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doi: 10.1371/journal.pone.0073925
The Chicken HS4 Insulator Element Does Not Protect the H19 ICR from Differential DNA Methylation in Yeast Artificial Chromosome Transgenic Mouse

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Abstract

Mono-allelic expression at the mouse IGF2/H19 locus is controlled by differential allelic DNA methylation of the imprinting control region (ICR). Because a randomly integrated H19 ICR fragment, when incorporated into the genome of transgenic mice (TgM), was allele-specifically methylated in somatic, but not in germ cells, it was suggested that allele-discriminating epigenetic signature, set within or somewhere outside of the Tg H19 ICR fragment in germ cells, was later translated into a differential DNA methylation pattern. To test if the chicken β-globin HS4 (cHS4) chromatin insulator might interfere with methylation imprinting establishment at the H19 ICR, we inserted the H19 ICR fragment, flanked by a set of floxed cHS4 core sequences, into a human β-globin locus YAC and generated TgM (insulated ICR' TgM). As controls, the cHS4 sequences were removed from one side (5HS4-deleted ICR') or both sides (pseudo-WT ICR’) of the insulated ICR’ by in vivo cre-loxP recombination. The data show that while maternally inherited transgenic H19 ICR was not methylated in insulated ICR' TgM, it was significantly methylated upon paternal transmission, though the level was lower than in the pseudo-WT ICR’ control. Because this reduced level of methylation was also observed in the 5HS4-deleted ICR' TgM, we speculate that the phenotype is due to VEZF1-dependent demethylation activity, rather than the insulator function, borne in cHS4. Collectively, although we cannot rule out the possibility that cHS4 is incapable of blocking an allele-discriminating signal from outside of the transgene, the epigenetic signature appears to be marked intrinsically within the H19 ICR.

Introduction

In genomic imprinting, a subset of mammalian genes are expressed exclusively from the paternally or maternally inherited chromosomes. This imprinted expression is controlled by allele-specific methylation of DNA blocks frequently found in the imprinted loci (DMR; differentially methylated region) [1–4]. The DMRs established during gametogenesis or during the post-fertilization period are called primary and secondary DMRs, respectively. Some primary DMRs acquire DNA methylation imprints in the male germ cells (paternal DMR), while many others become methylated specifically in the female germ cells (maternal DMR). Because de novo DNA methyltransferases, DNMT3A and 3L are involved in methylation acquisition in both germ lines [5,6], unique cis DNA sequences within or around the DMRs, in addition to hypothetical accessory molecules that confer allele discriminating activity to the DNMTs, are postulated to establish methylation imprinting in the germ line of each gender. To date, however, no apparent difference in cis DNA sequences between the paternal and maternal DMRs has been elucidated.

At the imprinted Igf2/H19 locus, differential DNA methylation of the H19 ICR is critical for imprinted expression of these genes and therefore, the segment is also called the H19 ICR (imprinting control region) [1,2] (Figure 1A). The mouse H19 ICR sequence is CpG-rich and carries four binding sites for CTCF, an insulator protein [7,8]. Preferential DNA methylation
of the $H19$ ICR is acquired in prospermatogonia [9,10] and is maintained in somatic cells after fertilization. CTCF binding to the $H19$ ICR confers enhancer blocking activity to the unmethylated maternal allele, which in turn prevents interaction between the $Igf2$ gene and its enhancers, located upstream of the $H19$ ICR and downstream of the $H19$ gene, respectively. On the methylated paternal allele, a lack of CTCF binding to the $H19$ ICR allows the enhancers to alternatively activate the $Igf2$ gene. Thus, methylation-sensitive CTCF binding to the $H19$ ICR is the molecular basis for imprint expression at the $Igf2/H19$ locus. Furthermore, deletion of the CTCF sites from the maternal $H19$ ICR causes its hypermethylation during the post-implantation period, revealing that CTCF-binding also plays an indispensable role in maintenance of the unmethylated state of the maternal $H19$ ICR [11]. Importantly, however, the CTCF sites are not required to protect the maternal $H19$ ICR from methylation in eggs or pre-implantation embryos. Therefore, the mechanism(s) by which the $H19$ ICR attracts and/or is protected from DNA methylation in an allele-specific manner is not yet determined.

As a first step in attempting to identify the sequences responsible for DMR formation in the $H19$ ICR, we inserted a 2.9-kb ICR fragment into a human β-globin yeast artificial chromosome (YAC) and generated transgenic mice (TgM) [12]. In somatic cells of these mice, the ICR fragment was hyperand hypo-methylated when paternally and maternally inherited, respectively, indicating that the 2.9-kb sequence harbored sufficient information to discriminate its parental origin. Surprisingly, however, the transgenic $H19$ ICR did not acquire methylation in the tests, demonstrating that establishment of imprinted methylation in germ cells is not essential and it can be established even after fertilization. We therefore presume that gametic and post-fertilization methylation imprinting events are separable, at least in TgM. Because the $H19$ ICR in a 147-kb bacterial artificial chromosome (BAC) transgene acquired methylation during spermatogenesis [13], sequences outside the 2.9-kb region were apparently essential for the gametic DNA methylation. For the transgene to be preferentially methylated after fertilization, the 2.9-kb $H19$ ICR itself or nearby sequences to the transgene integration sites must be prospectively marked by an epigenetic signature during gametogenesis (Figure 1B). In case the 2.9-kb ICR is intrinsically marked, its signature is unlikely to be DNA methylation [12–15]. On the other hand, if it is externally marked, signature could be a histone modification and/or differential DNA methylation. In either case, the mark must be eventually translated into a differential DNA methylation of the $H19$ ICR after fertilization. Histone modifications are candidate epigenetic signatures that could potentially be utilized to mark the parental alleles. In fact, paternally- and maternally-inherited $H19$ ICR sequences are associated with different histone marks in somatic cells [16]; of course, those differences may be merely reflecting the allelic DNA methylation status of the $H19$ ICR.

