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Epigenetic Regulation of Methylated CpG Binding Protein MBD1 and its Effect on Developmental Gene Expression in the Bovine Embryo

HuiMin Wang, XuDong Ao, ZhenFei Wang, Na Zhang,
HaiXia Gao, HaiDong Bai, YongLi Yue and HaiQuan Yu
The Key Laboratory of Mammal Reproductive Biology and Biotechnology,
Ministry of Education, Inner Mongolia University, 010021 Hohhot, China

Abstract: To elucidate the epigenetic regulation of methylated CpG binding protein (*MBD1*) gene expression and its effect on other developmental genes, the expression of MBD1 and the DNA methylation status of its regulatory region were investigated in differentiated bovine tissues, during oocyte maturation and in the preimplantation embryo. Firstly, *MBD1* gene expression was analyzed by RT-PCR in bovine heart, liver, kidney, testis and ovary and at different stages of oocyte maturation and embryonic development. Dynamic changes in DNA methylation in the regulatory region of MBD1 were detected by bisulfite-sequencing and chromosome immunoprecipitation was used to investigate the relationship between MBD1 and the pluripotency genes *Nanog*, *Oct4* and *H1foo* or the maternal gene *ZAR1*. The results showed that the transcription level of the *MBD1* gene was lower in heart and kidney and the DNA methylation levels of the MBD1 regulatory region were also lower in heart and kidney than in liver, testis and ovary. The transcription level of MBD1 incresed stably during the *in vitro* maturation of oocytes but began to decline dramatically from the 4 cells embryo stage. However, the DNA methylation levels of the MBD1 regulatory region were identical during either oocyte maturation or embryonic development. MBD1 protein spatiotemporally binded to the promoter regions of the pluripotency genes *Nanog* and *Oct4* and the maternal genes *ZAR1* and *H1foo*. These results indicated that during the bovine preimplantation embryo stages from the germinal vesicle oocyte to the blastocyst, transcription of MBD1 was not controled by the mechanism of promoter DNA methylation and MBD1 protein may participates in the transcriptional regulation of some key developmental regulatory genes.

Key words: MBD1, early embryonic development, epigenetic regulation, pluripotency genes, maternal genes

INTRODUCTION

DNA methylation is the chief mechanism involved in epigenetic regulation of the genome in eukaryotes. DNA methylation plays a significant role in gene regulation and is the major suppressor of gene transcription. One of the main mechanisms of suppression is the specific binding of repressors, known as methylated CpG binding proteins, to methylated DNA (Bachman *et al*., 2001). These proteins compete with transcription factors for binding to methylated DNA. The Methyl Binding Domain (MBD) family consists of methylated CpG binding proteins and plays a central role in DNA methylation-associated regulation of gene expression (Santos *et al*., 2002).

MBD1 is a member of the MBD family and contains a MBD domain, a Transcriptional Repression Domain (TRD) and multiple CxxC structures. The MBD can bind methylated CpG sites and recruits histone modification enzymes and the TRD is associated with suppression of transcription. When the TRD is lost, MBD1 will not suppress transcription (Wade, 2001). CxxC is the key factor involved in binding and suppressing nonmethylated DNA and is involved in suppression of both methylated and nonmethylated promoters (Fujita *et al*., 2000). In mouse, MBD1 can be detected in the chromosomes in oocytes in meiosis metaphase and is distributed uniformly in the cytoplasm and nucleus after fertilization. At the 8-16 cell stage, MBD1 is located in only the nucleus and decreases dramatically at the blastula stage. This is possibly due to changes in maternal zygotes (Ruddock-D'Cruz *et al*., 2008). However, there has been little study to date concerning the variation of expression of MDB1 and its relationship with other genes during bovine oocyte maturation and early embryo development. The experiments started at the early stages of embryo development and studied the epigenetic regulation of development-associated genes by MBD1. Researchers designed primers for the *MBD1* gene

Corresponding Author: HaiQuan Yu, The Key Laboratory of Mammal Reproductive Biology and Biotechnology, Ministry of Education, Inner Mongolia University, 235 West College Road, 010021 Hohhot, China

regulatory area and analyzed the methylation changes in this region in bovine heart, kidney, liver, testis and ovary using bisulfite sequencing. Researchers then investigated changes in MBD1 transcription using Reverse Transcription PCR (RT-PCR) and changes in the methylation status of the MBD1 regulatory area in oocytes and at different stages of embryo development. Finally, researchers analysed the binding patterns of MBD1 protein with the promoters of pluripotency and maternal genes by Chromatin Immunoprecipitation (ChIP), in order to investigate the potential transcriptional regulation of MBD1 to the these genes.

