DNA methylation and mRNA expression profiles in bovine oocytes derived from prepubertal and adult donors

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Abstract

The developmental capacity of oocytes from prepubertal cattle is reduced compared with their adult counterparts, and epigenetic mechanisms are thought to be involved herein. Here, we analyzed DNA methylation in three developmentally important, nonimprinted genes (SLC2A1, PRDX1, ZAR1) and two satellite sequences, i.e. ‘bovine testis satellite I’ (BTS) and ‘Bos taurus alpha satellite I’ (BtaS). In parallel, mRNA expression of the genes was determined by quantitative real-time PCR. Oocytes were retrieved from prepubertal calves and adult cows twice per week over a 3-week period by ultrasound-guided follicular aspiration after treatment with FSH and/or IGF1. Both immature and in vitro matured prepubertal and adult oocytes showed a distinct hypomethylation profile of the three genes without differences between the two types of donors. The methylation status of the BTS sequence changed according to the age and treatment while the methylation status of BtaS sequence remained largely unchanged across the different age and treatment groups. Relative transcript abundance of the selected genes was significantly different in immature and in vitro matured oocytes; only minor changes related to origin and treatment were observed. In conclusion, methylation levels of the investigated satellite sequences were high (>50%) in all groups and showed significant variation depending on the age, treatment, or in vitro maturation. To what extent this is involved in the acquisition of developmental competence of bovine oocytes needs further study.

Introduction

The use of bovine oocytes from prepubertal animals for embryo production in vitro could accelerate genetic gain and shorten generation intervals (Lohuis 1995). However, currently the average rates of viable blastocysts produced from prepubertal donor oocytes are lower than the use of adult oocytes (10–15 vs ~30%; Oropeza et al. 2004, Zaraza et al. 2010). Incomplete or deficient cytoplasmic maturation, altered protein synthesis, reduced oocyte size, and impaired metabolism in prepubertal oocytes have been proposed as possible mechanisms for the reduced developmental potential (Lonergan et al. 1994, Steeves et al. 1999, De Paz et al. 2001, Kauffold et al. 2005).

The intraovarian (i.o.) injection of insulin-like growth factor 1 (IGF1) has been shown to improve the developmental competence of prepubertal oocytes (Oropeza et al. 2004). Embryos produced from oocytes derived from IGF1-treated prepubertal donors showed similar developmental potential and a similar mRNA expression profile of genes critical in embryonic development as those produced from adult donors (Oropeza et al. 2004). Epigenetic factors were proposed to be involved in the acquisition of full developmental competence of bovine prepubertal oocytes, but have not yet been studied in detail (Oropeza et al. 2004, Zaraza et al. 2010).

DNA methylation at the CpG dinucleotides is one of the major epigenetic modifications in eukaryotic genomes. In somatic cells ~60–90% of the CpG dinucleotides are methylated, mainly in noncoding regions of the genome (Siegfried & Cedar 1997). During gametogenesis and early embryogenesis, the genome undergoes waves of demethylation and remethylation (Jaenisch 1997, Kageyama et al. 2007). In the first round of epigenetic reprogramming a sex-specific methylation pattern of certain loci, i.e. genomic imprinting, is established in the male and female germ lines. After fertilization, the second round of epigenetic reprogramming of the female and male genomes is essential for the activation or silencing of specific genes and creates the methylation pattern compatible with normal
Results
Calves were randomly assigned to four groups (calf, without treatment; calf FSH, i.m. FSH treatment; calf FSH + IGF1, i.m. FSH treatment + i.o. IGF1 injection; and calf FSH + IGF C, FSH + i.o. 0.01 M acetic acid injection). Cows were divided into two groups (cow, no treatment and cow FSH). Oocytes were retrieved by ovum pick-up (OPU) and used either directly for analysis or subjected to in vitro maturation (IVM). The numbers of aspirated follicles, retrieved oocytes, and oocytes used for experiments did not differ significantly between treatment groups (Table 1). Maturation rates did not differ significantly between groups (Table 1).

Methylation analysis
Methylation profile of two satellite DNA sequences
We examined the methylation status of two representative satellite DNA sequences, the BTS and BTzs that are indicative of global methylation dynamics, in the genome in prepubertal and adult bovine oocytes. For analysis of the BTS sequence, a 211 bp segment of the satellite sequence, including 12 highly conserved CpGs sites, was amplified by PCR from bisulfite-treated genomic DNA, and the resulting PCR products were individually cloned and sequenced (Kang et al. 2001, 2005). A 154 bp region was amplified by PCR which included nine CpGs for analysis of the BTzs sequence in immature and in vitro matured bovine oocytes.

The genome of immature oocytes displayed differential methylation for the BTS sequence between the experimental groups. In the group ‘cow’ the methylation of BTS I was 49.6% for immature oocytes, whereas methylation rates for the groups ‘calf’ (74.6%) and ‘calf FSH’ (69.8%) were significantly higher (Table 2). After IVM a significant difference was observed between the groups ‘calf’ (53.3%) and ‘calf FSH + IGF1’ (71.7%) (Table 2). Within the age and treatment groups, we observed significant differences (P<0.05) prior to and after IVM in groups ‘cow’ (49.6 vs 64.9%) and ‘calf FSH + IGF1’ (60.6 vs 71.7%) and ‘calf’ (74.6 vs 53.4%) (Table 2). A representative methylation profile for the BTS sequence is shown in Supplementary Figure 1, see section on supplementary data given at the end of this article.