The Dnmt3L protein, together with the Dnmt3A plays an essential role in methylation acquisition at the $H19$ ICR in prospermatogonia [6]. Interestingly, the Dnmt3L can bind to histone H3 in vitro and their association on chromatin is prevented by its dimethyl- or trimethyl-lysine-4 (H3K4me2 or H3K4me3) modifications [17]. In addition, interaction between the Dnmt3A and the histone H3 tail is also sensitive to methylation of the H3K4 [18–20]. Accordingly, it was reported that paternally methylated ICRs, including the $H19$ ICR, were associated with unmethylated H3K4 histone marks on the sperm chromatin [21]. Therefore, differential states of histone methylation established during gametogenesis may lead to preferential recruitment of DNA methyltransferase complexes to parental alleles and their differential DNA methylation status, even after fertilization.

In transgenic experiments, insulator sequences are used to 'protect' genes from the spread of heterochromatin. The core region of a prototypical vertebrate insulator sequence, the chicken β-globin hypersensitive site 4 (CHS4) consists of five footprint regions (FI–V). The enhancer blocking activity of the insulator is conferred by CTCF binding to FI [22], while chromatin boundary activity depends on USF1 binding to FIV [23], as well as VEZF1 to FI, FIII, and FV sequences [24]. It has been reported that USF1 can recruit histone methyltransferases, PRMT1 and SET7/9, as well as the histone acetyltransferases, p300/CBP and PCAF [25]. Accordingly, active histone marks, such as acetylated H3/4 and methylated H3K4 are enriched around the FIV site and thereby block invasion of the silencing histone mark, methylated H3K9. In addition, VEZF carries DNA demethylation or protection against methylation activity, which may be involved in maintaining the CHS4 in hypomethylation status [24].

As a first step to identify a primary deposition site(s) of an allele-discriminating signature, i.e. inside or outside the $H19$ ICR, we surrounded the ICR fragment with tandemly arrayed CHS4 core sequences (insulated ICR'), which was then used for generating YAC-TgM. Maternally inherited transgenic fragments were not methylated, indicating that placing the CHS4 core sequences on both sides of the $H19$ ICR had no apparent effect on its hypomethylation status. Although a paternally inherited and insulated ICR' became significantly methylated after fertilization, the level was seemingly lower than in the control YAC-TgM. Therefore, to examine whether the incomplete paternal methylation is due to blocking of a hypothetical epigenetic signal from outside the $H19$ ICR or to demethylation activity of the CHS4 sequences, CHS4 was removed from one or both sides of the $H19$ ICR by in vivo cre-loxP-mediated recombination (5'HS4-del ICR' and pseudo-WT ICR'). The level of DNA methylation in paternally inherited 5HS4-del ICR' fragment in somatic cells was similar to that in the insulated ICR' and lower than that in the pseudo-WT ICR' fragments. Therefore, whereas the CHS4 fragment may reduce the DNA methylation level of nearby CpG sites as a consequence of its DNA demethylation activity, it does not interfere with the post-fertilization methylation imprinting establishment at the transgenic $H19$ ICR, suggesting that the allele-discriminating epigenetic signature is likely to be marked intrinsically within the $H19$ ICR.
Figure 1. Experimental design. (A) Generation of Insulated ICR' transgenic mice (top). Genomic structure of the mouse Igf2/H19 gene locus. The H19 ICR fragment used in this study corresponds to nucleotides from 1126 to 3503 in the AF049091 sequences (GenBank). In the enlarged map, restriction enzyme sites (MluI, BsrGI, BssHII, XhoI), which were artificially introduced to facilitate cloning procedure, are shown adjacent to the CTCF binding sites (solid rectangles) of the H19 ICR (termed the ICR’). Nucleotides are numbered relative to the 5’ end (set at 1’) of the sequence (bottom). Chicken HS4 core fragment (cHS4c, 237 bp) carries five footprints, FI–FV. Tandem cHS4c fragments (I for insulator, gray rectangles) were floxed by a set of either loxP5171 (solid triangles) or loxP2272 (open) sequences and attached to either ends of the ICR’ fragment (termed the insulated ICR’), which was then introduced in reversed orientation 3’ to the LCR in the human β-globin locus YAC (A201F4.3 [27]). The entire β-globin locus is contained within two SfiI fragments (8 and 100 kbp). Transgene-specific probes used for long-range structural analysis in (C) are shown as solid rectangles. (B) Two possible models for marking epigenetic signature. The mark can be set inside (left) or outside (right) of the H19 ICR. (C) Long-range structural analysis of transgenes. DNA from thymus cells was digested with SfiI in agarose plugs, separated by pulsed-field gel electrophoresis, and blots were hybridized separately to probes in (A). (D) (left) Structure of the transgene after in vivo cre-loxP recombination. Recombination removed the 5’ or both (cHS4c) fragments from the parental insulated ICR’ locus and generated either the 5’HS4-del or the pseudo-WT loci, respectively. S, SacI; A, AvaII; X, XbaI (right). Tail DNA of each TgM subline was digested with XbaI/SacI (top) or AvaII (bottom), separated on agarose gels, and blots were hybridized with the probe.