MATERIALS AND METHODS

Reagents: The DNA Extraction kit was purchased from Qiagen (Hilden, Germany). The RNA extraction and RT-PCR kits were from TaKaRa (Dalian, China). The Chromatin Immunoprecipitation (ChIP) kit was from Life Technologies (Carlsbad, CA, USA). The ChIP grade anti-MBD1 antibody (ab3753) was from Abcam (Cambridge, England). All primers were synthesized by Shanghai BioAsia Biotechnology Co. (Shanghai, China). Unless otherwise stated, all other reagents used were purchased from Sigma (St. Louis, MO, USA).

Bovine tissues samples: Bovine heart, kidney, liver, ovary and testis were harvested and cut into 1 cm^3 pieces. The samples were washed three times with saline, frozen in liquid nitrogen and stored at -80°C.

Oocyte recovery and embryo production: Bovine ovaries were collected at a local abattoir and transported in a 0.9% sterile NaCl aqueous solution. Cumulus-Oocyte Complexes (COCs) were aspirated from 2-8 mm follicles with an 18-gauge needle syringe. COCs with at least five layers of cumulus cells were selected for *in vitro* maturation and embryo production as previously described (Blondin and Sirard, 1995). COCs were allowed to go through *in vitro* maturation after three washes with M199 (Gibco, Life Technologies Co., Grand Island, NY, USA) supplemented with 1% FBS (Sigma-Aldrich Co., St. Louis, MO, USA). Groups of 80-100 COCs were placed in one well of a 4 well plate with 1 mL maturation medium (M199 supplemented with 10% FBS, 0.38 mM sodium pyruvate, 0.5 mg mL^{-1} Follicle-Stimulating Hormone (FSH), 0.5 mg mL^{-1} Luteinizing Hormone (LH), 1 μg mL^{-1} estradiol, 0.075 mg mL^{-1} penicillin and 0.05 mg mL^{-1} streptomycin) covered with mineral oil (M1180; Sigma-Aldrich Co.). The 4 well plates containing COCs were incubated in a humidified atmosphere for 24 h at 38.5°C with 5% CO_2.

In Vitro Fertilization (IVF) was conducted following a standard protocol established in the lab. Briefly, after 10 sec of gentle shaking, a semen straw was thawed for 10 sec in a 37°C water bath. Sperm were then washed twice by centrifugation (first spin: 3,500 rpm; second spin: 3,000 rpm) for 5 min in 10 mL of Brackett and Oliphant (1975)'s (BO) sperm-washing medium containing 3 mg mL^{-1} of Bovine Serum Albumin (BSA, Sigma-Aldrich Co.) supplemented with 10 mM caffeine (Sigma-Aldrich Co.). The washed sperm pellet was then resuspended in BO sperm-washing medium at a concentration of 1.0×10^6 sperm mL^{-1} for subsequent IVF.

After *in vitro* maturation, bovine COCs were washed twice and transferred into a 50 μL drop of BO medium (30 oocytes/drop) containing 6 mg mL^{-1} of BSA (Sigma-Aldrich Co.) and 10 mg mL^{-1} of heparin (Sigma-Aldrich Co.) pre-equilibrated for 2 h at 38.5°C in 5% CO_2 in humidified air; 50 μL of sperm suspension was then added to each drop of medium. Oocytes were incubated with sperm for 6 h at 38.5°C in 5% CO_2 in humidified air. After IVF, embryos were further cultured in CR1 medium supplemented with 6 mg mL^{-1} of BSA (Sigma-Aldrich Co.) for 48 h at 38.5°C in 5% CO_2 in humidified air. Cleaved embryos were then selected and cultured for an additional 5 days in CR1 medium supplemented with 5% FBS on cumulus cell monolayers in an atmosphere of 5% CO_2 in air.

