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SHORT COMMUNICATION

Expression of DNA Methyltransferase Genes in Four-Cell Bovine Embryos Cultured in the Presence of Oviductal Fluid

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With 1 figure and 1 table

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Summary

The possible epigenomic effect of oviductal fluid on expression of DNA methyltransferase (DNMT) genes was examined in early bovine embryos (4-cell stage). Real-time quantitative PCR was performed to determine the relative expression of DNMT1, DNMT3a and DNMT3b transcripts in embryos cultured *in vitro* in the presence or absence of oviductal fluid. Expression of DNMT1 significantly increased when cultured with oviductal fluid, whereas DNMT3a and DNMT3b transcripts were unaffected by the addition of oviductal fluid. These results may help reveal the role of oviductal factors in the regulation of DNMT expression.

Introduction

Genomic DNA methylation, catalysed by DNA methyltransferases (DNMTs), is an important mechanism of epigenetic gene regulation during gametogenesis and early embryogenesis. Mammalian DNMTs include three members: DNMT1 that maintains methylation patterns of hemimethylated CpG dinucleotides after DNA replication, and DNMT3a and DNMT3b which establish the initial methylation pattern *de novo* (Hermann et al., 2004). mRNA expression and enzymatic activity of DNMTs are coordinatedly regulated during pre-implantation embryo development (Shi and Wu, 2009).

Early embryonic development is often considered as a process regulated by the embryo itself. However, the embryo environment also influences regulation of the global pattern of gene expression through epigenetically regulated mechanisms (Chanson et al., 2011). In the oviduct, the gametes and the early embryo undergo epigenetic changes including DNA methylation. After fertilization, the zygote experiences extensive DNA demethylation in order to re-programme its genome to a totipotent state after which DNA re-methylation takes place (Shi and Wu, 2009). Thus, in an early stage, the oviductal environment may have a significant impact on the expression of developmentally important genes in the embryo (Wrenzycki et al., 2005).

Genomic imprinting could be influenced by factors that form the physiological microenvironment in the oviduct. In bovine embryos, highest activation of the embryonic genome is at the 8–16-cell stage, whereas at the 4-cell stage activation occurs to a lesser extent (Kues et al., 2008). Therefore, to examine the influence of oviductal fluid on epigenetic factors, the mRNA expression level of maternal DNMT1, DNMT3a and DNMT3b was analysed in oviductal-stage bovine embryos (4-cell stage embryos), cultured *in vitro* in the presence of bovine oviductal fluid.

Materials and Methods

Oviductal fluid collection

Genital tracts from beef cows (*Bos taurus*) were obtained from a local slaughterhouse and transported on ice to the laboratory. The stage of the oestrous cycle was assessed by post-mortem visual examination of the ovarian morphology (Ireland et al., 1980) and oviducts at metaoestrus and early diestrus were separated from the tracts. The bovine oviductal fluid (bOF) was collected by aspiration using a 200 μl automatic pipette. The fluid obtained from 20 oviducts was combined, centrifuged at 7000 × ***g*** for 10 min at 4°C to remove cellular debris, fractionated and then stored at −80°C until used. Total protein concentration of the pooled bOF

samples was measured with a Micro BCA™ Protein Assay kit (Pierce, Rockford, IL, USA).

In vitro embryo production

Ovaries were obtained from 75 beef cows sampled in a local slaughterhouse and transported to the laboratory at ambient temperature in phosphate-buffered saline (PBS, pH 7.4). Cumulus oocyte complexes (COCs) were recovered by aspiration with an 18-G needle, and follicular fluid containing COCs was transferred to 130 mm Petri dishes for morphological selection. Maturation of selected COCs was carried out in groups of 30–40 in 400 μl drops of TCM-199 medium (Gibco 12340, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Natocor, Córdoba, Argentina), 0.05 IU/ml FSH (Puregon, Organon, Dublin, Ireland), 0.23 mmol/l of sodium pyruvate (Sigma P4562, St. Louis, MO, USA) and 50 μg/ml of gentamicin for 22–24 h at 38.5°C under a 5% CO_2 atmosphere. For fertilization, COCs were washed in HEPES-TALP (Parrish et al., 1986), placed in groups of 30–40 in 400 μl drops of IVF-TALP (Parrish et al., 1986) and then inseminated with swim-up purified sperm ($1–3 \times 10^6$/ml) pooled from two different bulls at 38.5°C and 5% CO_2 in humidified air. Cumulus cells were removed approximately 18 h post-insemination by vortexing them during 1 min in TCM-199 (Gibco) supplemented with 3 mg/ml bovine serum albumin (BSA, Sigma A6003). Presumptive zygotes were cultured under mineral oil (Sigma M8410) in groups of 20–30 in 400 μl drops of TCM-199 containing 0.23 mmol/l of sodium pyruvate (Sigma P4562), 50 μg/ml of gentamicin and 6 mg/ml of BSA (Sigma A6003) or 10% (v/v) bOF to reach a final concentration of 6 mg of oviductal proteins/mL. Plates were incubated at 38.5°C under a 5% CO_2 atmosphere and good quality 4-cell stage embryos were selected and collected approximately 48 h after insemination.

