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GONADAL PHYSIOLOGY

Characterisation of the methylation pattern in the intragenic CpG island of the IGF2 gene in *Bos taurus indicus* cumulus cells during in vitro maturation

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Abstract
Purpose The aim of this study was to characterise the methylation pattern in a CpG island of the IGF2 gene in cumulus cells from 1–3 mm and ≥8.0 mm follicles and to evaluate the effects of in vitro maturation on this pattern.
Methods Genomic DNA was treatment with sodium bisulphite. Nested PCR using bisulphite-treated DNA was performed, and DNA methylation patterns have been characterised.
Results There were no differences in the methylation pattern among groups (P>0.05). Cells of pre-IVM and post-IVM from small follicles showed methylation levels of 78.17±14.11 % and 82.93±5.86 %, respectively, and those from large follicles showed methylation levels of 81.81±10.40 % and 79.64± 13.04 %, respectively. Evaluating only the effect of in vitro maturation, cells of pre-IVM and post-IVM COCs showed methylation levels of 80.17±12.01 % and 81.19±10.15 %.
Conclusions In conclusion, the methylation levels of the cumulus cells of all groups were higher than that expected from the imprinted pattern of somatic cells. As the cumulus cells from the pre-IVM follicles were not subjected to any in vitro manipulation, the hypermethylated pattern that was observed may be the actual physiological methylation pattern for this particular locus in these cells. Due the importance of DNA methylation in oogenesis, and to be a non-invasive method for determining oocyte quality, the identification of new epigenetic markers in cumulus cells has great potential to be used to support reproductive biotechniques in humans and other mammals.

Keywords Bovine · Cumulus cells · IGF2 · Epigenetics · DNA methylation · In vitro maturation

Capsule DNA methylation pattern in the IGF2 gene of cumulus cells was not affected by the in vitro maturation process. Prospection of epigenetic markers associated to oocyte quality in cumulus cells is a non-invasive method to be used in the routine of IVF.

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Introduction

In vitro embryo production (IVP) is one of the most powerful reproductive techniques available to multiply animals quickly, contributing positively to animal breeding programs [38]. Although IVP has great potential, there is much room for improvement. One of the most important aspects of IVP is oocyte quality [25,33,36]. During mammalian oogenesis, oocyte starts growing and accumulating a specific mRNA and protein population that will be important to the end of this process and to drive the first embryonic cell divisions after fertilization. Furthermore, changes in DNA methylation and post-translational histone modifications happen. These

molecular events are essential to produce an oocyte with high embryonic development potential.

In this sense, many studies have been conducted to establish the parameters that can be used to select the most competent oocytes. Identifying molecular markers associated with oocyte quality can be an important step in improving IVP results. One of the parameters routinely used to infer oocyte quality is based on the number and compaction of cumulus cells [15,26,37]. However, morphological evaluation is insufficient to identify the most competent oocytes that possess the ability to carry a pregnancy to term [11,25,26]. Therefore, evaluation at the molecular level can be very useful for understanding the mechanisms involved in oocyte competence. A very important molecular event that must occur during gametogenesis is genome-wide epigenetic reprogramming [32,34], making the identification of epigenetic markers a useful tool for enhancing oocyte competence. DNA methylation is one of the most studied epigenetic changes. It is also known that DNA methylation reprogramming occurs during gametogenesis and that the genome of mature gametes is highly methylated. Moreover, a sex-specific methylation pattern must be established in genes that are imprinted in the gametes [5,29]. A previous study in our laboratory evaluated the DNA methylation pattern in bovine oocytes with different competencies. We showed that the methylation pattern in the IGF2 gene changed during in vitro maturation in competent oocytes [19]. Bidirectional molecular communication between the granulosa/cumulus cells and the oocyte is essential for the coordination of oogenesis and folliculogenesis, and it is required for correct follicular growth and the development of a competent oocyte [7]. Thus, characterising the DNA methylation pattern in cumulus cells during in vitro maturation can improve our understanding of the effect of in vitro conditions on epigenetic mechanisms and oocyte competence, leading to the generation of a good quality embryo.

In humans, embryonic cells and/or the polar body obtained by aspiration or biopsy can be used to assess molecular markers to predict the future possibility of disease [3,43]. This strategy can be very useful for both, human and bovine because it identifies embryos with higher potential of implantation.

