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

Mike Diederich, Tamara Hansmann¹, Julia Heinzmann, Brigitte Barg-Kues, Doris Herrmann, Patrick Aldag, Ulrich Baulain, Richard Reinhard², Wilfried Kues, Christian Weißgerber¹, Thomas Haaf¹ and Heiner Niemann

Institute of Farm Animal Genetics (FLI), 31535 Mariensee, Neustadt, Germany, ¹Institute of Human Genetics, Julius Maximilians University, 97074 Würzburg, Germany and ²Max Planck Institute for Plant Breeding Research, 50829 Cologne, Germany

Correspondence should be addressed to H Niemann; Email: heiner.niemann@fli.bund.de

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' (BT α S). 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 BT α S 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.

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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

development (Jaenisch 1997, Kageyama *et al.* 2007, Sato *et al.* 2007, Lees-Murdock & Walsh 2008).

Here, we investigated the potential impact of age (prepubertal vs adult) and hormonal treatment (FSH and IGF1) of the donor animal on: i) DNA methylation and ii) mRNA expression of developmentally important, nonimprinted genes in bovine oocytes. We applied the recently developed and validated limiting dilution approach and direct bisulfite sequencing (El Hajj *et al.* 2011, Heinzmann *et al.* 2011) to analyze the methylation profile of individual DNA molecules in three nonimprinted genes, including solute carrier family 2 member 1 (*SLC2A1*/facilitated glucose transporter), peroxiredoxin 1 (*PRDX1*), and zygotic arrest gene 1 (*ZAR1*) from prepubertal and adult oocytes, collected from donors with and without treatment with FSH.

In addition, we analyzed differences in DNA methylation of two satellite sequences, i.e. 'bovine testis satellite I' (BTS) and 'Bos taurus alpha satellite I' (BT α S) by classical bisulfite sequencing. Furthermore, we analyzed the mRNA expression levels of the above genes, indicative of the developmental potential of oocytes, including growth factor response (growth differentiation factor 9 (*GDF9*)), response to oxidative stress (*PRDX1*), a maternal factor (*ZAR1*), and transport of energy (*SLC2A1*).

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 BT α S 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 BT α S 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 'cow 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 BT α S 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 BT α S 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 1 Ovum pick-up and morphological quality of oocytes from calves and cows.

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

Table 2 Methylation pattern of 'bovine testis satellite I (BTS) sequence' ($X \pm S.E.M.$).

Group	Immature oocytes; methylation (%)	<i>In vitro</i> matured oocytes; methylation (%)
Cow	49.6 $\pm$ 3.5 ^{c,A}	64.9 $\pm$ 6.3 ^{a,b,B}
Cow FSH	69.8 $\pm$ 3.4 ^{a,b}	59.7 $\pm$ 3.7 ^{a,b}
Calf	74.6 $\pm$ 3.9 ^{a,A}	53.4 $\pm$ 5.4 ^{b,B}
Calf FSH	64.7 $\pm$ 3 ^{a,b,c}	63.3 $\pm$ 5.7 ^{a,b}
Calf FSH + IGF1	60.6 $\pm$ 3.4 ^{a,b,c,A}	71.7 $\pm$ 5.2 ^{a,B}
Calf FSH + IGF C	69.6 $\pm$ 5.9 ^{a,b,c}	62.5 $\pm$ 4.5 ^{a,b}

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 ($P \leq 0.05$).

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 *SLC2A1*, 17 CpGs for *PRDX1*, and 18 CpGs for *ZAR1*.

On average, we recovered 12.9 of the 120 target molecules per pool of immature and 12.3 per pool of *in vitro* matured oocytes (Table 4). For immature oocytes, we obtained 26.3 copies per gene in the group 'cow', 16.7 in 'cow FSH', 5.3 in 'calf', 18.3 in 'calf FSH', 11.3 in 'calf FSH + IGF1', and 8.0 in 'calf FSH + IGF C'. For *in vitro* matured oocytes, we obtained on average 26.3 copies in the group 'cow', 20.7 in 'cow FSH', 17.7 in 'calf', 7.7 in 'calf FSH', 13.7 in 'calf FSH + IGF1', and 12.7 in 'calf FSH + IGF C'. All recovered DNA molecules were demethylated, as expected for the *SLC2A1*, *PRDX1*, and *ZAR1* promoter regions in oocytes. None of 258 DNA molecules from immature and of 296 from *in vitro* matured oocytes showed >50% methylation,

indicating that epimutations (at least in the three studied genes) are extremely rare or lack completely in the analyzed groups. Consistent with our recent studies (El Hajj *et al.* 2011, Heinzmann *et al.* 2011), the rate of single methylated CpG sites in the three studied genes varied between 0 and 2.4% (data not shown). No significant differences ($P > 0.05$) existed between the different oocyte groups.