RT-PCR: MBD1 mRNA levels were detected by RT-PCR. Briefly, total RNA was extracted from 200 oocytes and 100 embryos, dissolved in diethylpyrocarbonate-treated water and quantified using a spectrophotometer. The cDNA was synthesized from 1 μg total RNA and PCR was carried out using the following primers: MBD1, sense 5'-AAAC AAGGCGTCCTCTGCTA-3', antisense 5'-CGGGTACC ATCTCCTGAAAA-3'; *GAPDH*, sense 5'-TTCAACGG CACAGTCAAGG-3', antisense 5'-ACATACTCAGC ACCAGCATCAC-3'. Each 50 μL PCR reaction mixture contained 1 μL cDNA template, PCR buffer, 1.25 U ExTaq DNA polymerase and 0.15 mM primers. The PCR conditions consisted of an initial denaturation step at 94°C for 2 min, followed by 35 cycles of denaturing at 94°C for 40 sec, annealing at 58°C for 40 sec and polymerization at 72°C for 1 min and a final extension at 72°C for 10 min. PCR products were separated by electrophoresis on a 1.5% agarose gel followed by ethidium bromide staining. The target bands were analyzed densitometrically using a GS-800 calibrated densitometer and gel analyzing software (Bio-Rad, Richmond, CA, USA) and the results were calculated as a ratio of the OD value relative to GAPDH.

Table 1: PCR primer sequences for chromatin immunoprecipitation

Genes	Forward primers (5'-3')	Reverse primers (5'-3')	Length (bp)	GenBank accession number
Nanog	GTGTTTCCCGTTTCCG	GAGCCATCCAGTCCAA	236	ENSBTAG00000021111
Oct4	TACCAGGCATCACAGTTT	AGGCAGTTCCCATTTAG	257	ENSBTAG00000020916
H1foo	ACCCAAATACACCCTG	AAGCGACAAAGAACAC	200	NM001035372
ZAR1	GGGACTGGTTCCATCTTC	AGCACTCTAGGGCATCGT	250	NW001495189

DNA methylation analysis: Total DNA was extracted from bovine heart, kidney, liver, ovary and testis using a DNA Extraction kit according to the manufacturer's protocol. DNA was digested with EcoRI, alkaline-denatured, treated with bisulfite and recovered as previously described (Hattori *et al*., 2004). For oocytes and embryos at different stages after *in vitro* fertilization, the samples were first embedded in low melting point agarose gel and the DNA was then treated as described above. PCR primers for bisulfite-sequencing were designed according to the published *MBD1* DNA sequence (GenBank ID: 540558) (sense 5'-ATGTAGAAAAGGGAAGAGGTAG-3' and antisense 5'-AAGTTGGGTTAGGAAAGTAT-3') and the PCR product was 464 bp. The PCR conditions were as follows: 94°C, 5 min for initial denaturation; 35 cycles of 94°C for 40 sec, 58°C for 40 sec and 72°C for 2 min with a final extension at 72°C for 10 min. The PCR products were recovered and inserted into a PMD18-T vector and transformed into competent *E. coli*. The recombinant vectors were identified and 10 clones of each sample were used for sequencing.

Chromatin Immunoprecipitation (ChIP) analysis: Oocytes at the Germinal Vesicle (GV) and Metaphase II (MII) stages and 8 cells stage embryos were collected and fixed for 10 min in 1% formaldehyde solution, resulting in DNA and protein crosslinking (Nelson *et al*., 2006). The crosslinking was then stopped by treating with glycine for 5 min at room temperature. Chromatin was subjected to 10 cycles of 14 pulses of ultrasonic shearing using an Omni Ruptor 250 ultrasonic homogenizer (Omni International, Marietta, GA, USA) set at a 40% power output. Samples were incubated with anti-MBD1 antibody for 30 min followed by centrifugation at 10,000 g for 10 min. The supernatant was incubated with streptavidin magnetic beads for 30 min at 4°C on a rotator (Labnet Int., Edison, NJ, USA). Immunoprecipitates were eluted from the beads by two consecutive elution steps with elution buffer and crosslinks were reversed at 65°C for 2.5 h. Proteinase K (20 μg mL^{-1}) was added and the samples were incubated for 1 h at 37°C. DNA samples were recovered and used for PCR with the primers listed in Table 1.

