Fate of methylated/unmethylated \( H19 \) imprinting control region after paternal and maternal pronuclear injection

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Abstract: The paternal-allele-specific methylation of the \( Igf2/H19 \) imprinting control region (ICR) is established during gametogenesis and maintained throughout development. To elucidate the requirement of the germline passage in the maintenance of the imprinting methylation, we established a system introducing a methylated or unmethylated ICR-containing DNA fragment (ICR-F) into the paternal or maternal genome by microinjecting into the paternal or maternal pronucleus of fertilized eggs, and traced the methylation pattern in the ICR-F. When the ICR-F was injected in a methylated form, it was demethylated approximately to half degree at blastocyst stage but was almost completely remethylated at 3 weeks of age. In the case of the unmethylated form, the ICR-F remained unmethylated at the blastocyst stage, but was almost half-methylated at 3 weeks of age. Interestingly, the paternally injected ICR-F was highly methylated compared with maternally injected ICR-F at 3 weeks of age, partially mimicking the endogenous methylation pattern. Moreover, introduction of mutations in the CTCF (CCCTC binding factor) binding sites of the ICR-F, which are known to be important for the maintenance of hypomethylated maternal ICR, induced hypermethylation of the mutated ICR-F in both paternal and maternal pronuclear injected 3-week-old mice. Our results suggest the presence of a protection-against-methylation activity of the CTCF binding site in establishing the preferential paternal methylation during post-fertilization development and the importance of germline passage in the maintenance of the parental specific methylation at \( H19 \) ICR.

Key words: CTCF, DNA methylation, genomic imprinting, \( H19 \) ICR, pronuclear injection

Introduction

Genomic imprinting is an epigenetic phenomenon that results in mono-allelic expression of imprinted genes based on parent-of-origin-specific DNA methylation. It is indispensable for mammalian development, growth and behavior [5, 7, 13]. Allele-specific DNA methylation is established at the germline level during oogenesis and spermatogenesis, and maintained throughout embryo development in somatic cells despite the wave of genome-wide epigenetic reprogramming [24, 25].

The imprinted expression of the mouse \( Igf2/H19 \) locus
is governed by the differential methylation of the imprinting control region (ICR) between paternal and maternal alleles [2, 6]. A hypomethylated ICR on the maternal allele functions as an insulator by binding of the CCCTC-binding factor (CTCF) protein to the four recognition motifs in the ICR, which prevents activation of the distal Igf2 gene from the shared enhancer located 3′ to the H19 gene and allows exclusive H19 expression. Conversely, a hypermethylated paternal ICR represses H19 gene transcription by inducing epigenetic changes at the H19 promoter and prevents CTCF from binding to the ICR, thereby allowing Igf2 expression. Thus, differential methylation of the H19 ICR between the parental alleles constitutes the central imprinting mechanism in this locus.

The H19 ICR is methylated by the DNMT3A-DNMT3L complex in prospermatogonia [12, 15, 28] and the paternal allele-specific methylation status is maintained following fertilization (Supplementary Fig. 1a). Maternal H19 ICR hypomethylation has been shown to be regulated depending on the CTCF binding sites [4, 16]. Indeed, a study in CTCF site-mutated mice demonstrated that maternally inherited mutant ICRs acquired aberrant methylation after implantation [26]. However, little is known about the mechanisms maintaining the methylation status of paternal H19 ICR after fertilization. In transgenic mouse lines, a 2.9-kb DNA fragment encompassing the whole H19 ICR and a shorter 2.4-kb H19 ICR fragment was shown to recapitulate the paternally methylated pattern in somatic cells after passage through the germline (Supplementary Fig. 1b) [8, 27]. Recently, paternal-specific de novo methylation was shown to be established in a DNMT3A- and DNMT3L-dependent manner as early as 2-cell embryos [18]. This indicates the existence of a mechanism regulating methylation of the H19 ICR after fertilization.

In this study, we established a system that can analyze the methylation status of the H19 ICR fragment introduced into the genome after fertilization, to know the effect of the germline passage in the maintenance of allele-specific methylation. The 2.9-kb H19 ICR [27] containing fragments (ICR-F), which were artificially methylated or unmethylated, were injected into the paternal or maternal pronucleus and the methylation level of the transgene was traced. When using unmethylated ICR-F, the methylation levels were higher in transgenic founder mice generated from paternal injections compared with maternal injections. However, no difference was observed using methylated ICR-F. These results indicate the presence of a mechanism that may add preferential paternal de novo methylation after fertilization, although germline passage was necessary for the maintenance of paternal specific imprinting.