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 + IGF C' (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 'cow', 'cow 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

Table 3 Methylation pattern of 'Bos taurus alpha satellite I (BTαS)' ($X \pm S.E.M.$).

Group	Immature oocytes; methylation (%)	<i>In vitro</i> matured oocytes; methylation (%)
Cow	66.4 $\pm$ 4.2	61.7 $\pm$ 2.1
Cow FSH	76.7 $\pm$ 4 ^A	52.5 $\pm$ 4.1 ^B
Calf	73 $\pm$ 2.7	70 $\pm$ 4.1
Calf FSH	72.8 $\pm$ 3.6 ^A	57.8 $\pm$ 2.7 ^B
Calf FSH + IGF1	66 $\pm$ 3.1	56.8 $\pm$ 2.2
Calf FSH + IGF C	68.9 $\pm$ 3.1	62.9 $\pm$ 3.9

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 ($P \leq 0.05$).

Figure 1 Schematic drawing of the main steps of limiting dilution bisulfite sequencing. (A) Immature and mature oocytes are collected by OPU or after IVF. 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, and Ns of the specific well on that plate. DNA from wells containing a PCR product is analyzed by direct bisulfite sequencing.

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

Table 4 Number of analyzed DNA molecules in three nonimprinted genes in bovine oocytes.

Oocyte group	Number of pools	Number of analyzed DNA molecules			All three genes		
		<i>SLC2A1</i>	<i>PRDX1</i>	<i>ZAR1</i>	Number of DNA molecules	DNA molecules per gene	DNA molecules per pool
Immature oocytes					258	12.9	14.3
Cow	4	33	18	28	79	19.8	26.3
Cow FSH	4	20	10	20	50	12.5	16.7
Calf	1	6	7	3	16	16	5.3
Calf FSH	5	26	16	13	55	11	18.3
Calf FSH+IGF1	3	18	5	11	34	11.3	11.3
Calf FSH+IGF C	3	12	1	11	24	8	8
Mature oocytes					296	12.6	15.4
Cow	5	33	18	28	79	15.8	26.3
Cow FSH	4	28	10	24	62	15.5	20.7
Calf	4	18	10	25	53	13.3	17.7
Calf FSH	4	7	5	11	23	5.8	7.7
Calf FSH+IGF1	4	17	6	18	41	10.3	13.7
Calf FSH+IGF C	3	13	10	15	38	12.7	12.7

Figure 2 Relative mRNA abundance of nonimprinted and developmentally important genes based on single oocyte preparations. Immature (black) and *in vitro* matured oocytes (gray). (a, b) Different superscripts within each treatment and gene indicate statistical significance ($P \leq 0.05$).

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 α 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 α 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 $47.6 \pm 23.2\%$ in adult rabbit oocytes, which is comparable with the results found for the BT α S 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, Thlie *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 (Duranton & 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 (Gurin *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 (≥ 2 nd 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 μ g 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 μ g rhIGF1 (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

Table 5 Overview of experimental design and treatment groups.

Group	No treatment (control)	FSH	FSH+IGF1	FSH+IGF C
Cow ($\geq$ 2nd lactation)	+	+	–	–
Calf (6–9 months)	+	+	+	+

second group were injected i.m. with 100 μ g 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 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 CS9000 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 (Servoprax, 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 $2\frac{3}{4}$ needles (Terumo, Eschborn, Germany) and a flexible tube system (2 mm $\times$ 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% heat-inactivated New Born Calf Serum (NBCS; PAA Laboratories, Coelbe, Germany), 36 μ g/ml Na pyruvate (AppliChem), 1 mg/ml glucose (Roth, Karlsruhe, Germany), 133 μ g/ml calcium chloride dehydrate (Fluka, Munich, Germany), 50 μ g/ml streptomycin (AppliChem), 6 μ g/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 μ m filter (Jürgens, Hannover, Germany), and washed with fresh PBS medium. Cumulus–oocyte–complexes (COCs) were flushed from the filter with PBS and retrieved under a stereomicroscope at $\times$ 50 magnification. COCs were collected in individual 50 μ l TCM-air drops, at pH 7.2 (TCM199; Sigma–Aldrich), containing 50 μ g/ml gentamicin sulfate (Sigma–Aldrich), 0.2 mM Na pyruvate (Sigma–Aldrich), 4.2 mM NaHCO₃ (Roth), and 1 mg/ml BSA (FAF; 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 pipetting 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 real-time 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 (FAF; Sigma–Aldrich). Subsequently, groups of five to ten washed COCs were placed in 50 μ l 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 μ l 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 pg 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 μ l 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 μ l 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 μ l with 10 $\times$ RT reaction buffer (Invitrogen), supplemented with 5 mM MgCl₂ (Invitrogen), 1 mM dNTP solution (Amersham Biosciences), 2.5 μ M 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 μ l 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 6 Primers used for quantitative real-time PCR.