doi: 10.1371/journal.pone.0073925.g001
Materials and Methods

Preparation of ICR’ fragment

To facilitate plasmid construction, following two oligonucleotides were phosphorylated, annealed, and ligated to SacI/KpnI-digested pBluescriptII/KS(+) vector, in which two BssHII sites were prospectively disrupted; 5’-GGGATCATGACGCTGACT-3’ and 3’-CATGGCCCTAGATCTTGGG-5’. The resultant vector, pBSIIK(+)_KBABS carried KpnI-BamHI-AvrII-BamHI-Sacl multi-cloning sites.

Five DNA fragments (I1 through I5) were PCR-generated by using following primer sets and murine H19 ICR DNA fragment as a template. Artificially introduced enzyme restriction sites (except for endogenous BglII sites) and CTCF recognition motifs with their flanking four nucleotides on both sides are underlined and italicized, respectively, and indicated in parentheses.  

\[ 5'\text{-GGTCTCTAGGGCCGAGATCTGGCTAGTTGGGAGTTATACGAAGTTAT-G} \] (AvrII-Kas-BglII) and 5’-GGGATCATGACGCTGACT-3’ (AvrII). 

\[ 5'\text{-AACCTGAGTCTGCTGTCATACATATACAATAACTCATA-TGGTCCG-3'} \] (XhoI-m4). I2: 5’-TAACCTGAGTCTGCTGTCATACATATACAATAACTCATA-TGGTCCG-3’ (XhoI) and 5’-GGGCCGGCATGGCTACGGCGGTGCGACGACCATACTCTCATATATCGTGGC-3’ (BssHII-m3). I3: 5’-GGGCCGGCATGGCTACGGCGGTGCGACGACCATACTCTCATATATCGTGGC-3’ (BssHII) and 5’-GGGCCGGCATGGCTACGGCGGTGCGACGACCATACTCTCATATATCGTGGC-3’ (BssHII). I4: 5’-CCTGCGCCGCTGTACATTCATACCTGCGCGCGCGCACCACCCTGTACATACATCCAGAAGA-3’ (BssHII-BsrGI-m2) and 5’-GGGCCGGCATGGCTACGGCGGTGCGACGACCATACTCTCATATATCGTGGC-3’ (BssHII). I5: 5’-CCTGCGCCGCTGTACATTCATACCTGCGCGCGCACCACCCTGTACATACATCCAGAAGA-3’ (BssHII-BsrGI-m2) and 5’-GGGCCGGCATGGCTACGGCGGTGCGACGACCATACTCTCATATATCGTGGC-3’ (BssHII).

The AvrII/XhoI-digested I1 and XhoI/AvrII-digested I5 fragments were simultaneously ligated with the AvrII-digested pBSIIK(+)_KBABS vector to generate pKBABS/I1+i+5. Then, XhoI/BssHII-digested I2 and BssHII/MulI-digested I4 fragments were simultaneously ligated with the XhoI/MulI-digested pKBABS/I1+i+5 vector to make pKBABS/I1+i+2+4+i+5. Finally, BssHII/BsrGI-digested I3 fragment was ligated with the BssHII/BsrGI-digested pKBABS/I1+i+2+4+i+5 vector to make pICR’. In each cloning step, correctness of DNA sequences were confirmed by DNA sequencing (DDBJ accession no. AB775805).

Preparation of Targeting Construct and Homologous Recombination in Yeast

A backbone plasmid for targeting construct, pH51 [26], carried a human β-globin H51 DNA fragment (from 13299 to 14250 nt; HUMHBB, GenBank) in the pRS306. The following double-stranded DNA (only the upper strand sequences are shown) was subcloned into HindIII site (at 13769 in HUMHBB) of the pH51 to generate pH51/IoxP5171-2272: 5’-AACCTGAGTCTGCTGTCATACATATACAATAACTCATA-TGGTCCG-3’ (HindIII-IoxP5171-BamHI-AvrII-BamHI-lopP2272-HindIII; restriction enzyme sites are underlined and IoxP sequences italicized). Single copy of chs4 core sequence (238 bp) was PCR-amplified as BglII-BamHI fragment by using the following primer set and pJC13-1 (generous gift from professor Gary Felsenfeld) as a template: chs4core-S2, 5’-AGCCCCGATCAGCGGGAGACGCCGCC-3’ (BglII) and chs4core-3A, 5’-ACTAGTGATCTTATTTCCCCCATC-3’ (BamHI). Following BglII/BamHI digestion, two copies of the core fragment were subcloned into modified pBluescriptII vector in tandem arrays (i.e. BglII-BamHI/BglII-BamHI configuration; pBluescriptGchHS4 double).