Statistical analysis: Three independent experiments were performed in triplicate. MBD1 mRNA expression data were analyzed using the one-way ANOVA. DNA methylation data from bisulfite sequencing were analyzed using the χ^2-test. All statistical analyses were performed with SPSS 14.0 for Windows software (SPSS Inc.). The $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Expression of MBD1 mRNA in tissues, oocytes and at different stages of embryonic development: MBD1 mRNA was detected by RT-PCR in oocytes, embryos at different stages and in five bovine tissues using GAPDH to normalize the expression and water as a negative control. The expression levels were analyzed with ImageJ Gray Scale Software and the results are shown in Fig. 1. The length of the PCR product detected by agarose gel electrophoresis corresponded to the anticipated length in all five bovine tissue samples (Fig. 1a). ImageJ analysis of three sets of PCR results for each sample confirmed that MBD1 was expressed in bovine heart, kidney, liver, testis and ovary. However, the expression level in heart and kidney was significantly lower than in the other tissues ($p<0.05$) and the transcription level was highest in ovary and testis (Fig. 1a and d).

Researchers then amplified the fragment from oocytes and embryos at different stages and the length of the PCR product again corresponded to the anticipated length (Fig. 1b and c). Analysis using ImageJ demonstrated that MBD1 was expressed in GV and MII oocytes and the expression levels gradually increased during the process of *in vitro* maturation (Fig. 1e). However, in the early embryos, MBD1 was only expressed at the 2 cells stage at approximately the same level as in oocytes at 24 h of *in vitro* maturation and was not detected from the 4 cells stage to the blastula stage (Fig. 1f).

Changes in DNA methylation of the *MBD1* gene regulatory area in different tissues, oocytes and *in vitro* zygotes: Researchers designed primers for the 5' regulatory area of MBD1, in order to perform PCR with bisulfite-treated genomic DNA and sequence the resulting fragments. After bisulfite treatment, the product amplified using these primers was 464 bp as anticipated.

The results of methylated sequence analysis for 12 CpG sites in 10 clones from each of the five tissues are shown in Fig. 2a. The rate of CpG methylation for MBD1 was lower in heart and kidney than in other tissues (49.17 and 30.00%, respectively), however, the levels in liver,

