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Non-CpG hypermethylation in placenta of mutation-induced intrauterine growth restricted bovine foetuses

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ABSTRACT

The existence of non-CpG methylation in mammalian DNA has mainly been observed in embryonic stem cells, but its functional significance is uncertain. We found an age-dependent non-CpG hypermethylation in DMR at the 3′ end of the *MIMT1* in the placenta of intrauterine growth restricted foetuses in cattle. Data suggest that this DMR play a role in epigenetic regulation of the PEG3 domain.

1. Introduction

The placenta epigenome is critical for normal placental function [1], with important programming events occurring during early pregnancy. After fertilisation, both paternal and maternal genomes undergo demethylation [2]. Establishment of the correct epigenetic pattern in the trophoblast is vital for formation of the foetal side of the placenta, and epigenetic factors play an important role in placental maturation and development [3].

Cytosine DNA methylation-driven transcriptional regulation plays a significant part in regulating the epigenome. Aberrant methylation patterns are associated with intrauterine growth restriction (IUGR) [4]. In humans, loss of methylation at the *H19* locus is found in Silver Russell syndrome [5]. In mice, hypermethylation of the *IGF2* differentially methylated domain leads to prenatal growth restriction [6]. In cattle, aberrant *PEG3*, *H19*, *IGF2*, *IGF2R* methylation patterns are associated with IUGR, late abortion and stillbirth [7–9].

We previously identified and characterised a deletion of the 3′ end of the *MIMT1* (MER1 repeat containing imprinted transcript 1) gene that, when inherited from the male parent, causes IUGR, late abortion and stillbirth in cattle [10–12]. *MIMT1* is a non-protein coding gene within the imprinted PEG3 (paternally expressed gene 3) domain. Most PEG3 domain genes are expressed during embryogenesis, but also in adult ovary, testis and brain [13–15]. Mouse *Peg3* is the most studied, and known to be involved in control of prenatal growth and development [16] and maternal nurturing behaviour [16].

Prenatal mortality observed in bovine $MIMT1^{Del/wt}$ foetuses is closely linked with IUGR and is probably a consequence of insufficient nutrient and oxygen supply through the placenta [17]. $MIMT1^{Del/wt}$ stillborn calves are typically ~50% undersized [10].

Many imprinted domains are controlled by imprinting control regions (ICR) and interactions between one or more non-protein coding genes and neighbouring protein-coding genes. It has been suggested that the PEG3 domain is regulated by long-range genomic interactions, with an evolutionary conserved region (ECR) in the middle part playing a key role [18]. This corresponds to the location of the *MIMT1* gene. The $MIMT1^{Del}$ allele thus provides a useful tool to investigate PEG3 regulation and the role of *MIMT1* in placental function.

In our earlier study, global DNA methylation analysis revealed markedly higher genome-wide methylation in cotyledons of $MIMT1^{Del/wt}$ compared to normal bovine foetuses [11]. Here we used the same tissue samples to investigate DNA methylation patterns of three well-conserved ECRs in *MIMT1* in cotyledons of $MIMT1^{Del/wt}$ bovine foetuses.

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0006-291X/

2. Materials and methods

2.1. Animals and tissue sampling

DNA methylation analysis was performed on samples of cotyledon (foetal placenta side) collected from 12 foetuses, six *MIMT1*$^{Del/wt}$ and six *MIMT1*$^{wt/wt}$. Foetuses were collected at two developmental stages: three *MIMT1*$^{Del/wt}$ and three *MIMT1*$^{wt/wt}$ at 43 ± 5 days; three *MIMT1*$^{Del/wt}$ and three *MIMT1*$^{wt/wt}$ at 89 ± 7 days. Samples were immediately frozen in liquid nitrogen and stored at −80 °C for further analysis.