The methylation level of the BTzs sequence was higher in immature oocytes than in in vitro matured oocytes, but did not differ significantly between groups and treatments (Table 3). Within groups, before and after IVM a significant decrease in methylation (P<0.05) was shown for ‘cow FSH’ (76.2 vs 52.5%) and ‘calf FSH’ (72.8 vs 57.8%). A representative methylation profile for the BTzs sequence is shown in Supplementary Figure 2, see section on supplementary data given at the end of this article.

Gene-specific methylation
Most gene-specific methylation analyses in oocytes have been performed with large pools of (100 or more) cells (Lucifero et al. 2002, Anckaert et al. 2010). However, when working with such small amounts of DNA, degradation and low complexity of bisulfite-converted DNA may lead to stochastic amplification of a single or only few molecules in the starting sample and may thus yield nonrepresentative results. To avoid amplification.

<table>
<thead>
<tr>
<th>Groups (no. of animals)</th>
<th>No. of aspirated follicles</th>
<th>Total no. of retrieved oocytes</th>
<th>No. of oocytes (class I and II)</th>
<th>No. of oocytes used for IVM (class I and II)</th>
<th>Matured oocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf (31)</td>
<td>954</td>
<td>725</td>
<td>225</td>
<td>124</td>
<td>50</td>
</tr>
<tr>
<td>Calf FSH (35)</td>
<td>1096</td>
<td>797</td>
<td>262</td>
<td>175</td>
<td>40</td>
</tr>
<tr>
<td>Calf FSH + IGF1 (30)</td>
<td>1212</td>
<td>995</td>
<td>287</td>
<td>180</td>
<td>50</td>
</tr>
<tr>
<td>Calf FSH + IGF C (27)</td>
<td>1201</td>
<td>923</td>
<td>263</td>
<td>162</td>
<td>50</td>
</tr>
<tr>
<td>Cow (26)</td>
<td>1287</td>
<td>827</td>
<td>273</td>
<td>182</td>
<td>42.8</td>
</tr>
<tr>
<td>Cow FSH (17)</td>
<td>912</td>
<td>659</td>
<td>204</td>
<td>102</td>
<td>50</td>
</tr>
</tbody>
</table>

Table 1 Ovum pick-up and morphological quality of oocytes from calves and cows.
bias and to recover as many DNA molecules as possible from small oocyte pools, we have used limiting dilution of bisulfite-treated DNA from ten cells each, combined with multiplex PCR and direct bisulfite sequencing of three representative nonimprinted genes. A schematic drawing of the basic steps of the protocol is shown in Fig. 1. The sequestration of individual DNA molecules from the starting sample in separate PCR reactions ensures that each generated bisulfite amplicon represents an individual DNA molecule in the starting sample.

Here, we analyzed promoter methylation of SLC2A1, PRDX1, and ZAR1 in bovine oocytes retrieved from prepubertal and adult donors. The GDF9 promoter sequence did not contain CpGs and was excluded from this analysis. Immature oocytes are arrested in meiotic prophase I and contain four paired chromatids (DNA molecules). After maturation, the oocyte is arrested in metaphase II with two chromatids in the oocyte and two in the attached first polar body. Thus, pools of ten immature or mature oocytes are endowed with 40 copies of each studied gene, implying that a maximum of 120 target DNA molecules are present for multiplex PCR with three genes. The amplicons for direct bisulfite sequencing included 14 CpG sites for multiplex PCR with three genes. The amplicons for direct bisulfite sequencing included 14 CpG sites for multiplex PCR with three genes. The amplicons for direct bisulfite sequencing included 14 CpG sites for multiplex PCR with three genes. The amplicons for direct bisulfite sequencing included 14 CpG sites for multiplex PCR with three genes.

### Table 2 Methylation pattern of ‘bovine testis satellite I (BTS) sequence’ (X ± s.e.m.),

<table>
<thead>
<tr>
<th>Group</th>
<th>Immature oocytes; methylation (%)</th>
<th>In vitro matured oocytes; methylation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow</td>
<td>49.6 ± 3.0 – A</td>
<td>64.9 ± 6.3 – A</td>
</tr>
<tr>
<td>Cow FSH</td>
<td>69.8 ± 3.4 – b</td>
<td>59.7 ± 3.7 – A</td>
</tr>
<tr>
<td>Calf</td>
<td>74.6 ± 3.9 – A</td>
<td>53.4 ± 5.6 – b</td>
</tr>
<tr>
<td>Calf FSH</td>
<td>64.7 ± 3.4 – a,c</td>
<td>63.3 ± 5.2 – a</td>
</tr>
<tr>
<td>Calf FSH+IGF1</td>
<td>60.6 ± 3.4 – a,c,A</td>
<td>71.7 ± 5.2 – A</td>
</tr>
<tr>
<td>Calf FSH+IGF C</td>
<td>69.6 ± 5.6 – a,b,c</td>
<td>62.5 ± 4.3 – a</td>
</tr>
</tbody>
</table>

Methylation pattern of ‘BTS sequence’ in immature and in vitro matured oocytes of different experimental groups. Significant differences within groups of immature and in vitro matured oocytes groups are characterized by small letters (a, b, c). Differences between immature and in vitro matured oocytes are characterized by capital letters (A, B). Different superscripts indicate significant differences (P ≤ 0.05).