RNA isolation and cDNA synthesis

Total RNA was isolated from five groups of pools of twenty 4-cell embryos obtained from oocytes collected on different days as described previously, using the RNAqueous® Micro Kit (Ambion, Darmstadt, Germany) according to the manufacturer's instructions. Embryos of each experimental group were cultured with the same oviductal fluid batch. The quality, purity and concentration (ng/μl) of isolated RNA were assessed using the Experion™ RNA HighSens system (Bio-Rad, Hercules, CA, USA). For synthesis of cDNA, 20 ng of total RNA was reverse transcribed with the iScript™ cDNA Synthesis Kit (Bio-Rad) using oligo (dT) and random hexamer primers following the manufacturer's instructions.

Table 1. Primers designed for real-time qPCR analysis

Gene	Primer sequence (5′-3′)	Amplicon length
DNMT1 (NM_182651)	Forward: GTACCAGTGCACCTTTGGCGT Reverse: GTGCGAACACATGCAACGGCT	134 bp
DNMT3a (XM_867643)	Forward: CTCCATAAAGCAGGGCAAG Reverse: TCATGTTGGAGACGTCGGTA	128 bp
DNMT3b (NM_181813)	Forward: AAGACCGGCCTTTCTTCTGGATGT Reverse: TGTGAGCAGCAGACACTTTGATGG	129 bp
GAPDH (NM_001034034)	Forward: AGATGGTGAAGGTCGGAGTG Reverse: GAAGGTCAATGAAGGGGTCA	117 bp

Real-time quantitative PCR

Gene expression was measured by qPCR using an iQ5 real-time PCR analysing system (Bio-Rad) and an SsoFast EvaGreen supermix with 4 μl of 1:8 diluted cDNA. Primer sequences used are described in Table 1. The expression of GAPDH mRNA was used as endogenous reference. The PCR programme consisted of an initial step of 1:15 min at 95°C, followed by 50 cycles of 15 s at 95°C and 30 s at 60°C for annealing and extension. At the end of each qPCR, melt curve analysis was performed for all genes to check the specificity of the products. The melting protocol consisted of 95°C for 1 min, followed by 60°C for 1 min, a step cycle with 70 repeats starting at 60°C for 10 s with a transition rate of +0.5°C/s and a final holding temperature of 25°C. A negative reverse transcription control was performed to check genomic DNA contamination. In addition, controls using RNase-free water instead of cDNA were carried out to prove that qPCR mixes were not contaminated with DNA. The amplification efficiency and correlation coefficient of a cDNA standard curve at five different 10-fold dilutions were determined for each gene. All target genes showed acceptable efficiency (80–100%) and correlation coefficients (close to 1.0). The relative expression was calculated using the formula $2^{-\Delta Ct}$ ($2^{-(\text{target Ct-GAPDH Ct})}$) (Schmittgen and Livak, 2008). Samples from each replicate pool were assayed in duplicate for each gene of interest and the reference gene. Results obtained for each gene in each pool of cDNA were normalized to GAPDH. Relative mRNA expression values between treatments (BSA or bOF supplement) were compared with Student's *t*-test. A probability <0.05 was considered statistically significant.