### Materials and Methods

#### Constructs

A DNA fragment including the mouse H19 imprinting control region (ICR) was cloned into pBluescript II SK (−) (Agilent Technologies Inc., CA, USA) as a 5.5-kb DNA fragment flanked by XbaI and EcoRI from a mouse genomic library constructed from a testis DNA (pXE). A mutant ICR with mutations in all four CTCF binding sites was created as previously described [26]. Briefly, five separate regions of the ICR were amplified with primers containing the mutant CTCF sites and a BbsI site at their 5′ ends. After digestion with BbsI, the fragments were ligated together to regenerate the complete sequence. The mutant ICR construct was generated by replacing the pXE Ncol-BamHI fragment with the Ncol-BamHI fragment containing the four mutant CTCF sites (pXE [m-CTCF]). The luciferase cDNA of pCpG-Luc (InvivoGen, CA, USA), containing no CpG, was replaced with EGFP cDNA at Ncol and Nhel sites. Finally, the 3-kb SacI-BamHI fragments from pXE and pXE (m-CTCF) were inserted into the blunted SpeI site of the pCpG-EGFP to include H19 ICR and EGFP cDNA as a non-imprinting fragment in a single transgene (pCpG-EGFP-SB and pCpG-EGFP-mutSB).

#### In vitro methylation

The pCpG-EGFP-SB and pCpG-EGFP-mutSB were methylated with CpG methyltransferase M.SssI (New England BioLabs, MA, USA) in vitro, as described previously [11]. Briefly, 40 µg plasmids were incubated with M.SssI in 350 µl reaction buffer by adding 1.75 µl of 32 mM S-Adenosyl-L-Methylation (New England BioLabs) every 2 h for 6 h at 37°C. After the reaction, the DNA was purified by phenol/chloroform extraction and ethanol precipitation followed by digestion with PacI (New England BioLabs). Methylation was confirmed by digestion with methylation-sensitive restriction enzyme HpaII (Thermo Scientific, MA, USA).

#### Animals

Wild-type B6D2F1 and ICR mice were purchased
from CLEA Japan (Tokyo, Japan) and Japan SLC (Shizuoka, Japan), respectively. All of the animal experiments described were approved by the Institutional Animal Care and Use Committee, Tottori University (permission number: 21–2–47 and 12-Y-4). All mice in this study received humane care in compliance with Tottori University’s guidelines for the care and use of laboratory animals in research, were fed ad libitum and housed in a room maintained at a constant temperature of 22°C, at 50% humidity and with a 12-h light-dark cycle.

**Microinjection**

The methylated or unmethylated *PacI* fragment of pCpG-eGFP-SB and pCpG-eGFP-mutsB was separated in a Seakem Gold agarose gel (TaKara, Shiga, Japan), and purified by Wizard SV Gel and PCR Clean-Up System (Promega Corp., Madison, WI, USA). DNAs were microinjected into the paternal or maternal pronuclei of B6D2F1 × B6D2F1 fertilized eggs. Paternal and maternal pronuclei were distinguished based on the location of the paternal pronucleus farther from the polar body and larger size compared with the maternal pronucleus.

**Preparation of genome DNA from blastocyst or mouse tail**

For blastocyst analysis, the embryos were incubated for 4 days at 37°C containing 5% CO₂ in air after microinjection. Embryos (5–10) were digested in 5 μl Digestion Buffer (Zymo Research, CA, USA) including 20 μg Proteinase K (Zymo Research) at 50°C for 1 h. The digested mixture was then heated at 72°C for 15 min for inactivation of proteinase K, followed by digestion with DpnI (New England BioLabs) at 37°C for 16 h to remove DNA fragments not incorporated into the embryonic genome.

To generate transgenic mice, two-cell embryos were transferred to pseudopregnant ICR female mice after microinjection. When the founder mice were born, the incorporation of the transgene was examined by PCR analysis using genomic DNA extracted from tail tissue of the founder mice using the following oligonucleotides: 5′-TGAACCCGATCGAGCTGAGGG-3′ and 5′-TC-CAGCAGGACCATGTGAC-3′.