### Table 3 Methylation pattern of ‘Bos taurus alpha satellite I (BTαS)’ (X ± s.e.m.),

<table>
<thead>
<tr>
<th>Group</th>
<th>Immature oocytes; methylation (%)</th>
<th>In vitro matured oocytes; methylation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow</td>
<td>66.4 ± 4.2</td>
<td>61.7 ± 2.1</td>
</tr>
<tr>
<td>Cow FSH</td>
<td>76.7 ± 4.8 – A</td>
<td>52.5 ± 4.1 – B</td>
</tr>
<tr>
<td>Calf</td>
<td>73.2 ± 2.7</td>
<td>70.4 ± 3.1</td>
</tr>
<tr>
<td>Calf FSH</td>
<td>72.8 ± 3.6 – A</td>
<td>57.8 ± 2.7 – B</td>
</tr>
<tr>
<td>Calf FSH+IGF1</td>
<td>66.4 ± 3.1</td>
<td>56.8 ± 2.2</td>
</tr>
<tr>
<td>Calf FSH+IGF C</td>
<td>68.9 ± 3.1</td>
<td>62.9 ± 3.9</td>
</tr>
</tbody>
</table>

Methylation pattern of ‘BTαS’ in immature and in vitro matured oocytes of different groups. Significant differences after IVM are characterized by capital letters (A, B). Different superscripts indicate significant difference (P ≤ 0.05).

**Determination of the relative mRNA abundance of developmentally important genes in bovine oocytes**

The relative mRNA abundance of the four developmentally important, nonimprinted genes (GDF9, SLC2A1, PRDX1, and ZAR1) selected for this study was investigated in immature and in vitro matured oocytes of the different age and treatment groups. No significant differences were observed for all four genes in immature oocytes. However, after maturation we observed significant reductions in transcript abundance for GDF9 and ZAR1 in all groups (Fig. 2). The ZAR1 transcript was reduced after IVM in hormonally treated animals compared with untreated animals.

Transcript levels of PRDX1 were significantly reduced (P < 0.05) after maturation in all groups with the exception of ‘calf FSH+IGF1’ (Fig. 2). A slight but nonsignificant increase of PRDX1 transcripts was observed in in vitro matured oocytes after hormonal treatment. SLC2A1 was significantly downregulated in the groups ‘calf’, ‘calf FSH’, ‘calf’, and ‘calf FSH’ after IVM. The observed difference in the transcript level for the groups ‘calf FSH+IGF1’ and ‘calf FSH+IGF C’ did not reach the level of statistical significance (Fig. 2).

### Discussion

Epigenetic mechanisms, specifically DNA methylation dynamics, have been implicated in the low developmental capacity of juvenile oocytes (Oropeza et al. 2004, Zaraza et al. 2010). In the study presented here, we investigated for the first time the influence of donor age and hormonal treatments on the general DNA methylation pattern of Juvenile bovine oocytes.
methylation dynamics and concomitantly the methylation status of selected genes, including SLC2A1, PRDX1, and ZAR1 in bovine oocytes. Moreover, the mRNA expression levels of these genes were determined in immature and in vitro matured oocytes to correlate transcript abundance with DNA methylation.

Several studies have reported that DNA methylation plays a crucial role in gametogenesis and early embryonic development (Cheung et al. 2009, Katari et al. 2009). Approximately 60% of the genes in the mammalian genome show a high CpG density in their promoters, called CpG islands (Antequera & Bird 1993). Usually, a low level of DNA methylation in the promoter region is associated with active transcription (Neumann & Barlow 1996), while high levels of methylation are correlated with low or no expression (Suzuki & Bird 2008). In contrast to the rest of the genome where most CpGs are methylated, CpG islands in 5’ cis-regulatory (promoter and first exon) regions of genes are normally unmethylated. Methylation of these CpG islands during the development or diseases is associated with gene silencing (Jaenisch & Bird 2003, Weber et al. 2007). Here, we found distinctive hypomethylation in all experimental groups regardless of maturation, age, or prior hormonal treatment for the developmentally important genes SLC2A1, PRDX1, and ZAR1, suggesting that the expression of these genes is not regulated by promoter methylation. Therefore, other mechanisms, i.e. gene body methylation (Maunakea et al. 2010), may be more important for regulating oocyte-specific efficiency of transcription. However, one has to take into account that the data presented here

![Figure 1](image)

**Figure 1** Schematic drawing of the main steps of limiting dilution bisulfite sequencing. (A) Immature and mature oocytes are collected by OPU or after IVM. Ten oocytes of a defined group are pooled. (B) DNA isolation and bisulfite conversion. (C) Dilution of the converted oocyte DNA. (D) The diluted DNA is distributed over 20 wells on a microtiter plate. Most wells contain either no or a single DNA target molecule (ideograms); few wells may contain two or more copies. In addition, six negative controls (N) are added. First-round multiplex PCR is performed with outer primers for the PRDX1, ZAR1, and SLC2A1 genes. (E) Second-round singleplex PCRs of the three studied genes in individual microtiter plates (indicated by different colors) using 1 μl multiplex PCR product as template and gene-specific inner primers. (F) Second-round PCR products are visualized on agarose gels. The color code of each lane indicates the plate (gene), numbers, andNs of the specific well on that plate. DNA from wells containing a PCR product is analyzed by direct bisulfite sequencing.