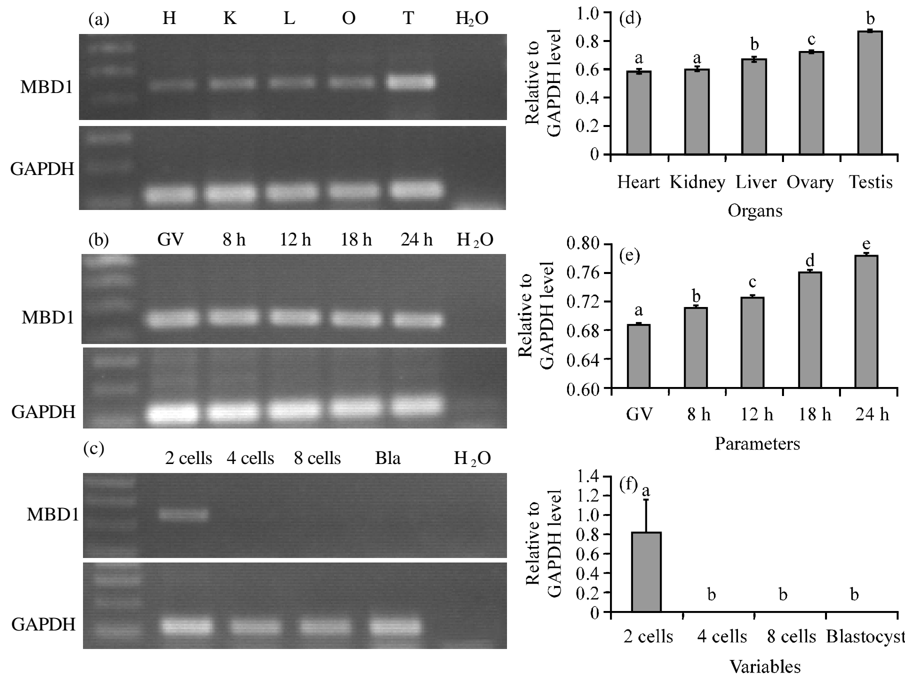

Fig. 1: Expression of MBD1 mRNA; MBD1 mRNA expression was detected by RT-PCR in; a) tissue samples from bovine Heart (H), Kidney (K), Liver (L), Ovary (O) and Testis (T); b) oocytes at different stages of maturation; c) embryos at different developmental stages. GADPH was used to normalize expression levels and water was used as a negative control. d-f) PCR results from three independent experiments were analyzed using ImageJ Software and the quantified results, expressed as OD ratios normalized to GADPH, respectively. Data shows mean±SD of three independent experiments. Different letters above the columns indicate $p<0.05$. GV: Germinal Vesicle; Bla: Blastocyst

ovary and testis were significantly higher (87.50, 81.67 and 79.17%, respectively; Fig. 2d). There were also differences between the tissues with respect to the particular sites which were methylated or unmethylated (Fig. 2a).

The methylation rate at each stage of oocyte maturation or development of the fertilized embryo *in vitro* remained constant at 83.33%. The methylated and unmethylated sites were identical at each stage. The CpG sites 443 and 696 bp upstream of ATG were both unmethylated but all other sites were methylated (Fig. 2b and c). No significant differences were observed between stages (Fig. 2e).

Binding patterns of MBD1 to pluripotency and maternal genes: Researchers selected the pluripotency genes *Nanog* and *Oct4* and the maternal genes *ZAR1* and *H1foo* to test the relationship between these genes and MBD1 protein by ChIP analysis. The results showed that MBD1 protein binded to the promoters of Nanog, Oct4 and H1foo but not to that of ZAR1 in GV oocytes. At the MII stage, MBD1 protein binded to the promoters of Nanog, H1foo and ZAR1 but not to that of Oct4. However, in 8 cells stage embryos, MBD1 protein binded to the promoter of H1Foo but not to those of the other three genes (Fig. 3 and Table 2). From these results, researchers deduced that MBD1 binds to the promoters of different genes at different stages of development to control their transcription.

DNA methylation is one of the major mechanism by which gene expression is regulated. Highly methylated DNA suppresses gene transcription. DNA methylation also plays an important role in the regulation of mammalian development and differentiation. Various methylation patterns are observed in different species and genetic backgrounds and also in different tissues of the same species (Chambers *et al.*, 2003). The study shows that the changes in the transcription levels of MBD1 between different tissues and different developmental stages of oocytes and *in vitro* zygotes is possibly due to differential methylation of the regulatory DNA in the *MBD1* gene.

In the five bovine tissues, the DNA methylation rate in heart and kidney was lower than in liver, testis and ovary; the transcription level of MBD1 was also lower in