**Bisulfite sequence methylation assay**

Methylation of the genomic DNA of transgenic mice and blastocysts was examined by bisulfite genomic sequencing method using the EZ DNA Methylation Direct Kit (Zymo Research) according to the manufacturer’s protocol. The bisulfite-treated DNAs were amplified by nested-PCR using primer pairs specific for the *H19 ICR* CTCF-binding site (CTCF1/2, nucleotides 1221 to 1977; CTCF3/4, nucleotides 2817 to 3497; GenBank accession no. AF049091) and GFP, using primer pairs as follows.

5′-GTAAATAGGGGTAGTTAATGGGT-3′, and 5′-ACTACATAAAACCCCCCTACTATAA-3′ for CTCF1/2 1stPCR. 5′-AAAAGTGTTGTGGTTATATAGGAGG-3′, and 5′-CCCTAACCCTATAACCCCATACAC-3′ for CTCF1/2 2ndPCR.

5′-CCCCAAAACCACCATATACTCAC-3′, and 5′-TTTGGATTGAGTTGTGAGGTG-3′ for CTCF3/4 1stPCR. 5′-AAAAACCAACATATAACTCCTAT-3′, and 5′-CTTGAGGAGGTATTATAAGG-3′ for CTCF3/4 2ndPCR. 5′-GTAATATTGTGTTGTGTTAAGGT-3′ and 5′-AAAACCAACATATAACTCCTAT-3′ and 5′-GGTGATATTATATAAGG-3′ for GFP 1stPCR, and 5′-CTTGAGGAGGTATTATAAGG-3′ and 5′-AAATATATATTATATTATATAAC-3′ for GFP 2ndPCR.

The reaction program consisted of 40 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The PCR products were gel-purified using the MonoFas (GL Sciences, Tokyo, Japan) and cloned into pGEM-T Easy Vector (Promega). To confirm that transformed cells contained the fragment of interest, colony-PCR was performed using M13 primers (RV: 5′-CAGGAAA-CAGCTATGC-3′ and M4: 5′-GTTTCCCTACACGAC-3′) and analyzed by agarose gel electrophoresis. PCR products were treated with ExoSAP-IT (GE Healthcare Life Science, Little Chalfont, UK) and directly sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) and an Applied Biosystems 3130 × 1 Genetic Analyzer (Applied Biosystems) using the above RV primer. For blastocyst, 18 clones were analyzed from two to three experimental groups, and for mice, 6 clones were analyzed in each mouse. We excluded clones with incomplete bisulfite conversion.

**TRITC-Dextran microinjection and immunohistochemistry**

TRITC-Dextran-lysine-fixable (4.5 mg/ml) was microinjected into the paternal or maternal pronuclei of B6D2F1 × B6D2F1 fertilized eggs. Injected eggs were
washed in PBS, fixed for 30 min in 4% PFA in PBS on ice and post-fixed in 2% PFA in PBS for 15 min on ice. Eggs were then washed with 0.05% Tween 20 in PBS and permeabilized with 1% Triton X-100 in PBS for 15 min. The eggs were blocked for 1 h in 3% goat serum and 0.05% Tween 20 in PBS and incubated overnight with anti-histone H3 trimethyl Lysine9 Rabbit pAB (1:500; Active Motif, CA, USA) antibody at 4°C. The following day, after washing, the signal was detected by incubating the eggs with Alexa Fluor 488 goat anti-rabbit IgG antibody (1:200; Molecular Probes, OR, USA) and 0.25 µg/ml DAPI for 1 h. Fluorescence images were captured as vertical sections using an Olympus FV1000D IX81 confocal laser scanning fluorescence microscope (Olympus Corp., Tokyo, Japan), stacked into one picture and pseudocolored using the Olympus FluoView FV1000 software.

Statistical analysis
Statistical analyses were performed using the nonparametric Mann-Whitney U test. Differences were considered significant at \( P<0.05. \)