2.2. Bisulphite sequencing

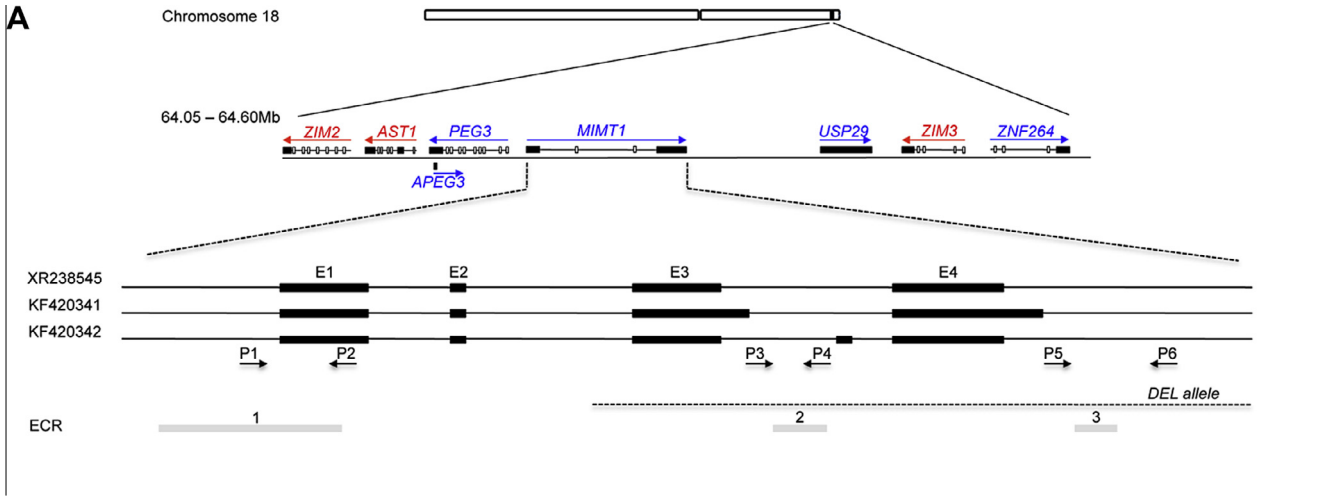
Genomic DNA was prepared by standard methods using proteinase K digestion and phenol–chloroform extraction. Bisulphite treatment of 500 ng genomic DNA was performed using the EpiTect Fast DNA Bisulphite Kit (Qiagen) according to the manufacturer's protocol. Bisulphite-treated DNA was amplified using a nested PCR strategy and the sequence determined. PCR primers were designed using BiSearch (http://bisearch.enzim.hu) and those used were: P1 – GGAGGATATTGGTAATTTATAGA; P2 – AACAAATA AAACTACTTAC; P3 – ATGGAGGATAGGGAGGAGTT; P4 – CCTAATCT AAAAAAATCCCA; P5 – TAGATTTAATGGTGTGGAAA; P6 – AACTA AAACCTTCTAAAATAAAC. Amplified PCR products were cloned into the pJET vector (Fermentas) and transformed into bacteria. Single colonies were sequenced and CpG methylation quantified using the QUMA tool (http://quma.cdb.riken.jp/).

2.3. 3′ rapid amplification of cDNA ends (3′ RACE)

3′ RACE analysis was performed using the FirstChoice® RLM-RACE kit (Ambion) according to the manufacturer's protocol. Total RNA was extracted from cotyledon tissue using Trizol reagent (Invitrogen). The sequence of two novel *MIMT1* transcripts has been deposited in the NCBI database under accession numbers KF420341 and KF420342.

3. Results and discussion

The PEG3 domain extends over 500 kb on bovine chromosome 18 (BTA18), between Megabase pairs 63.9 and 64.5 (based on the NCBI Btau_4.6.1 bovine genome assembly). The non-protein coding *MIMT1* gene is located in the middle part of the PEG3 domain (~200 kb), where we previously detected and characterised a 128 kb allele deletion [10,11]. Comparative genomic analyses revealed several ECRs within this region (Fig. 1), data consistent with that recently reported by others [18]. Three well-conserved regions were selected for *MIMT1* DNA methylation analysis: a putative ICR (imprinting control domain) in the promoter region



E

	Overall CpG methylation (%)		
	P1/P2	P3/P4	P5/P6
MIMT1$^{WT/WT}$	16,8	63,9	89,4
MIMT1$^{WT/DEL}$	10,5	70,0	100 ***

P-value: *** < 0.001

Fig. 1. Schematic presentation of *MIMT1* DNA methylation in bovine cotyledons. (A) Alternative mRNA transcripts detected in cotyledon using 3′ RACE. The GenBank mRNA sequence XR238545 was used as a reference. DNA methylation pattern in the *MIMT1*$^{wt/wt}$ and *MIMT1*$^{Del/wt}$ bovine foetuses at: (B) promoter region, ECR1; (C) intron 3, ECR2; (D) 3′ end, ECR3. (E) Summary of DNA methylation. ECR – evolutionary conserved region. P1–P6 – primer localisation.

(P1/P2, 336 bp, BTA18: 64052546–64052882 bp) termed ECR1; part of intron 3 (P3/P4, 290 bp, BTA18: 64090163–64090453 bp) termed ECR2; and a 3′ region (P5/P6, 269 bp, BTA18: 64131186–64131455 bp) termed ECR3.