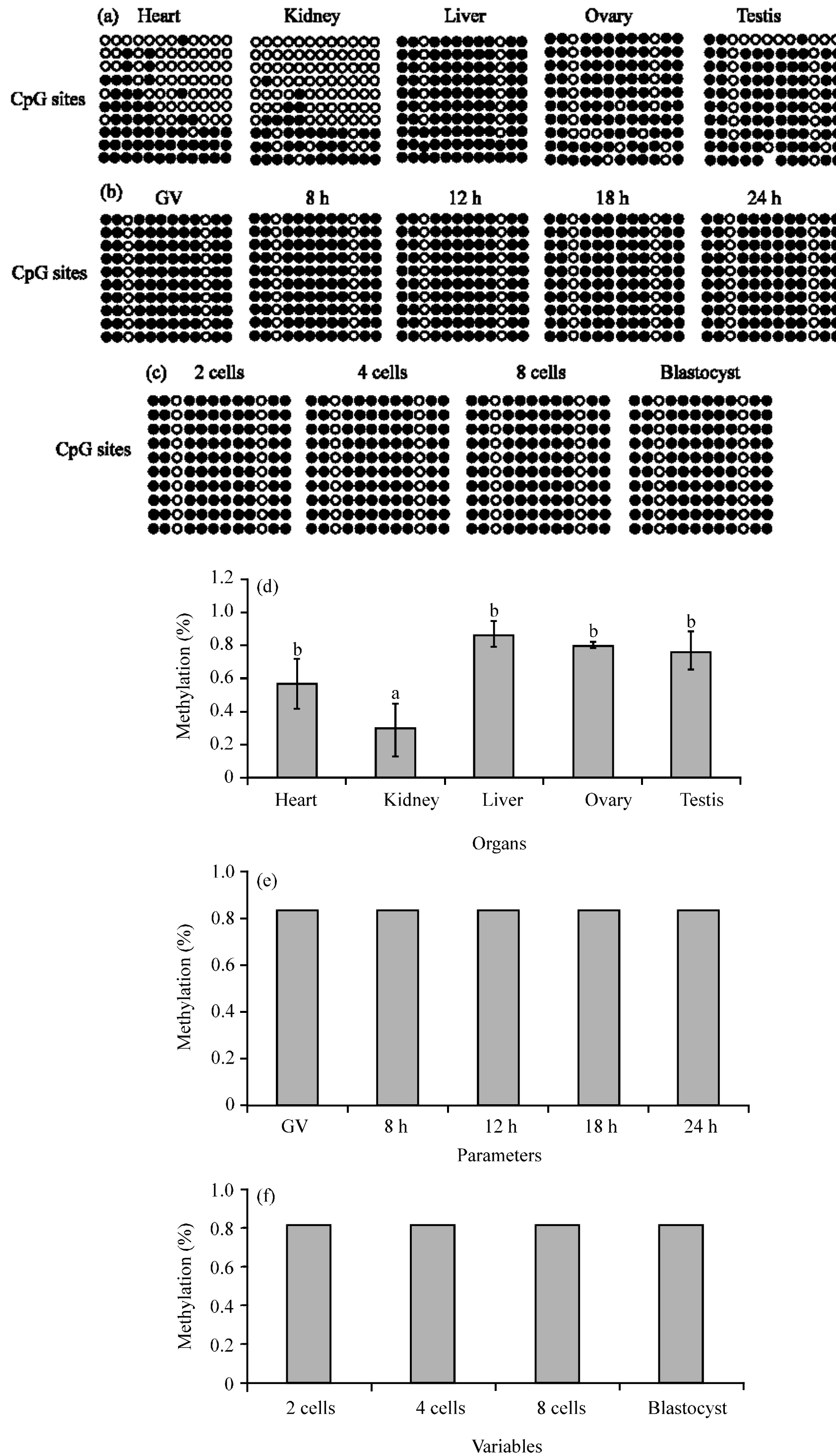

Fig. 2: Methylation status of MBD1; Bisulfate sequencing was used to analyze the methylation status of the regulatory area of the *MBD1* gene. The charts show the methylation status at 12 CpG sites in 10 clones from; a) five different bovine tissues; b) different stages of oocyte maturation; c) different stages of embryo development. Black circles represent methylated CpG sites, white circles represent unmethylated CpG sites; d-f) the percentage of methylation in tissues, oocytes and embryos, respectively. Different letters above the columns indicate $p<0.05$