Next, two kinds of tandem chs4 core sequences were PCR-generated by using above plasmid as a template and two combinations of primer sequences: chs4core-S2 and chs4core-3A-BspeI, 5’-ACTAGTGATCTCAGGAGATCTACAGCCC-3’ (BamHI/BspEI) or chs4core-5S2-BspeI, 5’-AGCCCCGATCAGCGGGAGACGCCGCC-3’ (BglII/BspEI) and chs4core-3A. These two fragments were digested with BglII/BspEI or BspEI/BamHI, respectively, and simultaneously subcloned into BamHI-digested pH51/IoxP5171-2272 to generate pH51/IoxP5171-2272/4xchs4.

Then, the following double-stranded DNA (only the upper strand sequences are shown) was subcloned into BspEI site of the pH51/IoxP5171-2272/4xchs4 to generate pH51/4xloxP/4xchs4:

\[ 5'\text{-TTCCGGAAGTTATAACCTTGTATAGTACACATATAAGCAGAAGTTATTTTGGTATCGAAGGATCGCTTCTCTCCATGTCGCACCTCGCCACTCTCGTCGATATACCTGCGCGGTGCGACGACCATACTCTCATATATCGTGGC-3'} \] (XhoI-Mulu-m1) and 5’-TTCCGGAAGTTATAACCTTGTATAGTACACATATAAGCAGAAGTTATTTTGGTATCGAAGGATCGCTTCTCTCCATGTCGCACCTCGCCACTCTCGTCGATATACCTGCGCGGTGCGACGACCATACTCTCATATATCGTGGC-3’ (XhoI-Mulu-m1).

Finally, AvrII DNA fragment excised from the pICR’ was introduced into the AvrII site (between the IoxP5171 and 2272 sequences) of the pH51/4xloxP/4xchs4 to generate pH51/4xloxP/4xchs4/ICR (-) targeting vector.

The targeting DNA was linearized with SpeI (at nt position 13670 in HUMHBB) and used for mutagenizing the human β-globin YAC (A201F4.3). Successful homologous recombination in yeast was confirmed by Southern blot analyses with several combinations of restriction enzymes and probes.

Generation of YAC-TgM

To generate TgM, purified YAC DNA was microinjected into fertilized mouse eggs from CD1 mice (ICR, Charles River Laboratories). Tail DNA from founder offspring was screened for homology by Southern blot analysis, using the following combinations of restriction enzymes and probes:
Methylation analysis by Southern blotting

Genomic DNA was prepared from tail-tip cells (1- to 2-weeks-old) or whole testes (2-month-old) of TgM using standard procedures. DNA was firstly digested by BamHI (for 3' portion of the H19 ICR), BamHI+BsrGI (middle portion), or BamHI+Sacl (chHS4 portion), then by methylation-sensitive HhaI enzymes. DNA was separated on an agarose gel, and transferred to a nylon membrane. The membrane was hybridized with the α-32P-labeled probes and subjected to X-ray film autoradiography.

Results

Generation of YAC-TgM

Imprinting control region of the Igf2/H19 locus in mouse is located from -4.4 to -2.0 kb of transcription start site of the H19 gene (H19 ICR; Figure 1A, top). To facilitate plasmid DNA construction, artificial restriction enzyme sites were introduced into the 2.4-kb H19 ICR fragment near the four CTCF binding sites. This modified sequence is referred to as the "ICR", hereafter (GenBank accession no. AB775805). In order to shelter this fragment from the spread of possible epigenetic signaling, such as DNA methylation and/or histone modifications (Figure 1B, right), tandemly-arrayed chicken HS4 core sequences, (chHS4c) were ligated to either side of the ICR' fragment. The chHS4c sequence used here carries all the footprint sites (FI–V) required for full insulator activity [28,29]. In order to delete the (chHS4c) sequences afterward as a rigorous control that would be transgene integration site independent, 5' and 3' (chHS4c) fragments were surrounded by a pair of loxP5171 or loxP2272 sequences, respectively (Figure 1A, bottom). This "insulated ICR'" fragment was inserted at 3' to LCR in the human β-globin locus YAC, which was then used to generate TgM. Long-range structural analyses of the transgene by Southern blotting revealed that three YAC-TgM lines (lines 23, 24 and 29) carried intact, single copies of the transgene (Figure 1A, C, and not shown).

To initiate in vivo cre-loxP recombination, parental YAC-TgM (insulated ICR', lines 24 and 29) were crossed with cre-TgM, and offspring were analyzed for recombination by Southern blotting (Cre-F0, data not shown). Pups that carried the recombined transgene were then crossed with non-Tg animals to remove the cre-containing transgene. Recombination removed the 5' (termed "5'HS4-del" allele) or both 5' and 3' ("pseudo-WT" allele) (chHS4c) fragments from the parental allele, which was confirmed by Southern blot analysis (Figure 1D and data not shown).

