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Methylation patterns in 5′ terminal regions of pluripotency-related genes in mature bovine gametes

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Summary

Gametogenesis is associated with DNA methylation and involves complicated and delicate gene regulation network in which stem cell marker genes exert their functions. Therefore, it is necessary to investigate DNA methylation profiles of those genes in mature gametes that have an effect on embryo development. However, to date, there are limited data available on these genes in mature gametes of bovine. Here we show methylation profiles in 5′ terminal regions of five pluripotency-related genes (*Oct4*, *Sox2*, *Nanog*, *Rex1* and *Fgf4*) in bovine mature gametes, based on the reasoning that the five genes harbour CpG islands in their own 5′ terminal regions, which are frequently the targets of DNA methylation. The results showed that *Oct4* and *Fgf4* exhibited significant hypermethylation in sperm compared with that in oocytes ($p < 0.01$), while *Sox2* and *Nanog* displayed relatively the same methylation levels between sperm and oocytes ($p > 0.05$). Additionally, *Rex1* showed a relatively high methylation level in sperm than in oocytes, although no significant differences were found ($p > 0.05$). In conclusion, bovine mature gametes exhibited two methylation profiles in terms of the five genes, one being non-sex-specific and the other being sex-specific.

Keywords: Bovine, DNA methylation, Oocyte, Pluripotency-related genes, Sperm

Introduction

The idea of 'epigenesis and development' coined by Waddington as early as 1957 (Patra *et al*., 2008) and which made the connection of epigenetics and development for the first time, suggested the important role of epigenetics in development. As for gametogenesis, this process involves overall demethylation followed by *de novo* methylation from primordial germ cells to mature gametes (Hajkova *et al*., 2002; Swales & Spears, 2005). Furthermore, it involves a complicated and delicate gene regulation network, in which stem cell marker genes exert important roles (Chambers *et al*., 2007; Geijsen & Jones, 2008; Kristensen *et al*., 2008; Pesce *et al*., 1998). Given that DNA methylation of the pluripotency-associated genes harbouring CpG islands in mature gametes could have an influence on embryogenesis, it is necessary to investigate their DNA methylation profiles in mature gametes. However, to date, there are limited data available on these genes in mature gametes of bovine. Here we chose five pluripotency-associated genes, namely *Oct4*, *Sox2*, *Nanog*, *Rex1* as well as *Fgf4*, as representatives and showed their methylation profiles in mature oocytes and sperm of bovine, based on the reasoning that the five genes harbour CpG islands in their own 5′ terminal regions, which are frequently the targets of DNA methylation.

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Table 1 Primer sequences

Gene	Primer sequence	T_m (°C)	Fragment size (bp)	GenBank accession no.
Oct4	F-5′-GATTTGGATGAGTTTTTAAGGGTT R-5′-ACTCCAACTTCTCCTTATCCAACTT	54.5	293	NC_007324
Sox2	F-5′-GTATTAAAAGAGTAAATTTAAGATTAAGTT R-5′-CAAATTAATAAACAACCATCCATATAAC	55.6	361	NW_001493904
Nanog	F-5′-TTTTTTAATTATAATTTGATGGGGT R-5′-CTAACACACCTTAAATAAACAAACC	52.4	288	NC_007303
Rex1	F-5′-AGTAGTTTGAGGATAGAGGTTAGGG R-5′-ACATACACCTAATAATCTAAAAAATCC	52.4	299	NW_001495362
Fgf4	F-5′-TTTATTTGAAGAAAGTGTATTAAGGGG R-5′-AATACAAATTCAAAAAAATCCTCCTC	52.4	223	NW_001494547

Materials and methods

In vitro maturation of oocytes

Holstein cow ovaries were collected from a local abattoir. The procedure of *in vitro* maturation of oocytes was carried out according to established methods in our laboratory (Hua *et al*., 2008). All the collected oocytes used for future work were of high quality in morphology and obtained from the same production line to eliminate the potential interference factors.

Collection of sperm

Bovine frozen–thawed semen was purchased from Keyuan Co., Ltd, Yangling, China. The preparation of sperm followed the previous method described (Wrenzycki *et al*., 2001). Capacitated sperm were used for DNA extraction.

Sodium bisulfite genomic sequencing

Extraction of genomic DNA from oocytes and sodium bisulfite treatment was combined using the EZ DNA Methylation-Direct™ Kit (Zymo Research). Genomic DNA from sperm was extracted with the E.Z.N.A.R Forensic DNA Kit (Omega), followed by quantitation using a NanoDrop™ ND-1000 spectrophotometer (Thermo Finnigan), and finally by sodium bisulfite treatment with the EZ DNA Methylation-Gold™ Kit (Zymo Research). All the procedures above were carried out following the manufacture's instructions strictly. The amplification of bisulfite-modified DNA was performed with ZymoTaq™ DNA polymerase (Zymo Research) in a reaction volume of 50 µl. Cycling conditions were 95 °C for 10 min followed by 40 cycles of 94 °C for 30 s, at an annealing temperature (T_m) for 40 s, then 72 °C for 30 s and a final extension of 7 min at 72 °C. The primer sets for the five genes were designed according to online software (http://www.urogene.org/methprimer/) with the exception of the Oct4 primers described previously (Lin *et al*., 2008). Details of primer sequences and T_m values are listed in Table 1. The locations of the CpG islands are indicated in Fig. 1. Given the sampling bias of PCR, three independent PCR reactions were performed. Next, the PCR products were purified using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research). Then, PCR products of three reactions were mixed together and cloned into a pMD18-T vector (TaKaRa), followed by verification using PCR. Finally, 10 colonies for each sample were sequenced.