However, the invasive process of obtaining a biopsy may negatively influence oocyte competence or embryo quality. Cumulus cell biopsies to assess molecular markers are an effective method for determining oocyte quality. A recent study investigated the association of apoptotic index in bovine cumulus cells of post-IVM COCs, using TUNEL, with COC morphology and meiotic competence. They did not find association neither to COC morphology nor to meiotic competence, concluding that the level of apoptosis in cumulus cells is not a good marker to oocyte quality after IVM [40]. As it is well established in the literature, epigenetic marks are susceptible to in vitro conditions. Therefore evaluating DNA methylation profile of imprinted genes in cumulus cells can be a useful strategy to discovery new molecular markers in this cell type.

Insulin-like growth factor 2 (IGF2) regulates cellular growth and differentiation in many species [9] and is a potent mitogen for foetal growth [12,13,17] and placental development [9,10]. Bovine IGF2 is an imprinted gene [16] predominantly expressed by the paternal allele. This gene is located on chromosome 29 [23] and contains 10 exons [4,14] and several differentially methylated regions (DMRs) [20]. A significant difference in the methylation levels in the last exon of the gene between the spermatozoa and the oocyte indicates the presence of an intragenic DMR [21]. This region is highly methylated in the spermatozoa [8,21], but it is hypomethylated in the oocyte [19,21]. According to the literature [28], this DMR plays a role in transcription initiation when it is methylated. Because of the importance of IGF2 in embryo development, characterisation of the DNA methylation pattern in this DMR in different tissues, especially in those related to reproduction, such as in the cumulus-oocyte complexes (COCs), is very useful in the development of reproductive techniques. For example, large offspring syndrome (LOS), observed when using reproductive techniques such as in vitro fertilisation and somatic cell nuclear transfer (SCNT), is associated with deregulation of the IGF2 and IGF2R genes [12,44].

The aim of this study was to characterise the methylation pattern in an intragenic CpG island located in the last exon of the IGF2 gene in *Bos taurus indicus* cumulus cells isolated from 1–3 mm and ≥ 8.0 mm follicles and to evaluate the effects of in vitro maturation on this pattern.

Materials and methods

Cumulus-oocyte complex recovery and in vitro maturation

Ovaries from Nellore (*Bos taurus indicus*) cows, that are aged between 30 and 60 months, were collected immediately after slaughter and transported to the laboratory in a saline solution (0.9 % NaCl) supplemented with penicillin G (100 IU/ml; Sigma Aldrich, St. Louis MO, USA) and streptomycin sulphate (100 mg/ml; Sigma Aldrich, St. Louis MO, USA) at 35–37 °C.

The follicles were dissected from the ovarian cortex at room temperature using scissors, scalpels and tweezers. During dissection, the follicles were kept in phosphate-buffered saline (PBS) with 10 % foetal calf serum (Sigma Aldrich, St. Louis MO, USA) at 36 °C. The follicles were measured using a stereomicroscope with a graduated eyepiece (OSM-4; Olympus, Tokyo, Japan) and were classified morphologically as small or large according to their diameter [6]. Follicles with a diameter of 1–3 mm were classified as small and representative of less competent oocytes, and those with a diameter ≥ 8.0 mm were considered large and representative of more competent oocytes, according to the model to study oocyte

competence that we have developed in our laboratory [6]. The COCs were released by rupturing the follicles. Only the 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, pre-IVM COCs were either denuded by repeated pipetting in PBS and the cumulus cells frozen at −80 °C until DNA extraction or submitted to in vitro maturation [19]. COCs were washed and transferred to 50 ml (≤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 Aldrich, St. Louis MO, USA), 0.1 IU/ml FSH (Sigma Aldrich, St. Louis MO, USA), 0.1 mg/ml L-glutamine (Sigma Aldrich, St. Louis MO, USA) and an antibiotic (amicacyn, 0.075 mg/ml). Following the maturation period, the COCs were incubated with 0.2 % hyaluronidase (Sigma Aldrich, St. Louis MO, USA) for 10 min and then denuded by repeated pipetting. The cumulus cells were frozen at −80 °C until DNA extraction. Only the cumulus cells from the oocytes that had extruded their first polar body (considered to be matured) were used in this experiment.

DNA isolation and sodium bisulphite treatment

Genomic DNA from cumulus cells was isolated using a salting out protocol. DNA treatment with sodium bisulphite was performed using the EZ DNA methylation kit (Zymo Research Corporation, Irvine, CA, USA) according to the manufacturer's protocol; however, the conversion temperature was changed to 55 °C. Bisulphite-treated DNA was diluted in 15 μl of distilled water and maintained at −20 °C until PCR amplification.

