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Sequences in the *H19* ICR that are transcribed as small RNA in oocytes are dispensable for methylation imprinting in YAC transgenic mice

Takuya Takahashi [a], Hitomi Matsuzaki [a], Shin-ichi Tomizawa [b,1], Eiichi Okamura [a], Tomoko Ichiyanagi [b], Akiyoshi Fukamizu [a], Hiroyuki Sasaki [b], Keiji Tanimoto [a,*]

[a] *Faculty of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki, 305-8577, Japan*
[b] *Division of Epigenomics, Department of Molecular Genetics, Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka, 812-8582, Japan*

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ABSTRACT

Allele-specific methylation of the endogenous *H19* imprinting control region (ICR) is established in sperm. We previously showed that the paternal *H19* ICR in yeast artificial chromosome (YAC) transgenic mice (TgM) was preferentially methylated in somatic cells, but not in germ cells, suggesting that differential methylation could be established after fertilization. In this report, we discovered small RNA molecules in growing oocytes, the nucleotide sequences of which mapped to the *H19* ICR. To test if these small RNA sequences play a role in the establishment of differential methylation, we deleted the sequences from the *H19* ICR DNA and generated YAC TgM. In somatic cells of these mice, methylation imprinting of the transgene was normally established. In addition, the mutant fragment was not methylated in sperm and eggs. These data demonstrate that sequences in the *H19* ICR that correspond to the small RNA sequences are dispensable for methylation imprinting in YAC TgM.

1. Introduction

Imprinted expression of a subset of genes in mammals is controlled by parent-of-origin-dependent DNA methylation of the differentially methylated regions (DMRs) (Ferguson-Smith, 2011). The DMRs are *de novo* methylated during gametogenesis (establishment), maintained in the somatic cells after fertilization, and then demethylated in primordial germ cells of embryos (Bartolomei, 2009; Sasaki and Matsui, 2008; Schaefer et al., 2007). While many DMRs are methylated maternally (Lucifero et al., 2002), a few, such as the *H19*, *Dlk1–Dio3*, and *Rasgrf1* DMRs, are paternally methylated (Li et al., 2004). Because differences in the structural features between maternally and paternally methylated DMRs have not been fully elucidated (Jia et al., 2007; Ooi et al., 2007), mechanisms specifying how DNA methyltransferases (DNMTs) recognize their specific targets in the appropriate germ line are, for the most part, obscure.

The reciprocal imprinting of the paternally expressed *insulin-like growth factor 2* (*Igf2*) gene and maternally expressed, non-coding *H19* gene is governed by the combined action of the *H19* DMR and a shared enhancer, located upstream and downstream of the *H19* gene, respectively (Bartolomei, 2009). Therefore, the *H19* DMR is also referred to as an imprinting control region (ICR). Four binding sites for the CTCF (CCCTC-binding factor) insulator protein in the *H19* ICR are responsible for imprinted *Igf2*/*H19* gene expression (Bell and Felsenfeld, 2000; Hark et al., 2000), as well as for maintaining the maternally inherited *H19* ICR in an unmethylated state (Schoenherr et al., 2003). Thus, although the mechanisms describing how imprinted methylation of the *H19* ICR is maintained and how imprinted expression is achieved in the locus are both reasonably well understood, the *cis* DNA sequences responsible for methylation imprinting establishment at the *H19* ICR have never been identified (Bowman et al., 2003; Hori et al., 2002; Katz et al., 2005; Szabo et al., 2004, 2006).

To test for possible autonomy of the *H19* ICR, we inserted a 2.9 kb *H19* ICR fragment into the human β-globin yeast artificial chromosome (YAC) and generated transgenic mice (TgM) (Tanimoto et al., 2005). In somatic cells, the transgenic *H19* ICR was preferentially methylated when paternally inherited, demonstrating that the 2.9 kb fragment carries sufficient information to establish differential methylation. Unexpectedly, however, the transgenic *H19* ICR fragment was almost completely devoid of methylation in sperm, indicating that methylation

Abbreviations: CTCF, CCCTC-binding factor; DMR, differentially methylated region; Dnmt, DNA methyltransferases; ICR, imprinting control region; Igf2, insulin-like growth factor 2; miRNA, micro RNA; piRNA, PIWI-interacting RNA; siRNA, small interfering RNA; TgM, transgenic mouse; YAC, yeast artificial chromosome.

* Corresponding author at: Faculty of Life and Environmental Sciences, University of Tsukuba, Tennoudai 1-1-1, Tsukuba, Ibaraki 305-8577, Japan. Tel./fax: +81 29 853 6070.