Results
Establishment of a system for discriminating between paternal and maternal pronuclei
Allele-specific DNA methylation at imprinted loci needs to be maintained throughout early development against genome-wide epigenetic reprogramming to allow for stable allelic expression in differentiated tissues. To focus on the post-fertilization mechanism maintaining the parent-of-origin-specific DNA methylation of imprinted loci, we microinjected DNA fragments into the paternal and maternal pronucleus in anticipation of integration into the paternal and maternal genomes after fertilization, respectively, and traced the methylation status. The maternal pronucleus is known to be smaller and closer to the polar body than the paternal pronucleus. Thus, we first confirmed the accuracy in discrimination between paternal and maternal pronuclei by injecting TRITC-labeled Dextran into the paternal or maternal pronucleus followed by immunostaining for trimethyl histone H3 lysine 9 (H3K9me3), which is apparent in the maternal pronucleus (Fig. 1a). When we injected TRITC-Dextran into the paternal pronucleus, TRITC signal was detected separately from H3K9me3 signal in 98% of the eggs (Figs. 1b and c). Upon injection into the maternal pronucleus, both signals colocalized in 96% of the eggs (Figs. 1b and c). These data suggest that it is possible to inject DNA fragments accurately and separately into paternal or maternal pronuclei.

Preparation of transgenic H19 ICR fragment
A 2.9-kb SacI/BamHI fragment of H19 ICR that includes four CTCF binding sites (Fig. 2a) has been described to contain sufficient information to recapitulate imprinted methylation at the normally non-imprinted β-globin locus after fertilization in transgenic mouse lines [27]. We inserted this fragment into a pCpG-EGFP vector that contains no CpG sites except for EGFP. The EGFP cDNA with CpG sites was used as a non-imprinted fragment, so that we could compare the regulation of imprinted DNA methylation of the ICR with EGFP. The plasmid was digested with PacI to prepare the transgene fragment (ICR-F), including the mCMV enhancer, 2.9 kb H19 ICR, hEF1 promoter, and EGFP cDNA (Fig. 2b). Furthermore, the ICR-F was prepared in methylated and unmethylated forms using SssI DNA methylase to mimic the DNA methylation status of endogenous H19 ICR in the paternal and maternal genome, respectively. The methylation status of the ICR-F was confirmed to be methylated over 95% in the 5’ segment of the ICR covering CTCF binding sites 1 and 2 (CTCF1/2), the 3’ segment covering sites 3 and 4 (CTCF3/4), and the latter half of the EGFP segment (Figs. 2b and c).

Analysis of blastocysts microinjected with H19 ICR into paternal or maternal pronucleus
The methylated and unmethylated ICR-F were microinjected into the paternal or maternal pronucleus, and the methylation status of the ICR-F was analyzed at blastocyst stage. Genomic DNA was extracted from several pools of 5–10 blastocysts, so that each pool contains approximately 1 transgenic blastocyst (the efficiency of transgenesis is known to be ~10% [3]), and subjected to DpnI digestion to eliminate the originally-injected DNA. After sodium bisulfite treatment, nested-PCR was conducted with transgene-specific primer sets to amplify DNA sequences covering CTCF1/2 and CTCF3/4 regions, of which methylation status was reported to be involved in regulation of the imprinted expression of H19 and Igf2 [4, 9]. When an unmethylated transgene fragment ICR-F was used for microinjection, transgenic CTCF1/2 and CTCF3/4 regions remained at low methylation level in both paternal and maternal
pronuclear injections (Fig. 3a). Conversely, in the case of methylated ICR-F, approximately half of the CTCF regions were unmethylated. The same tendency was observed regarding the methylation status of the eGFP region that was included in the ICR-F (Fig. 3b). These results suggest that part of the methylated H19 ICR fragments were exposed to global DNA demethylation during early development.

**Analysis of transgenic H19 ICR in mice**

We next evaluated the DNA methylation status of the ICR-F in the tail of the founder mice at 3 weeks of age. In transgenic mouse lines produced from microinjection of the unmethylated ICR-F, the ratios of methylated CpGs in the H19 ICR were high (Figs. 4a and b), compared with the blastocyst stage in which almost no methylation was detected (Fig. 3). This suggests that de novo methylation occurred after the blastocyst stage. We found...
that the CTCF1/2 region was heavily methylated in paternal injections compared with maternal injections, although the methylation ratio varied among mice. When the methylation level was analyzed focusing on CpGs, almost all of the CpGs in the CTCF1/2 region were methylated higher in paternal injections than in maternal injections, while few CpGs in the CTCF3/4 region displayed higher methylation (Fig. 4c). Conversely, more than 80% of the CpGs were methylated and no difference was detected between paternal and maternal injections in the EGFP region (Fig. 4b). These results indicate that the transgenic H19 ICR, especially the region covering
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CTCF1/2, acquired preferential paternal methylation after implantation in the transgenic founder mice. In the case of mice produced by methylated ICR-F injection, the H19 ICR was highly methylated compared with blastocysts, in which only approximately half of the ICR-F was methylated (Fig. 3). This suggests that de novo methylation occurred after blastocyst stage similar to unmethylated transgene injection. However, almost no difference was detected between paternal and maternal injections (Fig. 4b).