Gene	Primer sequences (5'–3')	Annealing temp./no. of cycles (°C)	Amplicon length (bp)	Accession no.
<i>GDF9</i>	F: CCGTATGGCTCTCCAGTTCAC R: TGGCACTGAGGAGTCAAGTTTC	60 40	69	NM_174681.2
<i>SLC2A1</i>	F: CAGGAGATGAAGGAGGAGC R: CACAAATAGCGACACGACAG	60 40	258	NM_174602.2
<i>PRDX1</i>	F: TCAAGCCTGATGTCCAGAAGAGC R: CCGTCCTGTCCCTACACCAC	60 40	173	NM_174431.1
<i>ZAR1</i>	F: AGACTAGATGCTCCTGCCAGT R: TCTTGACGGTGGGGCCGTTTAT	60 40	82	NM_001076203.1
Globin	F: GCAGCCACGGTGGCGAGTAT R: GTGGGACAGGAGCTTGAAAT	60 40	257	X04751

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.

Table 7 Primers for analysis of repeat sequences.

Repeat/binding site	Primer sequences (5'–3')	Annealing temp./no. of cycles (°C)	Amplicon length (bp)	GenBank
BTS sequence	F: AATACCTCTAATTCAAAC R: TTTGTGAATGTAGTTAATA	49 35	221	AH001157.1
BTαS sequence	F: GATGTTTTYGGGGAGAGAGG R: CCRATCCCCTCTAATAAAAACC	61 35	154	AJ293510.1
T7	ACT CAC TAT AGG GCG AAT TG	55		
SP6	ATT TAG GTG ACA CTA TAG AAT ACT C	35		

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 ‘cow’ (four immature/five IVM), five pools in the groups ‘calf’ (1/4), nine in ‘calf FSH’ (5/4) and eight in ‘cow FSH’ (4/4), seven pools in the group ‘calf FSH+IGF1’ (3/4), and six pools in the group ‘calf FSH+IGF C’ (3/3) were analyzed. Bisulfite sequencing after limiting dilution is compatible with the analysis of minimum amounts 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; Zymo Research) 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× PCR_x 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 pipeting. 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.

Gene	Primer	Sequence (5'–3')	Amplicon Length (bp)	Chromosomal Localization (bp)	Number of CpGs
SLC2A1	SLC2A1 OF SLC2A1 OR	GTTTTATAGGATTTTAGTTATTGGTTAG AAAAATCACACAACCAAAAAATTCTC	239	BTA 3, (–)-strand 110 220 764– 110 221 002	
	SLC2A1 IF M13/stuffer	TGTAAAACGACGGCCAGTCCACTCACT- CACCCACCCGTTTTATAGGATTTTAGT- TATTGGTTAG			
	SLC2A1 IR M13/stuffer	CAGGAAACAGCTATGACCGGGTGG- GAGGTGGGAGGGAAAACT- CAAAAAAATAAAAAATCC	213 (285)	110 220 790– 110 221 002	14
PRDX1	PRDX1 OF PRDX1 OR	TTGTTTTATATTTATTAGTTTATTGAAATAG AAAAAATTAATAAAAAACCCCTCAACT	307	BTA 3, (–)-strand 107 467 332– 107 467 638	
	PRDX1 IF M13/stuffer	TGTAAAACGACGGCCAGTCCACTCACT- CACCCACCCGTTTTATATTTATTAGTTTATT- GAAATAGGAT			
	PRDX1 IR M13/stuffer	CAGGAAACAGCTATGACCGGGTGG- GAGGTGGGAGGGCCCCCAA- CAAAAAAATC	220 (292)	107 467 415– 107 467 634	17
ZAR1	ZAR1 OF ZAR1 OR	AAAGTTTTTTTGTAGATTATTTAAGAAT AAAACAACCATCAATATACCCTCA	276	BTA 6, (+)-strand 69 751 593– 69 751 868	
	ZAR1 IF M13/stuffer	TGTAAAACGACGGCCAGTCCACTCACT- CACCCACCCGGGTGTGGAATATTTTAT- TATTAAGGT			
	ZAR1 IR M13/stuffer	CAGGAAACAGCTATGACCGGGTGG- GAGGTGGGAGGG AAAACAACCATCAA- TATACCCCTAC	230 (302)	69 751 639– 69 751 868	18
Sequencing primer M13/stuffer R		TGTAAAACGACGGCCAGTCCACTCACT- CACCCACCC			
Sequencing primer M13/stuffer F		CAGGAAACAGCTATGACCGGGTGG- GAGGTGGGAGGG			

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.

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 gene expression data (Siegel *et al.* 1988). Group comparison for the methylation analysis was performed using χ^2 -test or Fisher's test. In order to facilitate 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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