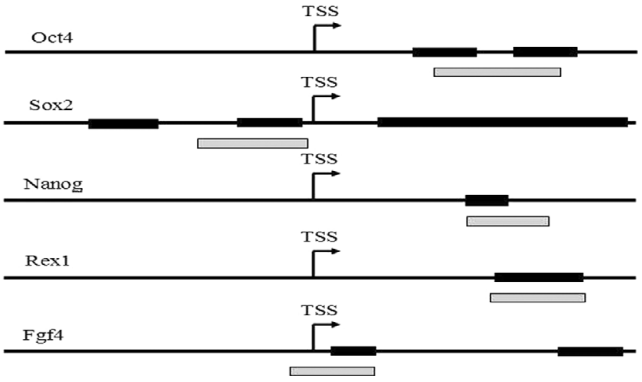

Figure 1 The locations of the CpG islands and amplified regions in the neighbourhood of transcription start sites (TSS) of five pluripotency-related genes (*Oct4*, *Sox2*, *Nanog*, *Rex1* and *Fgf4*). The black line represents the sequence surrounding TSS and spanning from –1 kb to +1 kb. The black box and the grey box indicate the CpG islands and the amplified regions for bisulfite analysis, respectively. The amplified regions of *Oct4*, *Sox2*, *Nanog*, *Rex1* and *Fgf4* are +399 to +691, −22 to −382, +450 to +737, +560 to +858 and −62 to +161, respectively.

Statistic analysis

DNA methylation levels of five genes were calculated in sperm and oocytes by BiQ-Analyzer software. Significant differences were determined using the

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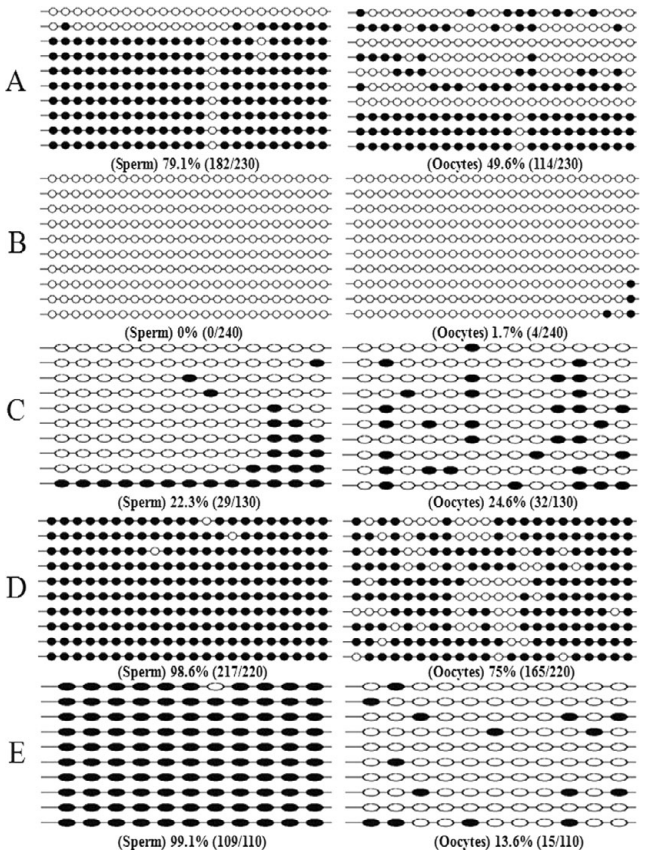

Figure 2 Methylation profiles of five pluripotency-related genes in mature gametes. (*A*), (*B*), (*C*), (*D*) and (*E*) respectively represent *Oct4*, *Sox2*, *Nanog*, *Rex1* and *Fgf4*. They respectively harbour 23, 24, 13, 22 and 11 CpG sites in the amplified CpG islands. Methylation levels are labelled below right. Each line and circle represents a sequencing result and a CpG site, respectively. Open and closed circles indicate unmethylated and methylated CpGs, respectively.

chi-squared test with statistical significance being accepted at $p < 0.05$.

Results and Discussion

Methylation profiles of five pluripotency-related genes

DNA methylation levels of *Oct4*, *Sox2*, *Nanog*, *Rex1* and *Fgf4* are shown in Fig. 2. Results showed *Oct4* and *Fgf4* exhibited significant hypermethylation in sperm compared with that in oocytes ($p < 0.01$) (Fig. 3), while *Sox2* and *Nanog* displayed relatively similar methylation levels between sperm and oocytes ($p > 0.05$) (Fig. 3). Additionally, *Rex1* showed a relatively high methylation level in sperm compared with oocytes, although no significant differences were found ($p > 0.05$) (Fig. 3).