Analysis of transgenic mutated H19 ICR

To confirm the significance of CTCF binding in establishing allele-specific DNA methylation of transgenic ICR-F, we prepared a transgenic fragment containing mutations in all four CTCF binding sites, m-CTCF (Fig. 5a) [26]. First, we injected ICR-F with m-CTCF in a methylated and an unmethylated form, and analyzed...
Fig. 4. Methylation analysis of the transgenic H19 ICR in somatic cells of the founder transgenic mice. (a) Bisulfite sequencing analysis of the ICR-F in transgenic founder mice that were obtained by injecting unmethylated DNA fragments into the paternal or maternal PN. The methylation status of the ICR-F was indicated as described in Fig. 2c. The results derived from a single founder mouse are represented as a cluster. Serial numbers of each transgenic mouse line are indicated to the left of the Pat-PN and Mat-PN columns. (b) Distribution of the methylation status of ICR-F among founder mice obtained by paternal or maternal pronuclear injection of unmethylated/methylated transgene. Methylation status of CTCF1/2, CTCF3/4, and EGFP regions are indicated separately in a combined box and scatter plot. Open rectangles and circles indicate methylation status of individual lines produced by paternal (P, n=6) and maternal (M, n=6) injection of unmethylated (Un) transgene, respectively. Closed rectangles and circles correspond to the mouse lines injected paternally (P, n=5) and maternally (M, n=6) by methylated (Me) transgene, respectively. The median (line within the box), interquartile range (edges of the box), and the range of all values (vertical lines) are shown. Outliers (all cases more distant than 1.5 interquartile ranges from the upper or lower quartile) were omitted; one outlier in Un-P (#143) and Me-M groups, respectively. Asterisks mark significant differences between the groups. As for EGFP, three to five mice were randomly chosen from each group for bisulfite analysis. (c) Methylation levels at individual CpG sites of ICR-F shown in (b). Paternally and maternally produced transgenic mice injected with an unmethylated ICR-F are compared. The data are indicated separately for CTCF1/2 and CTCF3/4 regions as bar graphs. Black and white bars indicate the average methylation levels among transgenic mouse lines derived from paternal and maternal injection, respectively. Solid bars indicate the location of CpGs included in the CTCF binding sites.
Fig. 5. Methylation analysis of mutated transgenic H19 ICR in somatic cells of the transgenic mouse lines. (a) Sequences of CTCF-binding sites in mutant (mut) H19 ICR compared with the wild-type (WT) ICR. CTCF-binding motifs and CpG dinucleotides are indicated in bold and underlined, respectively. Mutated nucleotides in the mutant ICR are shown in lowercase. (b) Bisulfite sequencing analysis of the mutated ICR-F in transgenic founder mice that were obtained by injecting unmethylated DNA fragments into the paternal or maternal PN. The methylation status of the transgenic ICR was indicated as described in Fig. 4A. (c) Distribution of the methylation status of transgenic mutated ICR-F among founder mice obtained by paternal or maternal pronuclear injection of unmethylated/methylated transgene. The m-CTCF1/2, m-CTCF3/4 and EGFP regions are indicated separately in a combined box and scatter plot. Open rectangles and circles indicate methylation status of individual lines produced by paternal (P, n=5) and maternal (M, n=6) injection of unmethylated (Un) transgene, respectively. Closed rectangles and circles correspond to the mouse lines injected paternally (P, n=3) and maternally (M, n=7) by methylated (Me) transgene, respectively. The median (line within the box), interquartile range (edges of the box), and the range of all values (vertical lines) are shown. Outliers (all cases more distant than 1.5 interquartile ranges from the upper or lower quartile) were omitted; one outlier in Un-M (#601) and Me-M groups, respectively. As for EGFP, three to seven mice were randomly chosen from each group for bisulfite analysis.
the methylation status at blastocyst stage. Although CTCF cannot bind to m-CTCF, methylation status was almost the same as that of wild-type ICR-F (methylation level of unmethylated ICR-F with m-CTCF was 2.2 and 4.5% for paternal and maternal injection, and methylated ICR-F with m-CTCF was 40.3 and 40.0% for paternal and maternal injection). This indicates that the methylation status of the ICR-F is independent of CTCF binding until the blastocyst stage, consistent with the endogenous H19 ICR [26]. On the other hand, when mutated ICR-F was injected in an unmethylated form and analyzed at 3-week-old mice, CpGs in the mutated CTCF1/2 and CTCF3/4 regions were hypermethylated (Figs. 5b and c), compared with ICR-F with no mutations (Figs. 4a and b). Moreover, there was no difference between paternal and maternal injections, although the methylation ratio varied among mice. In case of the methylated ICR-F with m-CTCF, the CpGs were also completely methylated. These results indicate that CTCF binding to the ICR-F is indispensable not only for maintaining a low methylation level but also for establishing the preferential methylation of the paternal ICR after implantation.