E-mail addresses: t-takahashi@tara.tsukuba.ac.jp (T. Takahashi), matsuzaki@tara.tsukuba.ac.jp (H. Matsuzaki), shinichi.tomizawa@babraham.ac.uk (S. Tomizawa), e_oka@tara.tsukuba.ac.jp (E. Okamura), t-ichiyanagi@bioreg.kyushu-u.ac.jp (T. Ichiyanagi), akif@tara.tsukuba.ac.jp (A. Fukamizu), hsasaki@bioreg.kyushu-u.ac.jp (H. Sasaki), keiji@tara.tsukuba.ac.jp (K. Tanimoto).

[1] Present address: Epigenetics Programme, The Babraham Institute, Cambridge, CB22 3AT, UK.

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imprinting in the transgenic *H19* ICR was established *after fertilization*, and independently of its methylation status in germ cells (Matsuzaki et al., 2009; Tanimoto et al., 2005). Subsequently, we found that the paternal transgenic *H19* ICR was already methylated at the blastocyst stage (Matsuzaki et al., 2010). These observations led us to assume that i) methylation imprinting establishment during gametogenesis and post-fertilization period is separable events and that ii) some unknown epigenetic mark other than DNA methylation might be established within the *H19* ICR in germ cells, which would eventually be translated into a differential methylation pattern after fertilization. Such a hypothetical epigenetic entity might function by actively recruiting Dnmts to the paternal allele and/or protecting the maternal allele from *de novo* DNA methylation.

Small non-coding RNAs, including small interfering RNAs (siRNAs), micro RNAs (miRNAs) and PIWI-interacting RNAs (piRNAs), play fundamental roles in the regulation of genomic activity by targeting their complementary sequences (Bonasio et al., 2010; Moazed, 2009). Recently, we have demonstrated that piRNA, targeting to the imprinted mouse *Rasgrf1* locus, was implicated in the *de novo* methylation of the *Rasgrf1* DMR in spermatogonia. However, because mice deficient for Piwi family genes (components of the piRNA-generating machinery) exhibited aberrant methylation imprinting only in the *Rasgrf1* DMR, but not in the *H19* DMR (Watanabe et al., 2011), it seems likely that methylation imprinting of the *H19* DMR is established by piRNA-independent mechanisms.

Recently, we determined the sequences of small RNAs (15–40 nucleotides in size) derived from mouse oocytes at 8 days after birth (Watanabe et al., 2008). In this current report, we found that some of these small RNA sequences mapped uniquely to the *H19* ICR. To specifically elucidate whether DNA segment of the *H19* ICR corresponding to those small RNA sequences is required for post-fertilization methylation imprinting, we deleted the sequences from the *H19* ICR fragment and used it to generate human β-globin YAC TgM. The mutant *H19* ICR was able to establish a methylation pattern that was indistinguishable from that in the wild-type fragment, demonstrating that these DNA sequences are either redundant or have no function in the context of a YAC transgene.

2. Materials and methods

2.1. Generation of YAC transgenic mice with the mutant ICR fragment

The oligonucleotide-directed PCR mutagenesis was performed on the plasmid pHS1/loxPw+/ICR− carrying the mouse *H19* ICR (from 833 to 3696 nt, Genbank accession no. AF049091) as a *Bam*HI restriction enzyme fragment (Tanimoto et al., 2005). Two PCR fragments were amplified by the following primer sets; ORI-5S1/ORI-3A1, 5′-TACCAGCCTA GAAAATGCATGTGT-3′/5′-GGTTCTGCAGCGGGAGTTGCCGCGTGGTGG-3′ and ORI-5S2/ORII-3A2, 5′-CGTCCTGCAGTAGGGCATGGTCTCCTTGCA-3′/ 5′-TTTGCCCACCAGCTGCTAGCCATC-3′ (*Pst*I sites are underlined) and digested with *Bsu*36I (at nt. 1721)/*Pst*I and *Pst*I/*Msc*I (at nt. 998), respectively. These fragments, together with the *Bsu*36I/*Msc*I-digested pHS1/loxPw+/ICR− were ligated to generate pHS1/loxPw+/ ICR−/mutOR-I. Subsequently, other two PCR fragments were amplified by following primer sets; ORIII-5S1/ORIII-3A1, 5′-GA TCCCTGACTTCTCCTAGTCTCT-3′/5′-CCTATAGGGCCCGGCCCAAATGCT GCCAACTT-3′ and ORIII-5S2/ORIII-3A2, 5′-GATATAGGGCCCTATGCTGCCA CCGCGCGGTAG-3′/5′-TTTTGCACTGTCCCGGAGAGCCTT-3′ (*Apa*I sites are underlined) and digested with *Avr*II (at nt. 3640)/*Apa*I and *Apa*I/*Bsp*EI (at nt. 2519), respectively. These fragments, together with the *Avr*II/ *Bsp*EI-digested pHS1/loxPw+/ICR−/mutOR-I were ligated to generate pHS1/loxPw+/ICR−/mutOR-I/III. As a result, OR-I and OR-III sequences were deleted and replaced with *Pst*I and *Apa*I restriction enzyme sites, respectively (Fig. 1B). The anticipated mutagenesis was confirmed by DNA sequencing.

