**Figure 1** Methylation pattern of the intragenic *IGF2* DMR in oocytes from different follicles. (**A**) Immature oocytes from 1- to 3-mm follicles. (**B**) Immature oocytes from ≥8.0-mm follicles. (**C**) Mature oocytes from 1- to 3-mm follicles. (**D**) Mature oocytes from ≥8.0-mm follicles. Each line represents one individual clone and each circle represents one CpG site (28 CpGs). Open circles represent unmethylated CpGs and black circles represent methylated CpGs. The arrow indicates the additional CpG site in this study compared with that of Gebert *et al.* (2006). (**E**) DNA methylation percentage (mean ± SEM; different letters represent statistically different means; $^{ab}P = 0.00201$).

# Discussion

Our results show that large follicles present a higher percentage of viable COCs than small follicles (40.5 versus 22% $P < 0.05$). These findings are in agreement with Ramos *et al.* (2006), whose study reported a higher quantity of Grade I COCs from larger follicles. It is important to point out that the percentage of viable COCs is not dependent on the efficiency of the *in vitro* embryo production system but is dependent on other factors, such as the physiological status of the oocyte donor animals. The low rate of good quality COCs from small follicles is probably due to their higher incidence of follicular atresia compared with large follicles. Indeed, at the moment of follicular aspiration and independent of the estral cycle phase, more than 85% of ovarian follicles are atretic (Castilho *et al.*, 2007).

After *in vitro* maturation, we showed that COCs from large follicles presented a higher maturation rate (60.6%) than those from small follicles (52.5% $P < 0.01$). However, nuclear maturation was considerably lower for both groups when compared with results usually obtained in our lab (83 and 40.2% for cleavage and blastocyst rates, respectively). Because we wanted to ensure collection of only the oocytes that had reached metaphase II without spending too much time on selecting oocytes for freezing, it is possible that we missed some oocytes that presented polar bodies that were not as evident as others. In this study, we considered mature oocytes to be only those that presented the first polar body. According to Edwards *et al.* (2005), the maturation rate was 83.9% when evaluated by staining the oocytes and 66% when evaluated using the presence of the polar body which is in line with our results in this study.

Follicular size and quality affect the capacity of *in vitro* development of bovine oocytes (Lonergan *et al.*, 1994; Sirard and Blodin, 1996), with COCs from larger follicles being more competent to undergo maturation *in vitro* than those from smaller follicles (Pavlok *et al.*, 1992; Yang *et al.*, 1998; Racedo *et al.*, 2008). The lower developmental capacity of the oocytes from small follicles may be due to intrinsic factors related to nuclear and/or cytoplasmic incompetence (Yang *et al.*, 1998). Maturational competence is progressively acquired during follicular growth through a range of cellular and molecular attributes that provide the oocyte with the ability to complete meiosis, be fertilized and undergo embryonic development. Therefore, reaching metaphase II of meiosis is only one aspect of maturation and does not necessarily signal oocyte competence (Crozet *et al.*, 1995; Dode *et al.*, 2000). The results from our laboratory have shown that oocytes from smaller follicles (1–3 mm) have a smaller diameter and that oocyte size did not change after the follicles had reached 3 mm in diameter. The group of oocytes from small follicles of 1–3 mm in diameter was also found to have lower cleavage and blastocyst rates (57 and 19%) than oocytes from large follicles (93 and 64%), probably because they had not completed their growth phase (Caixeta *et al.*, 2009). In addition, oocytes from follicles >6.0 mm in diameter were similar in size but had higher competence than those from follicles 3.0–6.0 mm in diameter (Caixeta *et al.*, 2009). These results confirm that factors other than full oocyte growth contribute to oocyte competence and that follicle size is a good model for oocyte competence. On the basis of our previous work we used oocytes from follicles of 1–3 mm and ≥8.0 mm diameters in the present study, representing oocytes with different developmental capacities.

During oogenesis and folliculogenesis, epigenetic and imprinting patterns are erased and re-established. It is possible that this reprogramming differs among the groups of oocytes we have used. To evaluate if the acquisition of competence is somehow related to the methylation patterns in oocytes, we have chosen to study the intragenic DMR (Gebert *et al.*, 2006) of the *IGF2* gene. Previous studies have shown that this DMR is highly methylated in spermatozoa and partially methylated in oocytes (Lopes *et al.*, 2003; Gebert *et al.*, 2006; Shim *et al.*, 2008; Han *et al.*, 2008; Gebert *et al.*, 2009).

After DNA sequencing, we identified 28 CpG sites in each of the clones we evaluated, which is in contrast to a report by Gebert *et al.* (2006) and GenBank accession no. X53553, which both only show 27. However, our result is similar to GenBank accession no. NM_174087.3, which also has 28 CpG sites. The difference in the number of CpG sites found between our study and the study of Gebert *et al.* (2006) may be due to the presence of a single nucleotide polymorphism. We speculate that this difference occurred because each study used a distinct *B. taurus* subspecies, although Gebert *et al.* (2006) did not mention the subspecies used.

