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DNA methylation dynamics of a maternally methylated DMR in the mouse *Dlk1–Dio3* domain

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ABSTRACT

The mouse delta-like homolog 1 and type III iodothyronine deiodinase (*Dlk1–Dio3*) imprinted domain contains three known paternally methylated differentially methylated regions (DMRs): intergenic DMR (IG-DMR), maternally expressed 3-DMR (*Gtl2*-DMR), and *Dlk1*-DMR. Here, we report the first maternally methylated DMR, CpG island 2 (CGI-2), is located approximately 800 bp downstream of miR-1188. CGI-2 is highly methylated in sperm and oocytes, de-methylated in pre-implantation embryos, and differentially re-methylated during post-implantation development. CGI-2, similarly to *Gtl2*-DMR and *Dlk1*-DMR, acquires differential methylation prior to embryonic day 7.5 (E7.5). Both H3K4me3 and H3K9me3 histone modifications are enriched at CGI-2. Furthermore, CCCTC-binding factor (CTCF) binds to both alleles of CGI-2 *in vivo*. These results contribute to the investigation of imprinting regulation in this domain.

1. Introduction

Imprinted genes exhibit allele-specific transcription in a parent-of-origin dependent manner [1]. Most known imprinted genes are clustered and co-regulated by regional control elements that are differentially methylated on the two parental chromosomes [2]. Identification of differentially methylated regions (DMRs) has greatly contributed to our understanding of imprinting regulation [3]. Allele-specific DNA methylation can be established either in the germline or post-fertilization (defined as germline and somatic DMRs, respectively) [4]. The *Dlk1–Dio3* imprinted domain on mouse chromosome 12qF1 contains three paternally expressed protein-coding genes, multiple maternally expressed long non-coding RNA genes, and numerous miRNAs and snoRNAs [5–10]. The expression of long non-coding RNAs and lots of miRNAs clustered in this domain is aberrantly silenced in most mouse induced pluripotent stem (iPS) cells, and is predictive for their developmental potential [11–13]. There are three known paternally methylated DMRs (IG-DMR, *Gtl2*-DMR and *Dlk1*-DMR) [14]. IG-DMR is a germline DMR that functions as the imprinting control region [15,16], *Gtl2*-DMR is a somatic DMR that spans the *Meg3*/*Gtl2* promoter [17,18].

A previous study found that, when received a randomly integrating adeno-associated virus (AAV) vector, mice developed hepatocellular carcinoma (HCC) that contained integrated vector genomes within the *Dlk1–Dio3* domain. All four insertion sites mapped to a 6-kb region, including the miR-341 locus [19]. Genes adjacent and telomeric to the AAV vector integration sites were up-regulated in all tumor samples examined [20]. Another study using a modified *sleeping beauty* transposon system also found this locus is involved in HCC [21]. Transcriptome analysis showed that transposon integration near miR-341 led to overexpression of numerous long non-coding RNAs and miRNAs in this domain [22]. These results suggest that insertional mutagenesis around miR-341 can cause overexpression of upstream or downstream genes. However, the underlying mechanism is not very clear. We hypothesized that there might be some unknown regulatory sequences in close proximity to miR-341. Interestingly, a previous study suggested a potential DMR is located near miR-341 (and upstream of *Rtl1* on the reverse strand) [23]. In this study, we focused on two small predicted CpG islands (CGI-1 and CGI-2) in intron 2 of *Meg8*. While CGI-1 (chr12:110849680-110849939, mm9) spans miR-341, CGI-2 (chr12:110850932-110851278) is located ~800 bp downstream of miR-1188 (Fig. 1). We performed detailed methylation analysis of CGI-1 and CGI-2 throughout mouse embryonic development, and found that CGI-2 is a maternally methylated somatic DMR.

2. Materials and methods

2.1. Ethics statement

All animal experiments were carried out according to the guidelines for the care and use of experimental animals approved by the National Institutes for Food and Drug Control of China (http://

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www.nicpbp.org.cn/sydw/CL0249/2730.html). Purchased mice were housed in standard plastic cages with a 12-h light–dark cycle in the Center for Experimental Animal of Harbin Institute of Technology. Pregnant mice were sacrificed by cervical dislocation to obtain mouse embryos from morula to E18.5. This study had received the approval to sacrifice the pregnant mice and use their embryos from the Ethical Committee of Harbin Institute of Technology.

2.2. Mouse samples

6–8 weeks old C57BL/6 J (BL6) and ICR (also called CD-1) mice were purchased from Vital River Laboratory Animal Technology Co. Ltd (Beijing, China). Early embryos and fetal tissues of F1 hybrids were obtained by reciprocally crossing BL6 and ICR mice. Breeding was performed overnight. The presence of a vaginal plug in the morning was defined as embryonic day 0.5 (E0.5). Spermatozoa were obtained from the vas deferens of adult male BL6 and ICR mice. MII oocytes were collected from the ovaries of superovulated 6–8 weeks old female BL6 and ICR mice, and washed in CZB medium containing hyaluronidase (Sigma–Aldrich) to remove the cumulus cells. Tissues used for methylation analysis and ChIP assays were isolated from E15.5 BIF1 (BL6♀ × ICR♂) and E18.5 IBF1 (ICR♀ × BL6♂) embryos.