<table>
<thead>
<tr>
<th>Oocyte group</th>
<th>Number of pools</th>
<th>SLC2A1</th>
<th>PRDX1</th>
<th>ZAR1</th>
<th>Number of DNA molecules</th>
<th>DNA molecules per gene</th>
<th>DNA molecules per pool</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immature oocytes</td>
<td>Cow</td>
<td>1</td>
<td>6</td>
<td>7</td>
<td>3</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Cow FSH</td>
<td>5</td>
<td>26</td>
<td>16</td>
<td>13</td>
<td>55</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Calf FSH</td>
<td>3</td>
<td>18</td>
<td>5</td>
<td>11</td>
<td>34</td>
<td>11.3</td>
</tr>
<tr>
<td></td>
<td>Calf FSH + IGF1</td>
<td>3</td>
<td>12</td>
<td>1</td>
<td>11</td>
<td>24</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Calf FSH + IGF C</td>
<td>12</td>
<td>10</td>
<td>15</td>
<td>28</td>
<td>96</td>
<td>12.6</td>
</tr>
<tr>
<td>Mature oocytes</td>
<td>Cow</td>
<td>5</td>
<td>33</td>
<td>18</td>
<td>28</td>
<td>79</td>
<td>15.8</td>
</tr>
<tr>
<td></td>
<td>Cow FSH</td>
<td>4</td>
<td>28</td>
<td>10</td>
<td>24</td>
<td>62</td>
<td>15.5</td>
</tr>
<tr>
<td></td>
<td>Calf</td>
<td>4</td>
<td>18</td>
<td>10</td>
<td>25</td>
<td>53</td>
<td>13.3</td>
</tr>
<tr>
<td></td>
<td>Calf FSH</td>
<td>4</td>
<td>7</td>
<td>5</td>
<td>11</td>
<td>23</td>
<td>5.8</td>
</tr>
<tr>
<td></td>
<td>Calf FSH + IGF1</td>
<td>4</td>
<td>17</td>
<td>6</td>
<td>18</td>
<td>41</td>
<td>10.3</td>
</tr>
<tr>
<td></td>
<td>Calf FSH + IGF C</td>
<td>3</td>
<td>13</td>
<td>10</td>
<td>15</td>
<td>38</td>
<td>12.7</td>
</tr>
</tbody>
</table>

**Table 4** Number of analyzed DNA molecules in three nonimprinted genes in bovine oocytes.
provide only a narrow window into methylation dynamics in immature and in vitro matured oocytes and age and/or hormonal treatment dependent methylation changes can have profound effects on functionality (Laird 2005, Wilkins-Haug 2009).

In order to achieve a more general overview of the methylation patterns of oocytes in all experimental groups, we investigated two repeat sequences: the BTS and the BT\textsuperscript{a}S sequence. Previously, a moderate methylation level (27.9–35%) was reported for bovine 1-cell embryos (Kang et al. 2001, 2005). Here, we showed hypermethylation (52–71%) in most groups of juvenile and adult oocytes, suggesting maternal epigenetic regulation of this specific repeat sequence during oogenesis and oocyte maturation. Methylation dynamics differed between groups for the BTS sequence. While the methylation level for untreated calves decreased significantly during IVM, the methylation rates increased after IVM for oocytes from untreated cows and calves treated with both FSH and IGF1. This suggests that the treatment of calves with FSH and IGF1 prior to OPU renders the epigenetic status of the oocyte closer to that of the adult untreated oocyte. Recently, O’Doherty et al. (2012) reported size-dependent methylation dynamics in growing bovine oocytes, possibly linking the acquisition of developmental competence with the acquisition of specific methylation.

Oocytes from all prepubertal and adult experimental groups uniformly showed reduced methylation levels for the satellite sequence BT\textsubscript{a}S after IVM. The methylation levels found in this study were higher than that reported previously for bovine matured oocytes (~5.5%; Kang et al. 2005). Whether or not the fertilization process affects the methylation status in a significant manner remains to be determined. In sheep oocytes, the level of DNA methylation was significantly decreased in prepubertal oocytes compared with their adult counterparts (Ptak et al. 2006).

It is crucial to discriminate between methylation of single CpG sites and methylation of the entire cis-regulatory region. A single or a few methylated CpGs within an overall nonmethylated promoter most likely represent a technical error during bisulfite conversion or a stochastic biological phenomenon without functional consequences. Although in some genomic regions methylation of a single CpG (i.e. in a transcription factor binding site) may interfere with gene regulation (Griswold & Kim 2001), it is usually the density of methylated CpGs rather than individual CpGs that switch a promoter on or off. Because individual CpGs cannot maintain methylation that is different from those of the neighboring CpGs, usually the entire promoter is either methylated or nonmethylated (Sontag et al. 2006, Weber et al. 2007). Consequently, only DNA molecules with >50% methylated CpGs can be considered as epimutations that are associated with gene silencing.