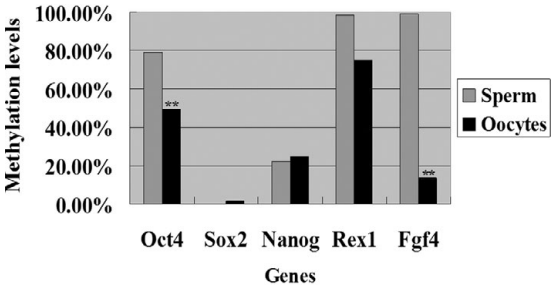

Figure 3 The diagram shows differences in DNA methylation levels of the five pluripotency-related genes between sperm and oocytes. **Extremely significant differences ($p < 0.01$).

With the completion of *de novo* methylation, the mature gametes harbour maximum overall DNA methylation level (Reik *et al*., 2003). However, our results showed hypermethylation of *Oct4*, *Rex1* as well as *Fgf4* and hypomethylation of *Sox2* and *Nanog* in sperm. According to a more recent report, 4% of the haploid genome in sperm is histone bound rather than protamine bound, with very significant overlap with hypomethylation regions and promoters for some developmental transcription factors (Hammoud *et al*., 2009), suggesting that *Sox2* and *Nanog* may be within the histone-enriched regions, while *Oct4*, *Rex1* and *Fgf4* may be within the protamine-enriched ones. Expectedly, the methylation levels of *Oct4* and *Sox2* in sperm of bovine were similar to that of human. However, surprisingly, the methylation level of *Nanog* showed the opposite result in sperm of bovine compared with that of human, which may be associated with differences between species. On the other hand, *Oct4* and *Rex1* exhibited the average methylation status (49.6%) and hypermethylation, respectively, while *Sox2*, *Nanog* and *Fgf4* displayed hypomethylation in oocytes, which may be due to the regulation of chromatin configuration to a certain extent. This aspect remains to be investigated. The above results suggested that it seems to be necessary for the given genes to maintain hypomethylation in mature gametes regardless of the global DNA methylation level, which may be beneficial for early embryo development and is consistent with the report on mouse that site-specific demethylation events occurred in mature germ cells (Kafri *et al*., 1992). Taken together, these results generally showed two methylation patterns, namely consistency and opposition, for the five genes we analysed in mature gametes, with the former being non-sex-specific and the latter sex-specific.

Through comparison between sperm and oocytes, it was observed that the methylation level of *Oct4* in oocytes significantly fails to reach the same as that in sperm ($p < 0.01$), although the average methylation

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status (49.6%) and hypermethylation existed in oocytes and sperm, respectively. As for *Rex1*, this gene also showed a relatively high methylation level in sperm than in oocytes, although no significant differences were found ($p > 0.05$). This finding may be explained by incomplete maturation of oocytes. However, the more reasonable explanation for the differences of methylation levels of *Oct4* and *Rex1* between sperm and oocytes is that the distinctions may reflect the time delay of demethylation. This idea is based on the fact that DNA in sperm and oocytes respectively is surrounded by protamines and histones. Differences in methylation may also affects the transition of protamines to histones in the male pronucleus after fertilization (Rousseaux *et al*., 2008), providing a relatively relaxed environment – namely from highly packaged to less packaged status, during which time demethylation of the paternal genome takes place prior to that of maternal genome and that both genomes reach the same minimum methylation level at the 8-cell or morula stage, depending on the species (Dean *et al*., 2003). As for *Sox2*, this gene remained unmethylated either in oocytes or in sperm, which coincided with the situations in fetal fibroblasts and in *in vitro* fertilized 8-cell embryos (data not shown), seeming that this analysed CpG island is not associated with the differentiation status; gametes, fetal fibroblasts and 8-cell embryos respectively represent three distinct differentiation situations. Additionally, *Nanog* possessed the similar low methylation levels not only in oocytes and sperm but also in IVF 8-cell embryos (data not shown), indicating that it may escape drastic demethylation action both in maternal and in paternal genomes from fertilization to the 8-cell stage (Dean *et al*., 2003). Finally, in contrast to the genes mentioned above, *Fgf4* exhibited another pattern, with hypomethylation in oocytes and hypermethylation in sperm, which is similar to the pattern of paternal imprinted genes, indicating that only the copy from paternal genome requires demethylation after fertilization. However, it should be taken into account that the present study was made only on *in vitro*-derived gametes and it is well known that *in vitro* conditions have profound effects on the epigenetic make-up of gametes. Therefore, results of this study would have to be confirmed in the future by analysis of *in vivo*-derived gametes.

Given that the methylation levels of gametes could have an effect on embryogenesis (Benchaib *et al*., 2005; Rousseaux *et al*., 2008; Hammoud *et al*., 2009), the contribution of the present study could serve as a theoretical basis for future work on bovine embryo development in assisted reproductive technologies, and provide a reference for methylation levels of donor cells used for nuclear transfer.

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