We first carried out 3′ RACE analysis and identified two novel *MIMT1* transcripts (*MIMT1_A*:KF420341 – 1427 bp; *MIMT1_B*:KF420342 – 426 bp) in the $MIMT1^{wt/wt}$ cotyledons and found that ECR1 region is transcribed (Fig. 1A). These two transcripts were not found in $MIMT1^{Del/wt}$ samples, consistent with paternal monoallelic expression. Bisulphite sequencing was then used to analyse 43 CpGs in ECR1, 10 in ECR2, and 10 in ECR3. ECR2 and 3 are located within the deletion region of the $MIMT1^{Del}$ allele. Eight to twelve single colonies per sample were quantified. We detected hypomethylation of ECR1 (16.8% for $MIMT1^{wt/wt}$, 10.5% for $MIMT1^{Del/wt}$, ns), close to mean methylation in ECR2 (63.9% vs. 70.0%, ns) and hypermethylation in ECR3 (89.4% vs. 100%, $P < 0.001$) (Fig. 1B–E). The CpG1, CpG2 and CpG5 sites in ECR3 showed the greatest difference in DNA methylation: 50% in $MIMT1^{wt/wt}$ and 100% methylation in $MIMT1^{Del/wt}$ samples (Fig. 1D). It may be relevant that a L1 retrotransposon element is located near the *MIMT1* 3′ end, retrotransposons are commonly silenced and hypermethylated in mammalian genomes [19]. The complete DNA methylation observed in $MIMT1^{Del/wt}$ foetuses indicates that we amplified the maternal allele. Genomic regions responsible for silencing the maternal allele would be highly methylated. The difference in methylation patterns between $MIMT1^{wt/wt}$ and $MIMT1^{Del/wt}$ foetuses as well as that we amplified hypermethylated maternal allele in $MIMT1^{Del/wt}$ foetuses suggests that ECR3 (at the *MIMT1* 3′ end) can be designated as maternal DMR (differentially methylated region) which may play a role in epigenetic regulation of this gene and possibly the PEG3 domain.

Further analysis of ECR3 revealed aberrant methylation of a non-CpG at the CAG site in the three younger $MIMT1^{Del/wt}$ foetuses (41 days), while the three older $MIMT1^{Del/wt}$ foetuses (91 days) were non-methylated in this region, as indicated by the sequence TAG. All $MIMT1^{wt/wt}$ cotyledon samples were non-methylated (Fig. 2). Three different bisulphite conversions and cloning experiments were performed and we can exclude that the observed difference is due to the incomplete bisulphite conversion. Non-CpG methylation has been observed in an L1 element in human embryonic fibroblasts [20]. Non-CpG DNA methylation represents 20–25% of global DNA methylation in human and mouse ES cell lines [21], and arises as result of Dnmt3 *de novo* methylation [22]. Recently, non-CpG methylation in somatic tissues such as brain, muscle and placenta was detected [23]. The age-dependent non-CpG methylation in the $MIMT1^{Del/wt}$ samples we observed could possibly be explained by the presence of stem cells and Dnmt3 enzyme activity at the earlier developmental stage [22,24]. Decreased Dnmt3 activity during placental development would reduce further *de novo* non-CpG methylation [22,25]. Loss