PCR amplification, cloning and bisulphite sequencing

Nested PCR using bisulphite-treated DNA was performed using primers cited in the literature [21]. PCR was performed in a total volume of 20 μl using 1 × Taq buffer, 2.0 mM $MgCl_2$, 100 mM of each dNTP, 0.5 mM of each primer, 1 U Platinum Taq polymerase (Life Technologies, Carlsbad, CA, USA) and 3 μl template DNA for the first round or 0.5 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 amplicons were recovered from an agarose gel and purified using the GenClean III kit (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's protocol. The purified products were cloned into the pGEMT-Easy vector (Promega, Madison, WI, USA) 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. The conversion of non-CpG cytosines was used to calculate the efficiency of the bisulphite treatment, and the methylation pattern in the CpG cytosines was used to identify clones from different DNA templates. Methylation patterns were analysed using CHROMAS software and DNAMAN 4.0 software; GenBank accession no. X53553 was used as a reference.

Statistical analysis

To compare the methylation patterns among groups of cumulus cells, 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 [24]. The methylation patterns were compared using the *t* test for data showing a normal distribution or the Mann–Whitney test for data that did not show a normal distribution. All data were compared using the Prophet Program, version 5.0 (BBN Systems and Technologies, 1996), and are shown as the mean ± SD.

Results

The cumulus cells from 60 COCs for each group (pre-IVM small, post-IVM small, pre-IVM large and post-IVM large follicles) were used for DNA isolation and methylation pattern evaluation. Based on the methylation patterns evaluated in the twenty-eight identified CpG sites in the last exon (Fig. 1), at least eight different alleles in the cumulus cells were found. The cumulus cells from pre-IVM and post-IVM COCs from small follicles showed methylation levels of 78.17±14.11 % and 82.93±5.86 %, respectively. The cumulus cells from pre-IVM and post-IVM COCs from large follicles showed methylation levels of 81.81±10.40 % and 79.64±13.04 %, respectively. Considering only the effect of in vitro maturation, independent of follicle size, the cumulus cells from pre-IVM and post-IVM COCs showed methylation levels of 80.17±12.01 % and 81.19±10.15 %, respectively. There were no differences in the methylation patterns among the various groups ($P > 0.05$) and all sequences showed a hypermethylated pattern (Fig. 1).

Discussion

There is extensive biochemical communication between the oocyte and the cumulus cells that surround it [1,18]. This functional contact during oocyte growth and maturation is essential for the oocyte to acquire maximal competence [2,39,42]. Because we have previously shown that the methylation patterns between more and less competent oocytes are different [19], we hypothesised that the identification of