Most repetitive DNA elements are densely methylated in somatic cells. Hypomethylation of centromeric satellite DNAs can lead to a decondensed chromatin structure and chromosomal (centromeric) instability (Miniou et al. 1994, Haaf 1995). On the other hand, demethylation of interspersed repetitive elements is frequently associated with the reactivation of retrotransposons and thus promotes genome instability by insertional mechanisms (Yoder et al. 1997). It was therefore not surprising that satellite sequences (and some other genomic sequences) are rather resistant against changes in DNA methylation and may have their own regulatory mechanisms. The high preservation of methylation marks at the BTS sequences during early embryo development suggests efficient maintenance of methylation in bovine embryos (Kang et al. 2005). Recently, it was shown that the methylation status of the oocyte correlates with follicle size, size of the oocyte,
and oocyte developmental capacity (Fagundes et al. 2011). While immature oocytes from follicles ≥ 8 mm showed significantly higher methylation than their matured counterparts from follicles of the same size category, oocytes from follicles 1–3 mm in diameter did not exhibit differences prior to and after maturation (Fagundes et al. 2011). In the present study, we used oocytes from follicles > 3 mm in size. Deviations from the physiological methylation profile were found in mouse embryos with deficient development and were attributed to the culture system and the different genetic background of the mouse strains (Shi & Haaf 2002). Hypomethylation of minor and major satellite sequences was shown for prepubertal mouse oocytes (Sanford et al. 1984, Monk et al. 1987). The centromeric satellite sequence (Rsat IIE) was moderately methylated with 47.6 ± 23.2% in adult rabbit oocytes, which is comparable with the results found for the BTrzS sequence in this study. These data indicate that species-specific differences exist with regard to satellite methylation during mammalian oocyte development.

Gene expression in the mammalian oocyte is subject to specific temporal and spatial control mechanisms. During oogenesis, oocytes synthesize substantial amounts of mRNA that are accumulated in the cytoplasm (Fair et al. 1995, Hyttel et al. 1997, Picton et al. 1998, Memili & First 2000). During maturation, fertilization, and early embryonic development the stored maternal mRNAs are essential for development, and transcription is resumed with the activation of the embryonic genome (Niemann & Wrenzycki 2000, Thélie et al. 2007). GDF9 is important for growth and proliferation of cells. The highest concentration of GDF9 transcripts was found in immature oocytes, while GDF9 abundance was reduced after maturation. Similar findings were reported previously and specifically the reduction in GDF9 mRNA transcript abundance was linked to the acquisition of developmental competence (Lonergan et al. 1994, 2003). While the mRNA content prior to and after IVM differed significantly for all treatment groups in this study, there were no significant differences between the groups of donors. Similar findings were made for ZAR1, an important maternal effect gene regulating early embryonic development (Duranthon & Renard 2001, Brevini et al. 2004). ZAR1 transcripts have been found in immature oocytes and in early embryos until major activation of the embryonic genome (Brevini et al. 2004, Nowak-Imialek et al. 2008).

An important nutrient for oocytes is glucose and its derivatives. Among others, it is transported into the cells by the glucose transporter 1 (SLC2A1; Morita et al. 1992). Although glucose cannot be utilized by the oocyte itself, the embryo until the two to four cell stage due to low hexokinase activity (Brinster 1965), SLC2A1 mRNA transcripts are present in immature and matured oocytes (Lequarre et al. 1997, Rajhans et al. 2010).

In vitro culture systems expose oocytes to a variety of environmental influences. The difference in mRNA levels for SLC2A1 in our previous study (Oropeza et al. 2004) in two to four cell embryos from untreated and IGF1-treated calves was not reflected in the retrieved oocytes in this study. Different antioxidant systems are active to protect oocytes from reactive oxygen species (Guérin et al. 2001). PRDX1 expression was detected in immature and matured bovine oocytes and zygotes. It was significantly higher in matured adult oocytes compared with oocytes from prepubertal oocytes (Romar et al. 2011). In this study no significant influence of age and/or treatment was found regarding PRDX1 expression.

These data demonstrate that neither gene-specific expression nor methylation levels were different in the various treatment or age groups. Only IVM reduced abundance of the analyzed transcripts. In contrast to individual promoters, the methylation levels of the investigated satellite sequences were high (>50%) in all groups and showed significant variation depending on the age, treatment, or IVM. Although we did not detect methylation changes in the three studied genes, the observed changes in satellite DNA methylation suggest a role of DNA methylation in the acquisition of developmental capacity of bovine oocytes, which needs to be explored in future studies. The in-depth understanding of DNA methylation and gene expression dynamics in oocytes is critical for developing improved IVP protocols for oocytes from prepubertal and adult donors.

Materials and Methods

Animals and treatments

All animal experiments were conducted in compliance with German Animal welfare regulations. A total of 105 prepubertal calves (6–9 months of age) and 43 cows (≥ 2nd lactation) from the experimental herd of the Institute in Mariensee were used for these experiments. Only healthy animals with adequate development of the reproductive tract were included in the experiments. Oocytes were collected over 3-week periods at intervals of 3–4 days by ultrasound-guided OPU after the initial removal of the dominant follicle 4 days prior to the first OPU session (Zaraza et al. 2010).

Calves were randomly assigned to four groups, i.e. calf, without treatment; calf FSH, i.m. FSH treatment; calf FSH + IGF1, i.m. FSH treatment + i.o. IGF1 injection; and calf FSH + IGF C, FSH + i.o. 0.01 M acetic acid injection (Table 5). Calves in groups 2, 3, and 4 all received one i.m. injection of 65 μg FSH (Stimufol; kindly provided by J F Beckers, University of Liege, Belgium) in order to promote the follicular growth 48 h prior to each OPU session. At the same time, groups 3 and 4 additionally received i.o. injections of 6 μg rHGF1 (R&D Systems, Wiesbaden, Germany) or 0.01 M acetic acid as vehicle control respectively at two distinct localizations of each ovary (Table 5).

Cows were randomly assigned to two treatment groups. Cows in the first group remained untreated (cow); cows in the
second group were injected i.m. with 100 μg FSH (Stimufol) 48 h prior to each session (cow FSH). This is the standard dose of FSH for healthy adult female cows subject to superovulatory treatment.