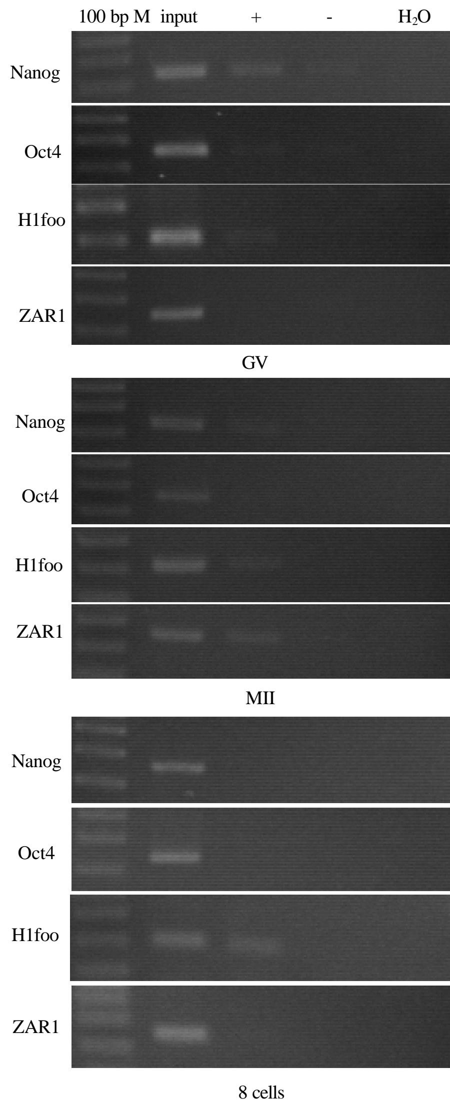

Fig. 3: Results of chromatin immunoprecipitation (ChIP). ChIP was carried out using antibody to MBD1 and PCR primers for the promoters of *Nanog*, *Oct4*, *H1Foo* and *ZAR1* genes. Plus (+) and minus (-) indicate the presence and absence of antibody, respectively; water was used as a negative control. GV: Germinal Vesicle; M: Markers; MII: Metaphase II stage. Three ChIP analysis was performed and one representative result was shown

heart and kidney compared with other tissues. Neither the methylation rate of MBD1 regulatory DNA nor the expression of MBD1 mRNA showed any significant changes during oocyte maturation. The methylation rate of MBD1 regulatory DNA also remained unchanged at different stages of embryonic development but the expression level of MBD1 dropped dramatically from the 4 cells stage; no expression was detected at the 8 cells stage. The research suggests that the regulation of DNA methylation affects the transcription of MBD1. However, the transcription levels of MBD1 did not always correspond with the DNA methylation pattern of the 5' regulatory area, suggesting that the transcription of MBD1 is affected by both DNA methylation and other regulatory factors.

Table 2: Results of chromatin immunoprecipitation

Stages	Nanog	Oct4	H1foo	ZAR1
GV	+	+	+	-
MII	+	-	+	+
8 cells	-	-	+	-

'+': Linkage of MBD1 with related gene; '-': No linkage of MBD1 with related gene; GV: Germinal Vesicle; MII: Metaphase II

To date, research has not clarified how DNA methylation affects gene expression. Possible mechanisms of gene inactivation and suppression of transcription by highly methylated CpG sites are as follows (Kurosaka *et al*., 2004; Mitsui *et al*., 2003): the highly methylated promoter area (including transcription factor binding sites) inhibits the accession of gene promoters by transcription factors; highly methylated Methyl-CpG-binding Proteins (MeCP) lead to inactivation of the gene by Histone Deacetylases (HDAC). MeCPs can specifically bind to methylated DNA; this family includes MBD1, MBD2, MBD3, MBD4 and MeCP2. These proteins can bind to methylated DNA in competition with transcription factors, leading to changes in chromosome structure affecting gene expression. The study tested the influence of MBD1 on the expression of Nanog, Oct4, H1foo and ZAR1 which are maternal or pluripotency genes.