DNA Methylation Status of the H19 ICR and the (chHS4c), Sequences in Insulated ICR' TgM

We first examined the DNA methylation status of the H19 ICR fragment in tail-tip somatic cells of the insulated ICR' TgM (Figure 2). Because the BamHI and BsrGI sites are unique to the transgene, the transgenic and endogenous ICs can be distinguished by Southern blotting (Figure 2A). We analyzed cytosine methylation in the recognition sequences of the methylation-sensitive HhaI enzyme using the "i5 probe", which detects the 3' portion of the transgenic H19 ICR (corresponding to 5' portion of the endogenous H19 ICR) sequences (Figure 2A). In the F1 and F2 generations (top panels in Figures 2B and C), the maternally-inherited transgenic H19 ICR' (Tg) was hardly methylated, while it was variably methylated upon paternal inheritance. Because half of the endogenous H19 ICR fragment (presumably the paternal allele) was resistant to HhaI digestion (endo, Figure 2B–E), the paternally inherited endogenous allele was likely to be highly methylated in the same samples. This allele-preferential methylation pattern was also observed in the following generations (Figure 2D and E). To assess methylation status in the middle portion of the H19 ICR, the DNA was digested with the methylation-sensitive HpaII enzyme and analyzed using the "middle probe" (bottom panel in Figure 2C) for Southern blotting. The DNA methylation status in this region of the transgenic H19 ICR' fragment was similar to that seen in the 3' portion of the fragment.

We next examined the methylation status of the (chHS4c2) fragments by Southern blot analysis. The (chHS4c2) sequences attached to either 5' or 3'-ends of the ICR' can be distinguished by their sizes when genomic DNA is digested with BamHI and ScaI (Figure 2A). Southern blot analysis using a combination of HhaI and the chs4 probe revealed that these fragments were hypo-methylated irrespective of whether they are paternally or maternally inherited (middle panel in Figure 2C).

To determine the methylation status of the transgene in more detail, we conducted bisulfite sequencing of the DNA regions covering the CTCF sites 1/2 (3' portion) of the H19 ICR and the (chHS4c2) sequences (Figure S1). Based on the Southern blot results (Figure 2C and D), highly (No. 311 and 312), partially (No. 307 and 308), and poorly methylated (No. 313) DNA samples from the paternally-inherited TgM, as well as that from the maternally-inherited TgM (No. 205 and 206) were individually pooled and subjected to analysis. Consistent with the Southern blot results, H19 ICR' sequences were variably methylated in paternally-inherited transgenes, while they were hypomethylated upon maternal transmission. In contrast, the (chHS4c2) sequences were infrequently methylated regardless of their parental inheritance, suggesting that the (chHS4c2)
Figure 2. DNA methylation status of the insulated ICR’ fragment in somatic and germ cells (line 29). (A) Partial restriction enzyme map of the Insulated ICR’ transgene. Fragments from the transgenic (Tg) and endogenous (endo.) H19 ICR can be distinguished by their sizes because of a transgene-specific BamHI (B) or BsrGI (Bs) sites (circled). Methylation-sensitive HhaI or HpaII sites in the SacI (S)-BamHI, BamHI, or BamHI-BsrGI fragments (horizontal lines) are displayed as vertical lines. Probes are shown as gray rectangles. (B, C) Genomic DNA from a tail-tip of TgM (Insulated ICR’, F1 (B) and F2 (C) generations), inheriting the transgene either paternally (Pat.) or maternally (Mat.) was analyzed by Southern blotting (upper panel). DNA was digested with BamHI in the presence (+) or absence (-) of HhaI and analyzed by the I5 probe shown in (A) to analyze 3’ portion of the transgenic H19 ICR’. Parental fragments are marked by asterisks. (middle) DNA was digested with BamHI+SacI in the presence (+) or absence (-) of HhaI and analyzed by the chS4 probe. (bottom) DNA was digested with BamHI+BsrGI in the presence (+) or absence (-) of HpaII and analyzed by the middle probe to analyze middle portion of the H19 ICR. (D, E) Methylation status of the 3’ portion of the H19 ICR in F3 (D) and F4 (E) generations was analyzed (by I5 probe). (F) Genomic DNA from testis of the TgM was analyzed by I5 (top panel), chS4 (middle), or middle (bottom) probes. (G) Pedigree depicting a paternally-methylated insulated ICR’ transgene. Male and female (hemi-zygous) transgenic individuals are denoted as squares and circles, respectively. In the Southern blot data of 3’ portion of the transgenic H19 ICR’ sequences, uncut (top), completely-cut (bottom), and other (intermediate) bands were quantified by Phosphorimager and methylation status of each sample was determined as follows. When ratio of uncut/total (top +intermediate+bottom) bands exceeds 50%, the sample was defined as hyper-methylated and represented by solid marks. When ratio of completely-cut/total bands exceeds 50%, the sample was defined as hypo-methylated (open marks). In other cases, the sample was defined as partially-methylated (shaded marks). Testis samples in (F) were obtained from male individuals marked by dots.

doi: 10.1371/journal.pone.0073925.g002
sequences harbor anti-methylation protective or demethylation activities.