No differences in the methylation pattern were observed between immature oocytes from small follicles and immature oocytes from large follicles (Fig. 1A and B; $P = 0.152$). Comparison of methylation pattern between metaphase II oocytes from small (52.58 ± 8.17%) and large (28.93 ± 10.26%) follicles showed a tendency for lower methylation in large follicles ($P = 0.0717$; Fig. 1C and D). When we compared immature and mature oocytes in each group of follicles, we observed a notable difference in changes in the methylation pattern during the maturation period. More competent oocytes were demethylated during maturation and were hypomethylated when they reached metaphase II (28.93 ± 10.26%) in comparison with immature ones (77.38 ± 6.76%; $P = 0.00201$; Fig. 1B and D). However, the less competent oocytes had no obvious changes in methylation status. Therefore, we speculate that this behaviour is due to the *de novo* methylation process that occurs at the end of oogenesis. Oocytes from large follicles have already reached full size and are more competent than oocytes from small follicles (Caixeta *et al.*, 2009). Therefore, we believe that the genomes of those immature but competent oocytes are already more methylated, even in this DMR, which is normally expected to be only partially methylated in the completely mature female gamete (Gebert *et al.*, 2006, 2009). It is possible that there is global and non-specific *de novo* methylation in the genome followed by a demethylation process in this DMR before fertilization, which would occur during the maturation process. Our hypothesis is supported by Imamura *et al.* (2005), who cited demethylation activity immediately before fertilization.

Taken together, our results suggest that there is a *de novo* methylation process in this DMR during oogenesis before the initiation of oocyte maturation. Afterwards, during *in vitro* maturation and only in competent oocytes, methylation is diminished in regions that are expected to be unmethylated or hypomethylated in female gametes. Therefore, imprinted patterns may be completely established during the maturation process. This is in agreement with the results reported by Tada *et al.* (2000) that during oogenesis, in fully grown oocytes, the genome receives an epigenetic mark on the X chromosome to avoid the inactivation of the maternal X chromosome after fertilization. Moreover, this statement is also supported by El-Maarri *et al.* (2001), who commented that imprinted marks may be completely established after fertilization, and by other studies (Gebert *et al.*, 2009; Park *et al.*, 2009) that have shown alterations in DMR methylation patterns during preimplantation development in bovines and swine.

Results on the percentage of hypermethylated sequences corroborate the methylation pattern data mentioned at the beginning of this discussion. Immature oocytes from small follicles presented less hypermethylated clones (53.85%; 7/13) than immature oocytes from large follicles (91.66%; 11/12). Mature oocytes from large follicles had the least hypermethylated clones (40%; 4/10), consistent with the expected hypomethylation pattern of this region in the female gamete (Gebert *et al.*, 2006, 2009).

*IGF2* is an imprinted gene, with the paternal allele being transcriptionally active. The DMR studied is expected to be highly methylated on the paternal allele to initiate the transcription process and promote high levels of gene expression (Murrell *et al.*, 2001). In mature oocytes from large follicles, methylation was 28.93% (Fig. 1D), which was slightly higher than the 16% reported in bovines (Gebert *et al.*, 2006) and the 10.1% (Park *et al.*, 2009) and 19.3% (Han *et al.*, 2008) reported in swine. Regarding hypermethylated sequences, we identified 40% (4/10), while Gebert *et al.* (2006) showed 22.22% (4/18). Therefore, we are convinced that this DMR is expected to be partially methylated in female gametes, as cited by Gebert *et al.* (2009). We believe that the differences between our results and those of Gebert *et al.* (2006) may be due to the origin of the oocytes. The aforementioned authors used oocytes from variously sized follicles that were obtained by slicing the ovaries, not from dissected and measured follicles that ensured the recovery of oocytes

with different levels of competence, as was done in the present study. Furthermore, we speculate that the differences mentioned above occurred because the composition of the maturation medium used in each study was different and each study used a different *B. taurus* subspecies. The medium composition is known to affect DNA methylation patterns and gene expression profiles (Wrenzycki *et al.*, 1999; Khosla, *et al.*, 2001; Young *et al.*, 2001; Rinaudo and Schultz, 2004). Another important aspect to be considered is that in both studies, the presence of a first polar body was used as a parameter of maturation. The presence of the polar body is a marker only of nuclear maturation, not of cytoplasmic maturation. In our study, we used only COCs with homogeneous granulated cytoplasm and at least five layers of compact cumulus cells to mature while Gebert *et al.* (2006) placed all COCs to mature. We believe that these parameters may be related to oocyte quality and possibly to DNA methylation patterns.

Finally, changes in the methylation pattern of the *IGF2* DMR during *in vitro* maturation were different between incompetent and competent oocytes, and this characteristic may be useful as a molecular marker in studies of oocyte competence, potentially contributing to an improvement of *in vitro* embryo production.

## Authors' roles

All authors had substantial contributions to this paper. N.S.F., F.C.R.: DNA isolation, PCR amplification, cloning and bisulphite sequencing. V.A.M.-L.: DNA isolation, sodium bisulphite treatment, PCR amplification, cloning and bisulphite sequencing. E.S.C.: oocyte recovery and *in vitro* maturation. G.M.M.: oocyte recovery and *in vitro* maturation. E.O.M., M.A.N.D.: design of experiment. M.M.F.: design of experiment and analysis and interpretation of data.

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