Results

Effect of bOF on gene expression levels of DNMT enzymes

Messenger RNA for DNMT1, DNMT3a and DNMT3b was detected in 4-cell embryos cultured with a supplement

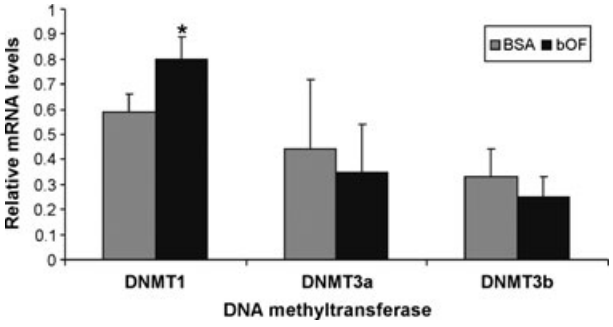

Fig. 1. Relative gene expression levels of DNMT1, DNMT3a and DNMT3b in 4-cell embryos cultured in TCM-199 supplemented with bovine serum albumin (grey columns) or bovine oviductal fluid (black columns). Bars indicate standard deviation (SD). *Shows significant differences between treatments.

of either BSA or fresh bOF. Transcript levels for DNMT1 were significantly higher in embryos cultured in medium supplemented with bOF ($P < 0.05$) compared with BSA (Fig. 1). In contrast, no significant difference in the relative expression of DNMT3a and DNMT3b was observed between the analysed groups (Fig. 1).

Discussion

The present study analysed the mRNA expression level of three DNMTs in 4-cell bovine embryos cultured in medium supplemented with BSA or fresh bOF. According to our results, the DNMT1 mRNA expression level significantly changed in 4-cell embryos cultured in medium supplemented with bOF compared to medium supplemented with BSA. The increase in DNMT1 mRNA did not seem to be related to the DNA demethylation process that occurs in the nuclear re-programming of embryos up to the 8–16-cell stage in cattle (Dean et al., 2003). During early development, DNMT1 is regulated to support demethylation and normal re-programming. It has been proposed that DNA methylation is passively lost due to the retention of DNMT1 in the cytoplasm until major genome activation, when it enters the nucleus (Lodde et al., 2009). A crucial event during embryonic development is the maintenance of DNA methylation marks of imprinted genes despite DNA demethylation (Kurihara et al., 2008). Therefore, our findings may suggest a relationship between the higher relative expression of DNMT1 messenger in experimental embryos and a suitable accumulation of DNMT1 transcripts that will be translated into protein in the cytoplasm when the translocation to the nucleus is required. In addition, no significant difference was observed between the relative gene expression of DNMT3a and DNMT3b in embryos cultured in the two media assayed, probably indicating little influence of oviductal factors on their mRNA's transcription. An effect of bOF factors on DNMT3a/b gene expression can not completely be discarded, because our study focused on a stage of embryonic development at which most genes experience a process of active demethylation.

Even though *in vitro* produced embryos during their early development do not need contact with the female reproductive tract to regulate their own cell division and differentiation, *in vitro* embryo development improved after addition of oviductal factors (Avilés et al., 2010). Rizos et al. (2007) reported that isolated mouse oviduct (IMO) supports the development of bovine embryos from the zygote to blastocyst stage in organ culture. Bovine blastocysts cultured in the presence of IMO showed different gene expression patterns of Glut-1, Cx43, E-cad, Oct-4, Sur, Sox, G6PDH, Na/K and IFN-τ compared with blastocysts cultured in the absence of IMO, inferring that IMO probably influences gene expression. Interestingly, the expression pattern of the genes studied in embryos derived from the IMO was similar to that of *in vivo* obtained embryos (Rizos et al., 2007). In the current study, the effect of oviductal fluid on the expression of DNMT1, DNMT3a and DNMT3b genes during development of 4-cell embryos was analysed *in vitro*. Our results suggest that factors presents in the oviductal fluid would influence the increase in mRNA expression of DNMT1. Lonergan et al. (2003) showed that transcript abundance of developmentally important genes between *in vitro* and *in vivo* (in ewe oviduct) cultured embryos differed, suggesting a strong influence of the oviduct environment on gene expression in pre-implanted embryos.

In conclusion, our findings suggest that oviductal fluid could affect the expression of epigenetic-related genes involved in DNA methylation during epigenetic re-programming of the embryonic genome.

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Conflict of Interests

The authors declare that they have no competing interests.

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