Finally, we examined the methylation status of the transgene in the testis DNA of the insulated ICR' TgM by Southern blotting (Figure 2F). The transgenic H19 ICR' (both 3' and middle regions), as well as the (cHS4c)_2 sequences were unmethylated. The weak methylated Tg band seen in the No. 204 and 312 individuals (those inheriting the transgene paternally) probably represent somatic cell DNA contamination, because the band was visible neither in the No. 207 nor 313 individuals (inheriting the transgene maternally). These results demonstrated that methylation imprinting in the transgene was established after fertilization, as has been shown in our previous reports [12,15,30].

By integrating the methylation status of individuals determined by Southern blot analyses of the H19 ICR' transgene (determined by IS probe), pedigree of the insulated ICR' TgM (line 29) was depicted in Figure 2G. The results demonstrated that the transgene was hypomethylated when maternally inherited. In contrast, it was methylated after paternal transmission, although the level of which was variable, indicating that the paternal phenotype was not fully penetrant.

We also analyzed two other insulated ICR' lines (lines 23 and 24 in Figures S2 and S3, respectively) and obtained essentially the same results.

DNA methylation status of the H19 ICR sequence in pseudo-WT ICR' TgM

In our previous reports [12], we did not observe such a variegated DNA methylation level in the paternally inherited transgenic H19 ICR fragment. We therefore decided to ask if the phenotype observed in the insulated ICR' transgene was elicited by the flanking (cHS4c)_2 sequences or by inserting the artificial enzyme sites in to the H19 ICR' sequences. To that end, we removed both 5' and 3' (cHS4c)_2 sequences from the insulated ICR' transgene by in vivo cre-loxP recombination to generate pseudo-WT ICR' allele (Figure 1D) and examined their DNA methylation statuses at the same chromosomal integration site (Figure 3 and Figure S4). In somatic cells of the pseudo-WT TgM, methylation level of 3' portion of the paternally inherited H19 ICR' in somatic cells (Figure 3B–D and Figure S4B and D) was apparently more uniform than that of the insulated ICR' TgM (Figure 2 and Figures S2 and S3) and most of them exhibited hypermethylation. DNA methylation level of the maternally inherited pseudo-WT transgene (Figure 3C, D and Figure S4A–C), as well as that in the testis (Figure 3E and Figure S4E) were low, which was indistinguishable from those in the insulated ICR' TgM (Figure 2 and Figures S2 and S3).

These results demonstrated that the (cHS4c)_2 fragments bordering the H19 ICR' sequence on both sides somehow interfered with methylation acquisition or carry a demethylation activity at least at the paternally inherited H19 ICR' sequence, which caused its variegated DNA methylation.

DNA methylation status of the H19 ICR sequence in 5'H5S4-del ICR' TgM

We hypothesized that an allele-discriminating epigenetic signature might be found outside the H19 ICR' and yet still affects H19 ICR' function (Figure 1B, right). Chromatin boundary activity of the chicken HS4 sequences has previously been suggested to be elicited by impeding the spread of repressive epigenetic signatures, either suppressive (H3K9me) histone modifications and/or DNA methylation of chromatins [23]. By employing such activities, (cHS4c)_2 fragments lying on both sides of the H19 ICR' could interfere with methylation imprinting establishment by preventing encroachment of any epigenetic signature (parental mark) on both sides of the H19 ICR', resulting in variable methylation levels of the paternally inherited H19 ICR', as was seen in the insulated ICR' TgM. If this were the case, full methylation imprinting might be restored by removing either one of the (cHS4c)_2 fragment from the insulated H19 ICR'. To test this concept, we screened for TgM (line 29) that had undergone partial in vivo cre-loxP recombination, and found one having only the 5'(cHS4c)_2 sequence deleted (5'H5S4-del ICR', Figures 1D and 4A). Southern blot analysis of these mutant animals revealed that paternally inherited 5'H5S4-del ICR' transgene in the tail somatic DNA was still variably methylated (Figure 4B–D), while a maternally inherited transgene (Figure 4C and D), as well as DNA from testis (Figure 4E) were hypomethylated. Because this methylation pattern was similar to that observed in the insulated ICR' TgM (Figures 2G, 4F, S2F, S3F, and 5), we conclude that the variegated DNA methylation at the paternal H19 ICR' was likely to be caused by demethylation activity, but not the insulator activity, of the (cHS4c)_2 sequence.