The pHS1/loxPw+/ICR−/mutOR-I/III plasmid was linearized with *Spe*I and used to mutagenize the human β-globin YAC (A201F4.3) (Tanimoto et al., 1999). Successful homologous recombination in yeast was confirmed by Southern blot analysis with several combinations of restriction enzyme and probes.

To generate TgM, purified YAC DNA was microinjected into fertilized eggs from CD1 mice (ICR; Charles River Laboratories). Tail-tip DNA of founder offspring was screened by PCR to identify TgM, and the transgene copy number was determined by qRT-PCR. Structural analysis of the YAC transgenes was done as described previously (Tanimoto et al., 2000).

Animal experiments were performed in a humane manner and approved by the Institutional Animal Experiment Committee of the University of Tsukuba. Experiments were conducted in accordance with the Regulation of Animal Experiments of the University of Tsukuba and the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

2.2. Methylation analysis of the transgene by Southern blotting

Genomic DNA was prepared from tail-tip cells (7-day-old TgM) or whole testis (1- to 2-month-old TgM), using standard procedures. DNA was digested either with *Bam*HI in the presence or absence of methylation-sensitive *Hha*I enzyme (for analyzing around the CTCF site 1) or with *Eco*T22I in the presence or absence of methylation-sensitive *Bst*UI enzyme (for the CTCF site 4), fractionated on an agarose gel, and transferred to nylon membranes. Membranes were hybridized with α-^{32}P-labeled probes (Fig. 2B), and subjected to X-ray film autoradiography.

2.3. Methylation analysis of the transgene by bisulfite sequencing

Tail-tip genomic DNA, prepared from 7-day-old TgM was digested with *Xba*I and treated with sodium bisulfite using EZ DNA methylation Kit (cat No. D5001, Zymo Research). MII oocytes were collected from oviducts of superovulated adult TgM, removed from cumulus cells by treatment with hyaluronidase, and washed with M2 medium and then phosphate buffered saline. Thirteen to twenty-seven oocytes were embedded in one agarose bead. The agarose beads were treated with sodium bisulfite as described previously (Matsuzaki et al., 2009).

Transgenic *H19* ICR-specific nested PCR, cloning, and sequencing analysis were performed as described previously (Matsuzaki et al., 2009). Subregions of the mutant *H19* ICR (CTCF 43, CTCF 21 and CTCF 1 shown in Figs. 2E and 4D) were amplified by nested PCR. The first-round PCR primers are as follows: LCR-MA-5S1, 5′-TATAGATGTTTTAG TTTTAATAAG-3′ and ICR-MA-3A15, 5′-ACCAACCAATATAACTCACTA TAA-3′ for the CTCF 43 region; ICR-MA-5S1 5′-GAATTTGAGGATTATGT TTAGTGG-3′ and BGLB-MA-3A1 5′-TCTCGTCAAACCACCTTCATTAAC-3′ for the CTCF 21 region; ICR-MA-5S13 5′-GGTGATTTATAGTATTGTTA TTTG-3′ and BGLB-MA-3A2 5′-CTCCTAAAAATAAATTAACCAACC-3′ for the CTCF 1 region.

The second-round PCR primers are as follows: ICR-MA-5 S4, 5′-GAA TTTGGGGTATTTAAAGTTTTG-3′ and ICR-MA-3A14, 5′-AAAACATAAAA ACTATTATATACA-3′ for the CTCF 43 region; ICR-MA-5S2, 5′-TTAAGGA TTAGTATGAATTTTTGG-3′ and ICR-MA-3A1, 5′-AACATAACAATACTATA ACCATAC-3′ for the CTCF 21 region; ICR-MA-5S13 and ICR-MA-3A2, 5′-AACAATACTAAATCTACCTAAAAC-3′ for the CTCF 1 region.

2.4. Semiquantitative reverse transcription-PCR (RT-PCR)

Total RNA was prepared from phenylhydrazine-induced anemic adult (2-month-old) spleens using ISOGEN (Nippon Gene) and converted to cDNA with ReverTra Ace (Toyobo). The real-time PCR reactions were performed in a 20 μl mixture containing 1/10 volume