2.3. DNA extraction and bisulfite sequencing analysis

Genomic DNA samples were isolated from E15.5 and E18.5 tissues and mature sperm by standard method using proteinase K (Roche, Mannheim, Germany) digestion, RNase A (Fermentas) treatment, and followed by phenol/chloroform extraction and ethanol precipitation. Bisulfite modification of the isolated DNA was performed using the EZ DNA methylation-Gold kit (Zymo Research, cat# D5005, Orange, CA, USA) according to the manufacturer's instructions. Bisulfite-treated samples were amplified by nested PCR using Zymo*Taq*™ DNA Polymerase (Zymo Research, cat# E2001). Primer sequences, SNP information and PCR conditions are listed in Table S1. PCR products were subcloned into pMD19 T-Vector (TaKaRa, cat# 3271, Dalian, China). Plasmids were isolated from selected clones and sequenced by the ABI PRISM 3500 Genetic Analyzer (Applied Biosystems, CA, USA) using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

2.4. Methylation analysis in early embryos

Before subjected to bisulfite treatment, numerous oocytes or several early embryos at each of the stages were pooled, the numbers are as follows (partially referred to [18]): ~200 ICR oocytes, ~140 BL6 oocytes, eleven BIF1 or thirteen IBF1 morulae, eight BIF1 or six IBF1 blastocysts, four BIF1 or five IBF1 whole embryos at E5.5, two BIF1 or three IBF1 whole embryos at E6.5, one BIF1 or two IBF1 whole embryos at E7.5. BIF1 and IBF1 embryos were separately analyzed at once. All pooled samples were directly subjected to proteinase K digestion and bisulfite treatment using the EZ DNA methylation-direct kit (Zymo Research, cat# D5020). PCR and cloning procedures are the same as described above.

2.5. Chromatin immunoprecipitation (ChIP) assays

ChIP experiments were performed using a ChIP assay kit (Upstate Biotechnology, cat# 17-295) according to the manufacturer's instructions. Briefly, ~50 mg liver or placenta tissue was homogenized in 1 × PBS containing protease inhibitors (1 mM PMSF, 1 μg/μl pepstatin, and 1 μg/μl aprotinin), treated with formaldehyde to final concentration of 1% at 37 °C for 10 min, and resuspended in SDS lysis buffer. The cross-linked DNA was sheared by sonication to 200–1000 bp in length. After centrifugation, the whole-cell extract was diluted and precleared with protein A agarose slurry to reduce non-specific background. For immunoprecipitation, 5 μg of anti-Tri-Methyl-Histone H3 (Lys4) (Cell Signaling Technology, #9751), 5 μg of anti-Histone H3 (tri methyl K9) (Abcam, ab8898), 5 μg of anti-CTCF (Upstate Biotechnology, 07-729) or 5 μg of normal rabbit IgG was added, and the mixture was incubated overnight at 4 °C with rocking. Next morning, the immune complexes were collected, washed in a series of buffers and reverse cross-linked. DNA was purified through phenol/chloroform extraction and used as templates for PCR amplification. PCR products were analyzed by running on 1.5% agarose gel with ethidium bromide staining, purified and directly sequenced.

3. Results

3.1. Methylation analysis of CGI-1 and CGI-2 in mid to late gestation somatic tissues

To find CGIs in the vicinity of mouse miR-341 and miR-1188, the online software CpG Island Searcher (http://cpgislands.usc.edu/) [24] was used with the following parameters: minimum length 200 bp, GC content greater than 50%, and ratio CpG observed/expected greater than 0.60. As a result we found two CGIs, named CGI-1 and CGI-2 (Fig. 1). To determine the methylation patterns of CGI-1 and CGI-2, bisulfite sequencing analysis

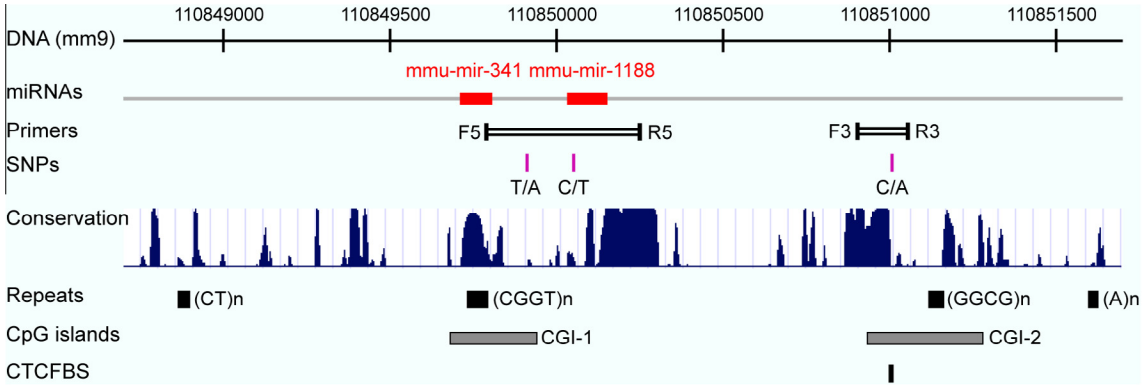

Fig. 1. Schematic representation of the loci of miR-341/miR-1188 and CGI-1/CGI-2. The precursor sequences of miR-341 and miR-1188 are located 227 bp apart. While CGI-1 overlaps miR-341, CGI-2 is located ~800 bp downstream of miR-1188. SNPs T/A and C/A are used to distinguish the two parental alleles of CGI-1 and CGI-2, respectively. CGI-1 contains (CGGT)n tandem repeats, and CGI-2 contains (GGCG)n tandem repeats. The conservation chromatogram was downloaded from the UCSC Genome Browser. Primers F3/R3 flank the third predicted CTCF binding site within CGI-2.