Discussion

Differential DNA methylation of the H19 ICR, which is normally established during gametogenesis, is essential to normal embryonic development in the mouse [31,32] and human [33], in part because it is utilized to discriminate parental alleles and to control mono-allelic expression of the Igf2 and H19 genes. Curiously, however, we and others have reported that gametic methylation of the H19 ICR is not mandatory to establish its DMR status and that this status can surprisingly be established even after fertilization [12–14], even in the situation where both paternal and maternal alleles coexist in a single cell, perhaps by propagating some unknown epigenetic signature that is set in the germ cells. Although the 2.9-kb H19 ICR fragment in TgM was unable to instruct its own methylation acquisition in sperm (our unpublished result), the ~140-kb BAC was capable of doing so [13]. Deductively then, establishment of germ-line methylation appears to require sequences lying outside the boundaries of the 2.9-kb H19 ICR fragment. It is therefore reasonable that the transgenic H19 ICR also requires a hypothetical epigenetic mark that resides outside the 2.9-kb H19 ICR region on the paternal chromosome to control post-fertilization methylation imprinting (Figure 1B, right). Because these randomly integrated H19 ICR fragments (into the mouse genome) acquired methylation imprinting at chromosomal sites where a DMR was not found.
Figure 3. Methylation status of the pseudo-WT ICR' fragment in somatic and germ cells (line 29).  
(A) Partial restriction enzyme map of the ICR' (pseudo-WT) transgene. (B–D) Southern blot analysis of the transgene in F1-3 generations. Genomic DNA was prepared from a tail-tip of TgM (pseudo-WT TgM) and the 3' portion of the transgenic H19 ICR' was analyzed (BamHI+HhaI) as described in the legend to Figure 2B and C. (E) Genomic DNA was prepare from testis of the TgM and the 3' portion of the transgenic H19 ICR' was analyzed (BamHI+HhaI) as described in the legend to Figure 2B and C. (F) Pedigree depicting a paternally-methylated pseudo-WT ICR' transgene is shown as described in the legend to Figure 2G.

doi: 10.1371/journal.pone.0073925.g003
Figure 4. Methylation status of the 5'HS4-del ICR' fragment in somatic and germ cells (line 29). (A) Partial restriction enzyme map of the ICR' (5'HS4-del) transgene. (B–D) Southern blot analysis of the transgene in F1-3 generations. Genomic DNA was prepared from a tail-tip of TgM (5'HS4-del TgM) and the 3’ portion of the transgenic H19 ICR’ was analyzed (BamHI+HhaI) as described in the legend to Figure 2B and C. (E) Genomic DNA was prepared from testis of the TgM and the 3’ portion of the transgenic H19 ICR’ was analyzed (BamHI+HhaI) as described in the legend to Figure 2B and C. (F) Pedigree depicting a paternally-methylated 5'HS4-del ICR' transgene is shown as described in the legend to Figure 2G.

doi: 10.1371/journal.pone.0073925.g004
Figure 5. Summary of the methylation levels in each transgenic mouse line. The vertical axis of the bar graph represents the percentage of individuals showing distinct degree of DNA methylation at the transgenic H19 ICR fragment (3’ portion), which was summarized from pedigrees of each TgM line. For each 100% stacked column, the number of individuals analyzed in each pedigree (Fig. 2-4 and Fig. S2-S4) was set at 100 (%) and DNA methylation levels were divided into three categories (highly-, partially- and poorly-methylated). P, paternal transmission; M, maternal transmission. doi: 10.1371/journal.pone.0073925.g005
addition, because the H19 ICR, remain associated with histones [21,34,35]. We therefore hypothesized that histone modifications within, or outside of (not necessarily restricted to β-globin sequences), the H19 ICR during spermatogenesis might direct allele-specific DNA methylation after fertilization.

Di- and tri-methylated lysine 9 methylation of histone H3 (H3K9me2 and -me3, respectively) are involved in heterochromatin protein 1 (HP1) recruitment and heterochromatin propagation. Because the H3K9me3 modification facilitates DNA methylation in non-mammalian model organisms [36] and the same mark is associated with methylated (paternal) H19 ICR regions [16,37,38], we presumed that the H3K9me3 epigenetic mark might be set during spermatogenesis and then translated into subsequent DNA methylation after fertilization in the TgM. Because it was reported that H3K9me3 modification was completely depleted in the H19 ICR during primordial germ cells (PGC) development [10] and was not found at the H19 ICR in spermatocytes or round/elongating spermatsids [16], we surmised that this repressive histone mark may be set outside the H19 ICR and only later spread into the ICR sequences in later stages of spermatogenesis, prior to fertilization. In addition, because the H19 ICR is marked by dimethyl H3K9 in sperm, Nakamura et al. proposed that the paternal H19 ICR is protected from genome-wide DNA demethylation during early embryogenesis through its’ interaction with the PGCG7 protein mediated by the H3K9me2 tail [35]. In accord with this notion, the level of DNA methylation at the H19 ICR in the paternal allele was diminished in somatic cells of Pgc7-null mutant mice. Therefore, the H3K9me2 modification found in mature sperm might also serve as an allele-specific DNA methylation signal at the H19 ICR after fertilization.

It has been shown previously that constitutive hyper-acetylation of H3K9 at the chs4 insulator excludes its further modification by di- and tri-methylation [23]. Furthermore, chs4 is purported to inhibit encroachment of H3K9 trimethylation by recruiting the H3K4me3 activating mark into nearby chromatin in a USF-dependent manner [25]. To ask whether two copies of the chs4 fragment (1.2 kb x 2), (ChβGI)2, carrying a similar number of CpGs as the H19 ICR as well as two CTCF-binding sites would generate proper DMR activity, Szabo et al. replaced the endogenous H19 ICR with the (ChβGI)2 in mice [39]. The (ChβGI)2 sequence did not acquire methylation neither in sperm nor in eggs, indicating that the fragment lacks cis sequences that can direct DNA methylation acquisition during gametogenesis. In addition, the mutant sequence was hypomethylated in somatic cells regardless of its’ paternal origin, indicating that the (ChβGI)2 did not acquire an epigenetic mark that can be subsequently translated into differential DNA methylation status. We therefore decided to test whether post-fertilization methylation imprinting at the H19 ICR is disturbed by placing the chs4 chromatin boundary at both ends by prohibiting the hypothetical encroachment of repressive histone modifications from the H19 ICR neighborhood.