Ultrasound-guided equipment and oocyte retrieval

Ultrasound-guided OPU was performed as previously described (Oropeza et al. 2004, Zaraza et al. 2010). Briefly, ovaries were visualized using a C59000 ultrasound system (Picker, München, Germany) and a 6.5 MHz ultrasound transducer (Picker model, EUP-F-331). The ultrasound transducer was protected by a sanitary cover (Servopraz, Wesel, Germany) and was coated with lubricant (Bovivet gel; Kruuse, Langeskov, Denmark) before insertion into the vagina. Follicles 3–17 mm in diameter were aspirated using disposable 20G 2½ needle (Terumo, Eschborn, Germany) and a flexible tube system (2 mm X 1 m) attached to a vacuum pump (IVF Ultra quiet; Cook Veterinary Products, Moenchengladbach, Germany) set to 8.3–8.4 hPa. The collected oocytes were flushed from the system after aspiration of four to five follicles, with Dulbecco’s PBS medium (AppliChem, Darmstadt, Germany) containing 1 mg/ml BSA (Fraction V; Sigma–Aldrich), 50 mg/ml Na pyruvate (AppliChem), 1 mg/ml glucose (Roth, Karlsruhe, Germany), 133 μg/ml calcium chloride dehydrate (Fluka, Munich, Germany), 50 μg/ml streptomycin (AppliChem), 6 μg/ml penicillin G (AppliChem), and 2.2 IU/ml Na heparin (AppliChem). The fluid from each animal was kept in separate 50 ml tubes, passed through a 50 m filter (Jürgens, Hannover, Germany) and was washed with fresh PBS medium. Cumulus-oocyte-complexes (COCs) were flushed from the filter with PBS and retrieved under a stereomicroscope at ×50 magnification. COCs were collected in individual 50 μl TCM-air drops, at pH 7.2 (TCM199; Sigma–Aldrich), containing 50 μg/ml gentamicin sulfate (Sigma–Aldrich), 0.2 mM Na pyruvate (Sigma–Aldrich), 4.2 mM NaHCO₃ (Roth), and 1 mg/ml BSA (FAS; Sigma–Aldrich) for each animal. Subsequently, the quality of the COCs was evaluated based on the homogeneity of the cytoplasm and the surrounding cumulus cells and only COCs morphologically classified into classes I and II (Looney et al. 1994, Goodhand et al. 1999) were used in this study.

Treatment of COCs

One group of COCs was immediately treated with PBS containing 1 mg/ml BSA (Fraction V; Sigma–Aldrich) and 0.1% hyaluronidase (Sigma–Aldrich) for 5 min at 39 °C followed by vortexing at 1400 r.p.m. for 5 min to remove adhering cumulus cells; any remaining cumulus cells were removed by careful pipeting of the oocytes. Denuded oocytes were washed three times in TCM-air and three times in PBS containing 0.1% polyvinyl alcohol and then frozen at −80 °C in pools of ten for gene-specific methylation analysis, in pools of five for DNA repeat analysis or singly for quantitative realtime PCR (qPCR).

A second group of randomly selected oocytes were subjected to IVM as described recently (Heinzmann et al. 2011). Briefly, COCs were washed three times in TCM199 at pH 7.4 supplemented with 0.2 mM Na pyruvate (Sigma–Aldrich), 25 mM NaHCO₃ (Roth), 1 mg/ml BSA (FAS; Sigma–Aldrich). Subsequently, groups of five to ten washed COCs were placed in 50 μl drops of supplemented TCM199 containing 10 IU/ml of equine chorionic gonadotropin and 5 IU/ml of human chorionic gonadotropin (Suigonan; Intervet, Unterschleissheim, Germany) under silicone oil (Serva, Heidelberg, Germany) and incubated at 39 °C in a humidified atmosphere of 5% CO₂ in air for 24 h. Successful maturation was confirmed after the removal of cumulus cells under the stereomicroscope by visualization of the first polar body. COCs were treated according to the protocol for immature oocytes above and frozen at −80 °C until further use.

mRNA isolation and RT

The relative transcript levels of four developmentally important genes were investigated on single cell basis. Poly(A)⁺ RNA was recovered from single oocytes using Dynabeads mRNA DIRECT Kit (Invitrogen; Heinzmann et al. 2011). Briefly, 40 μl lysis/binding buffer (100 mM Tris–HCl (pH 8.0), 500 mM LiCl, 10 mM EDTA, 1% lithium dodecyl sulfate, and 5 mM dithiothreitol) were added to the frozen single oocytes (n=15 per group) and incubated at room temperature for 10 min. For normalization purposes 1 μg rabbit globin mRNA (BRL, Gaithersburg, MD, USA) was added as an exogenous standard. This is necessary as degradation and deadenylation of transcripts in the growing and maturing oocyte inhibit the use of endogenous housekeeping genes for normalization and normalization to an exogenous standard has been successfully used in previous studies from our laboratory and others (Wrenzycki et al. 2001, Thélie et al. 2007, Niemann et al. 2010). A total of 5 μl pre-equilibrated Dynabeads Oligo d(T)₂₅ were added to the lysate and binding of mRNA to the magnetic beads was facilitated by incubation at 25 °C on a shaker for 15 min. Subsequently, a Dynal MPC-E-1 magnetic separator was used to separate the poly(A)⁺ RNA bound to the beads. The beads and mRNA were washed twice as described (Wrenzycki et al. 2001) and finally mRNA was eluted by incubation in 11 μl sterile water for 2.5 min at 68 °C. The recovered mRNA was immediately transcribed into cDNA (RT).