Nanog and Oct4 are both important pluripotency genes. Nanog plays an important role in regulating the self-renewal of inner cell mass during embryo development and maintains the pluripotency of the ectoderm by inhibiting its differentiation to primitive endoderm (Bachman *et al*., 2001; Sharma *et al*., 2009). Oct4 also maintains the pluripotency of embryo stem cells and their ability to self-renew. An *in vitro* study showed that the transcriptional signal of these genes is very weak at the 2, 4, 8 cells and even 16 cells stages but increases at the morula and blastula stages (Tate and Bird, 1993). The results from ChIP showed that MBD1 did not bind to the Nanog or Oct4 promoters at the 8-16 cells stage in bovine embryos, suggesting that the transcriptional inhibition of Nanog and Oct4 starts to be released from this stage, in preparation for their expression at the morula and blastula stages.

According to this analysis, although, the expression of the two pluripotency genes increased from the morula and blastula stages, the associated epigenetic regulation factors which bind to the promoter areas began to

dissociate at an earlier development stage, facilitating the conversion from an inactive chromosome conformation to an active state.

H1foo is a specific binding protein in oocytes and it is a subtype of the linker histone H1. H1foo is key to the maturation of oocytes, conformation of sperm chromosomes and reprogramming of the embryo (Tanaka *et al*., 2003). H1foo is only expressed in specific tissues and stages during embryo development and its expression pattern is almost the same in different species. *In situ* hybridization and immunostaining of oocytes and early mammalian embryos showed that H1foo mRNA and protein expression starts to occur in primitive follicles and increases as the follicles mature (Tanaka *et al*., 2001). However, during the oocyte maturation and embryo development, H1foo mRNA and protein expression decreases dramatically. The expression of *H1foo* mRNA in 2, 4 and 8 cells stage zygotes is only 41, 28 and 7% of the levels in GV stage oocytes, respectively and almost disappears in morula and blastula stage zygotes (McGraw *et al*., 2006). Using ChIP assay, researchers showed here that MBD1 protein binded to the H1foo promoter in GV and MII oocytes and 8-16 cells stage embryos. This suggests that MBD1 protein participates in the transcriptional suppression of H1foo during bovine oocyte maturation and embryo development.

ZAR1 is a specific ovarian maternal effect gene. A related study has shown that the expression of ZAR1 mRNA is in a state of dynamic change during bovine oocyte maturation which is high at GV stage and decreases as the oocyte matures. The previous study has shown that in bovine embryos, ZAR1 transcription is high at the 2, 4 and 8 cells stages and decreases at the morula and blastula stages (Xin *et al*., 2009). In this study, researchers demonstrated that MBD1 protein binded to the promoter of ZAR1 in MII oocytes but not in GV oocytes. MBD1 protein dissociated from ZAR1 promoter again in 8 cells stage embryos. Taken together, these data indicate that MBD1 participates in the transcriptional suppression of ZAR1 during oocyte maturation and later dissociates from the promoter to make way for transcriptional factors in early merogenesis. The binding of transcription factors with the ZAR1 promoter then reactivates ZAR1 transcription.

Research shows that as well as chromosome remodeling, massive selective suppression of genome transcription occurs during the maturation of oocytes, in parallel with massive RNA and protein synthesis and accumulation. It is important to produce abundant maternal energy for recovery from meiosis and for the fertilization and development of early embryos. At the same time, suppression of transcription of the total genome in oocytes occurs at a precise time, pre-ovulation, providing the opportunity for a silent start of transcription (De La Fuente and Eppig, 2001; Tan *et al*., 2009). H1foo and ZAR1 transcription are both suppressed and the dynamic link between MBD1 and the two genes demonstrates the importance of MBD1 in this process, involving regulation of the structure of the chromosome. During embryo development, plentiful supplies of maternal material stored during oocyte maturation are consumed and zygote genes are activated to induce embryo development. The study shows that Nanog and Oct4 are regulated by MBD1 during the development of the embryo, through dynamic binding to their promoters to suppress transcription.

CONCLUSION

The data supports the hypothesis that during embryo development, MBD1 protein binds to the promoters of specific genes at particular times, leading to transcriptional suppression of these genes. Other members in the MBD family may regulate the expression of other genes by a similar process. This means the transcriptional regulation of some key developmental regulatory genes by MBD1 is both selective and spatial.

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