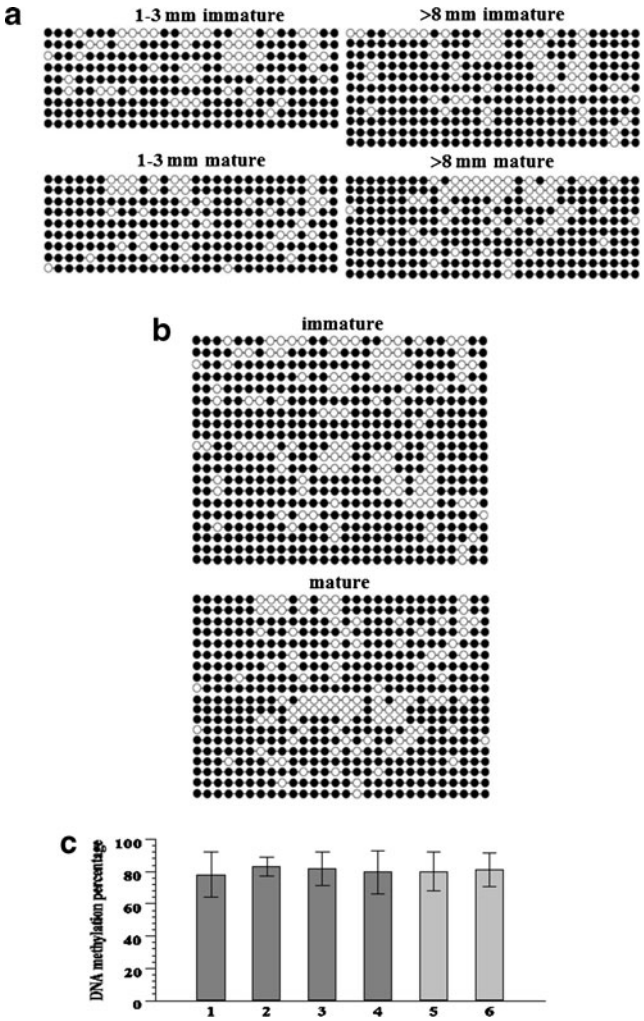

Fig. 1 Methylation pattern of the intragenic IGF2 DMR in *Bos taurus indicus* cumulus cells. **a** Methylation levels in immature and in vitro matured cumulus cells from 1–3 mm and >8.0 mm follicles. **b** Methylation levels in immature and in vitro matured cumulus cells. Each line represents one individual clone, and each circle represents one CpG site (28 CpGs). Open circles represent unmethylated CpGs, and black circles represent methylated CpGs. **c** Percentage of DNA methylation (mean ± SD; $P>0.05$); 1–6: 1–3 mm immature, 1–3 mm mature, >8.0 mm immature, >8.0 mm mature, immature pooled, and mature pooled cumulus cells, respectively

alterations in cumulus cells could be a non-invasive technique for determining oocyte quality. Thus, we evaluated the DNA methylation pattern in the CpG island located in the last exon of the bovine IGF2 gene in cumulus cells from pre- and post-IVM COCs collected from small (less competent) and large (more competent) follicles. The CpG island evaluated in this study showed a hypermethylated pattern in cumulus cells from both small and large follicles, before and after in vitro maturation (Fig. 1). This result demonstrates that the methylation pattern of this region is not similar to that imprinted of somatic cells, which have one methylated allele and one unmethylated allele, depending on the parent from whom each allele originates [32]. This result is intriguing because we expected to find an imprinted pattern of this CpG island in cumulus cells, considering that this region is hypermethylated in spermatozoa [8] and hypomethylated in oocytes [19,21]. However, it has been demonstrated in the literature that an imprinted pattern can be established in a tissue-specific manner, whereby a gene can be imprinted in one tissue and not in another [31]. Therefore, based on our results, we can affirm that this CpG island is not imprinted in bovine cumulus cells. We also have to consider that there are other CpG islands present in this gene that may have different methylation patterns [21,22,41].

In two previous studies from our group [8,19], we evaluated the methylation pattern for the same genomic region evaluated in this study in oocytes and spermatozoa and found that oocytes are hypomethylated and that spermatozoa are hypermethylated. These results are in agreement with studies [21,27,30,35] showing that this DMR is highly methylated in spermatozoa and hypomethylated in oocytes.

Moreover, in one study, the methylation pattern in oocytes from large follicles changed after in vitro maturation, demonstrating that epigenetic reprogramming extends throughout oocyte maturation [19], a result that is different from what we found in this study. Taken together these information, it is clear that this DMR conclude its methylation reprogramming during oocyte maturation [19] and that this region is not imprinted in bovine cumulus cells, showing a very stable pattern of methylation, as expected by a somatic cell [32].

Many studies in the past decade have shown that the maturation of oocytes in vitro and the in vitro culture of cells and embryos may alter DNA methylation patterns, resulting in altered gene expression and a change in cell quality. Therefore, it is important to evaluate methylation patterns in cumulus cells, which can be obtained non-invasively, to identify the influence of in vitro conditions and the level of competence on epigenetic patterns and, consequently, on oocyte quality. Our results showed that neither oocyte competence [6] nor in vitro maturation affected DNA methylation of the CpG island studied. However, it is possible that the methylation patterns in other CpG islands can be influenced by in vitro conditions because the stability or resistance to change of methylation patterns can vary in different regions of the genome [34].

In conclusion, the methylation levels of the cumulus cells of all groups evaluated were higher than that expected from the imprinted pattern of somatic cells. As the cumulus cells from the pre-IVM follicles were not subjected to any in vitro manipulation, the hypermethylated pattern that was observed may be the actual physiological methylation pattern for this particular locus in this cell type. Because of the importance of epigenetic reprogramming in oogenesis and its susceptibility to in vitro conditions, as well as the importance of having a non-invasive method for determining oocyte quality, we believe that the identification of epigenetic markers in cumulus cells has great potential. Similar studies should be extended to

other CpG islands in the cumulus cell genome, which will contribute to the identification of new epigenetic markers that can be used to support reproductive biotechniques in humans and other mammals.

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

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