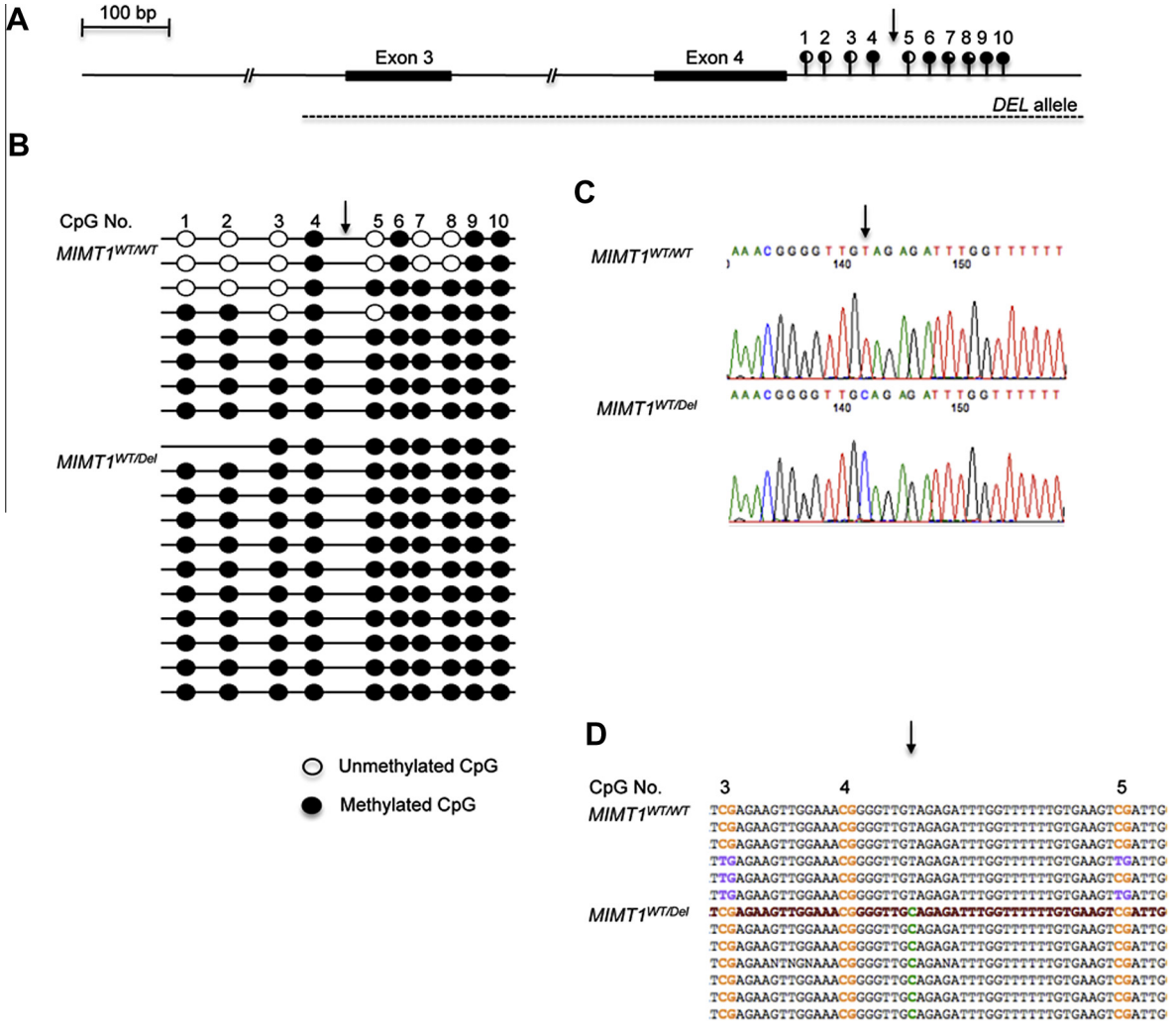


Fig. 2. Non-CpG DNA methylation in DMR at the *MIMT1* 3′ end in bovine cotyledons. (A) Partial genomic structure of *MIMT1*. (B) Representative DNA methylation plots from one $MIMT1^{wt/wt}$ and $MIMT1^{Del/wt}$ sample. (C) Bisulphite sequencing chromatograms showing CAG/TAG site (arrow). (D) Bisulphite nucleotide sequence alignment.

of non-CpG methylation has also been observed in mitotically dividing cells during male germ-cell development [26]. In the early placenta of *MIMT1*$^{Del/wt}$ foetuses we found amplification of the methylated CAG trinucleotide only. However because of the heterogeneous nature of the tissue, we cannot exclude the presence of very low level unmethylated TAG. Alternatively, preferential amplification of bisulphite treated templates can occur in the same primer pair due to biases during PCR amplification and molecular cloning [27]. We however observed either methylated or unmethylated amplification within the same sample, not a mixture, and therefore believe that our results are technically valid.

This study has highlighted that certain well-conserved methylation sites at the *MIMT1* 3′ end region, putatively DMR, may be involved in the epigenetic regulation of the bovine PEG3 domain. More extensive methylation mapping for the entire PEG3 domain is required to confirm its regulatory role. However, the location of this putative DMR is consistent with recent findings showing a long range genomic interaction between the middle part of the PEG3 domain and the *Peg3* promoter in foetal mouse brain [18]. Our work also raises the intriguing possibility of a role for non-CpG methylation in IUGR. This is of particular interest because non-CpG DNA methylation has not yet been functionally associated with a defined phenotype. Recent studies show that non-CpG methylation can be detected at higher resolution, providing a launching point to investigate the function of this newly characterised phenomenon. Further work should also be directed to decipher the epigenetic role of non-CpG methylation in the diverse cell types of the early placenta.

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