RT was performed in a volume of 20 μl with 10 × RT reaction buffer (Invitrogen), supplemented with 5 mM MgCl₂ (Invitrogen), 1 mM dNTP solution (Amersham Biosciences), 2.5 μl random hexamer primers (Applied Biosystems, Darmstadt, Germany), 20 U RNAsin (Applied Biosystems), and 50 U murine leukemia virus reverse transcriptase (Applied Biosystems) in a total of 11 μl mRNA sample. RT was performed in a thermal cycler with a program of 25 °C for 10 min for primer annealing and then 1 h at 42 °C for elongation and finally 5 min at 99 °C. The eluted cDNA was directly used for qPCR.

Table 5 Overview of experimental design and treatment groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>No treatment (control)</th>
<th>FSH</th>
<th>FSH + IGF1</th>
<th>FSH + IGF C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow (≥ 2nd lactation)</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Calf (6–9 months)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Quantitative real-time PCR

qPCR was performed as described recently by our laboratory (Niemann et al. 2010, Heinzmann et al. 2011). Twenty microliter reactions were set up in 96 well optical plates (Applied Biosystems). Each well contained 10 μl 2× Power SYBR Green PCR Master Mix (Applied Biosystems), 0.2 μM each of the forward and reverse primers, 2 μl cDNA (equivalent of 0.1 oocyte), and 6.4 μl H2O. The primer sequences and fragment sizes used for mRNA transcript analyses are summarized in Table 6.

An ABI 7500 Fast Real-Time System (Applied Biosystems) was used on a program consisting of 10 min at 95 °C (cDNA denaturation and Taq polymerase activation), and 40 cycles at 95 °C for 15 s, 60 °C for 1 min, and a final slow heating cycle to obtain the dissociation curves.

The cDNA dilution standards of pooled oocyte mRNA from adult cows and rabbit globin cDNA were included on every plate to provide standard curves for each gene. Standard curves were used to calculate the relative amount of each target gene to be normalized to the signal of the added globin mRNA. Sequence Detection Software 1.3.1 (Applied Biosciences) was used for quantification.

Methylation analyses

Repeat analyses

The developmental stage-specific methylation profiles of the bovine repeat sequences, BTS DNA, segment 2 (GenBank: J00032.1), and BTαS DNA (GenBank: AJ293510.1) were analyzed in pools from all experimental groups using pools of five immature or five in vitro matured oocytes (Kang et al. 2005). DNA extraction and bisulfite conversion were performed simultaneously using the EZ DNA Methylation-Direct Kit (Zymo Research, Irvine, CA, USA). Briefly, oocytes were treated with 13 μl M Digestion Buffer, 1 μl Proteinase K, and 12 μl H2O at 50 °C for 20 min and subsequently centrifuged for 5 min at 10 000 r.p.m. in a bench top centrifuge. Bisulfite conversion was performed in a thermal cycler with a program of 8 min at 98 °C, 3.5 h at 64 °C and 4 °C for storage after addition of 130 μl CT Conversion Reagent to 20 μl of the supernatant. Cleanup of the converted DNA was performed using the ZYMO-Spin IC column and buffers provided in the kit according to the manufacturer’s instructions. After final elution, the DNA was stored at −20 °C until further use. Analysis of the bisulfite-treated oocyte DNA was performed according to Kang et al. (2005) with slight modifications. Briefly, satellite sequence-specific PCR fragments were amplified using the primers and PCR conditions summarized in Table 7.

Successful amplification was confirmed by agarose gel electrophoresis and PCR products were cleaned up using the Wizard SV Gel and PCR Clean-Up System Kit (Promega) according to the manufacturer’s instructions. PCR products were ligated into the pGEM-T easy vector using the pGEM-T easy vector system (Promega) according to the manual and transformed into Escherichia coli XL10-Gold cells (Stratagene, Santa Clara, CA, USA).

Individual clones were picked and directly used for amplification of the insert using the universal T7 and SP6 primers (Table 7). These primers were also used for subsequent sequencing. Twenty-four bacterial clones per analyzed pool were sequenced and the BiQ Analyzer (MPI for Informatics, Saarland, Germany) was used for data analysis.
Gene-specific methylation