When inherited maternally, the transgenic H19 ICR as well as the chs4 sequences were hypomethylated in somatic cells of the insulated ICR’ TgM (Fig. 2 and Fig. S1-S3). When paternally inherited, the chs4 portion of the insulated ICR’ transgene was again unmethylated, as was expected from the result of Szabo et al. [39]. However, the H19 ICR segment of the transgene was significantly methylated, although its level was somehow lower than that of a pseudo-WT transgene (Fig. 5). We therefore envisioned two possible explanations for the observed phenotypes: 1) the epigenetic signature (such as the H3K9me3) was set outside the transgenic fragment and its spread into the H19 ICR sequence was obstructed by the chs4 chromatin insulator, and thus its transformation to complete DNA methylation was partially prohibited; 2) although the epigenetic signature was set within the H19 ICR, its conversion into the appropriate DNA methylation status or perhaps DNA methylation per se was inhibited by the surrounding chs4 sequences. To distinguish between these two possibilities, we removed the chs4 sequences only from the 5’ side of the transgene (5’HS4-del ICR’), because, in this situation, the hypothetical external epigenetic signature would presumably spread into the H19 ICR. In those TgM, we found a similar (somewhat reduced) level of H19 ICR methylation upon paternal transmission, a result most consistent with the second possibility. However, it is also possible that the signature spreads only uni-directionally, eg. the H3K9me mark may spread in from 3’ side of the ICR’, where histone association in the sperm has been reported in the endogenous human β-globin locus [40]. It is also formally possible that allele-restricted transcription may occur across the H19 ICR [10,41] at the ectopic sites and this “external signal” might guide post-fertilization methylation imprinting, since the chs4 insulator is not capable of blocking such transcriptional run through. In addition, although mRNAs for Usf1, Usf2, and Vezf1 are shown to be present in the embryonic testes at E15.5 (GenitoUrinary Development Molecular Anatomy Project; http://www.gudmap.org.), we cannot exclude the possibility that chs4 insulator activity may not be fully established at the time when this hypothetical epigenetic signal spreads (i.e. the latest stage of spermatogenesis), leading to variegated DNA methylation at the insulated H19 ICR.

Following the report by Szabo et al. [39], a similar experiment was repeated using the mutant chs4 sequence, (mChβGI)2, lacking the USF and VEZF1 binding sites, both required for chromatin boundary activity of the insulator [42]. In those mutant mice, the sequences were partially methylated (32%) in sperm germ cells, suggesting that, although the CpGs in chs4 can potentially acquire DNA methylation, the USF and/or VEZF1 sites protect much of the chs4 from DNA methylation in the sperm. Because it has been reported that VEZF1 sites are capable of mediating protection from de novo DNA methylation [24], we postulated that placing VEZF sites (in chs4) on one or both sides of the H19 ICR fragment in the YAC-TgM caused its partial demethylation in the paternal allele. Alternatively, the H3K4me3 mark associated with the USF site in chs4 might cause the hypomethylation of nearby sequences, since Dnmt3L preferentially interacts with...
unmethylated H3K4 and induces de novo DNA methylation [17].

We assume that establishment of methylation imprinting at the H19 ICR during gametogenesis and early embryogenesis are separable events [15]; the latter is normally masked because of the former at the endogenous locus. Based on the results reported here, we infer that the epigenetic signature to direct post-fertilization methylation imprinting is likely to be set within the H19 ICR and that it does not require "spread-in" of additional information from the neighborhood. However, because methylation acquisition at the endogenous H19 ICR during spermatogenesis requires sequences outside of the 2.9-kb region [12,13,15], the spread of external epigenetic marks into the H19 ICR may be the basis for its gametic DNA methylation. Unfortunately, we were unable to examine the effect of inserting cHS4 on gametic DNA methylation of the H19 ICR, because transgenic H19 ICR does not acquire DNA methylation in germ cells [12]. If gametic methylation is affected by introducing the cHS4 on both sides of the endogenous H19 ICR, that would provide significant insight into the underlying molecular mechanisms of methylation imprinting establishment at the endogenous H19 ICR. In addition, by obstructing methylation acquisition at the endogenous H19 ICR during gametogenesis, we may be able to assess a functional role, if any, for the post-fertilization methylation activity at the endogenous locus.

Acknowledgements

We thank Dr. Doug Engel (University of Michigan) for critically reading the manuscript.

Author Contributions

Conceived and designed the experiments: EO KT. Performed the experiments: EO HM KT. Analyzed the data: EO AF KT. Contributed reagents/materials/analysis tools: AF. Wrote the manuscript: EO HM KT.

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