Methylation patterns of the promoter regions of the developmentally important genes SLC2A1, PRDX1, and ZAR1 were analyzed in immature oocytes and in in vitro matured oocytes retrieved from follicles >3 mm by OPU. For SLC2A1, the analyzed CpG sites lie between −225 and −384 bp relative to the transcription start site (TSS) of the mRNA, while the CpG sites of PRDX1 and ZAR1 are located between −160 and +11 bp and −192 and −15 bp respectively relative to the mRNA–TSS. In total, nine pools of oocytes in the group ‘calf’ (1/4), nine in ‘calf FSH’ (5/4) and eight in ‘cow FSH’ (4/4), five pools in the groups ‘calf’ (four immature/five IVM), five pools in the groups ‘calf FSH’ (four immature/five IVM), five pools in the group ‘calf FSH’ (3/3) were diluted to a final volume of 520 μl bisulfite-treated DNA (EZ DNA Methylation-Direct Kit; conversion rate was 95–100%). Ten microliters of DNA from pools of only a few cells (El Hajj et al. 2011, Heinzmann et al. 2011). In the present study, the bisulfite conversion rate was 95–100%. Ten microliters of bisulfite-treated DNA (EZ DNA Methylation-Direct Kit; conversion rate was 95–100%) were diluted to a final volume of 520 μl with PCR master mix (see below) and subsequently distributed into 20 PCR tubes (26 μl each). In a first amplification step, multiplex PCR containing the outer primers for the three genes was performed. Subsequently, gene-specific single PCRs with nested primers were used to amplify single DNA molecules. The sequencing performance of the CG-poor DNA was enhanced by tagging the inner primers with a stuffer sequence (Han et al. 2006) and further improved by additionally tagging the stuffer with an M13-tag. All primers used in multiplex and gene-specific PCR are summarized in Table 8. Briefly, the multiplex PCR mastermix contained 12% 10× ammonium buffer (200 mM ammonium sulfate, 750 mM Tris–HCl, and 0.1% Tween20), 240 μM dNTPs (100 mM dNTP Set; Invitrogen), 2.4 mM magnesium sulfate, 2.4× Enhancer (10× PCRx Enhancer Solution; Invitrogen), 0.4 μM of each outer primer, and 1 U Platinum Taq (Invitrogen) per each 25 μl of mastermix. Six negative controls were run (6×26 μl mastermix) to detect possible contamination during pipetting. For the second round of PCR, FastStart Taq Polymerase (Roche Diagnostics) was used according to the amplification protocol (Heinzmann et al. 2011).

Table 8 Primer sequences for direct bisulfite sequencing.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5’→3’)</th>
<th>Amplicon Length (bp)</th>
<th>Chromosomal Localization (bp)</th>
<th>Number of CpGs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SLC2A1 OR</td>
<td>AAAATTCACACAAAAAATTCCT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLC2A1 IF M13/stuffer</td>
<td>TGTGAAAAGAGCTATGACCCCGTTTACCTTATGAGTTTATGTTA</td>
<td>213 (285)</td>
<td>110 220 790–</td>
<td>11 221 002</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SLC2A1 IR M13/stuffer</td>
<td>CAGGAAATACCGACCCCCCGTTTACCTTATGAGTTTATGTTA</td>
<td>307</td>
<td>BTA 3, (−)-strand</td>
<td>107 467 332–</td>
</tr>
<tr>
<td>PRDX1</td>
<td>PRDX1 OF</td>
<td>TTATTTATTATTATTATGTTTATATGTTAAGATAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PRDX1 OR</td>
<td>AAAATTCACACAAAAAATTCCT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRDX1 IF M13/stuffer</td>
<td>CAGGAAATACCGACCCCCCGTTTACCTTATGAGTTTATGTTA</td>
<td>220 (292)</td>
<td>107 467 415–</td>
<td>107 467 634</td>
<td></td>
</tr>
<tr>
<td>PRDX1 IR M13/stuffer</td>
<td>CAGGAAATACCGACCCCCCGTTTACCTTATGAGTTTATGTTA</td>
<td>276</td>
<td>BTA 6, (+)-strand</td>
<td>69 751 593–</td>
<td>69 751 868</td>
</tr>
<tr>
<td>ZAR1</td>
<td>ZAR1 OF</td>
<td>AAATTTTTTTGTTATATTATTTATACATGCAAATTG</td>
<td>230 (302)</td>
<td>69 751 639–</td>
<td>69 751 868</td>
</tr>
<tr>
<td></td>
<td>ZAR1 OR</td>
<td>AAAATTCACACAAAAAATTCCT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZAR1 IF M13/stuffer</td>
<td>CAGGAAATACCGACCCCCCGTTTACCTTATGAGTTTATGTTA</td>
<td>276</td>
<td>BTA 6, (+)-strand</td>
<td>69 751 593–</td>
<td>69 751 868</td>
</tr>
<tr>
<td>ZAR1 IR M13/stuffer</td>
<td>CAGGAAATACCGACCCCCCGTTTACCTTATGAGTTTATGTTA</td>
<td>302</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

This table provides the primer sequences used for direct bisulfite sequencing of the studied genes, the length and chromosomal localization of the amplified segments (according to NCBI release of Btau_4.0, August 2010), and the number of CpG sites in the amplicons. O, outer primer; I, inner primer; F, forward primer; and R, reverse primer. Inner primers are tagged with an M13 (italic) and stuffer (bold) sequence. Amplicon length without brackets: original bovine bisulfite specific primed amplicon length; amplicon length in brackets: actual full amplicon length including M13/stuffer tag.

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The general PCR conditions were as follows: an initial denaturation step at 95 °C for 4 min was followed by 31–35 cycles of 95 °C for 30 s, primer-specific annealing temperature for 30 s and 72 °C for 45 s, and a final extension step at 72 °C for 5 min. Multiplex PCR was performed with an annealing temperature of 54 °C for 35 cycles. In the gene-specific second round of PCR, SLC2A1 and ZAR1 products were amplified at an annealing temperature of 58 °C for 31 cycles and PRDX1 at 54 °C for 32 cycles. Sequencing of the resulting PCR products was done on an ABI 3130xl automated sequencer.

### Statistical analysis

Kruskal–Wallis one-way ANOVA was used to analyze the methylation analysis was performed using comparison for the methylation analysis was performed using \( \chi^2 \)-test or Fisher’s test. In order to facilitate comparison between comparison between groups the ‘pairwise.prop.test’ (www.r-project.org) was used. For all tests, \( P \leq 0.05 \) was considered as being statistically significant.

### Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-12-0134.

### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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