### Discussion

Germline passage is known to be important in establishing the allele-specific methylation and expression patterns of imprinted genes [14, 17, 28]. Recently, Matsuzaki et al. demonstrated that the transgenic H19 ICR fragment was de novo methylated from pronuclear toward blastocyst stage in a DNMT3A- and DNMT3L-dependent manner only when it was paternally inherited [18]. This indicated that differential epigenetic marks between paternal and maternal alleles, such as chromatin structure and histone modifications, are established during gametogenesis to maintain the allele-specific methylation status after fertilization. In our study, a weak but preferential paternal de novo methylation was observed after implantation without passage through the germline cells (Supplementary Fig. 1c). Combined with the results reported by Matsuzaki et al. [17], the de novo methylation would be added after implantation through around 10 dpc during embryogenesis. Moreover, the de novo methylation of ICR-F might be obtained by recognizing the allele-specific epigenetic marks, which is established at the pronuclear stage by unknown mechanisms. Although the germline passage was clearly important in completely maintaining the paternal-specific methylation of H19 ICR, a CTCF dependent mechanism protecting ICR-F against de novo methylation could exist to support the maintenance of the specific methylation after implantation.

We showed that H19 ICR, included in the ICR-F transgene fragment, underwent de novo methylation preferentially in transgenic mice produced by paternal pronuclear injection compared with maternal pronuclear injection. The preferential paternal methylation was particularly detected in the CTCF1/2 region, consistent with the study showing that this region plays a central role for introducing paternal allele-specific DNA methylation [23]. On the other hand, almost no difference was observed between paternal and maternal injection utilizing methylated ICR-F. This might be due to inhibition in binding of the CTCF, which regulates preferential paternal methylation after implantation, because half of the ICR-F was already methylated at the blastocyst stage (Fig. 3a). Moreover, mutation in the CTCF binding sites resulted in hypermethylation of the H19 ICR, leading to disappearance of the difference between paternal and maternal injection, as well as the endogenous H19 ICR [26]. Non-imprinting EGFP region in the ICR-F was constitutively methylated, confirming that the ICR-F was regulated as an imprinting region (Fig. 4b). Our results suggest that paternal pronuclei are able to imprint the ICR-F with some marks that will ultimately lead to CpG methylation. We suppose the marks are not methylation because the fragment remains unmethylated in blastocysts. The mechanism would be elucidated by microinjecting ICR-F with various epigenetic modulators (inhibitors and activators) that target changes to DNA methylation and chromatin remodeling proteins.

As for EGFP region, which was included in the transgenic ICR-F as a non-imprinted fragment, the methylation level in 3-weeks-old mice produced by unmethylated ICR-F with m-CTCF injection was low (Fig. 5c), compared with unmethylated ICR-F with no mutation (Fig. 4b). However, the difference was not statistically significant. The range of the methylation level among the mice injected by unmethylated ICR-F with m-CTCF was wide, leading to low methylation level. The low methylation level of EGFP in unmethylated ICR-F with m-CTCF injection might be the effect that mutated-ICR was preferentially methylated than EGFP region due to the inhibition of CTCF-binding. To block the effect between H19 ICR and EGFP region, an insulator sequence
such as chicken HS4 [18] would be needed.

We observed a wide range of differences in the methylation levels of H19 ICR in the ICR-F between the individual transgenic mouse lines, although preferential de novo methylation occurred in paternal injection after implantation using the unmethylated transgene. Immunostaining for H3K9me3 after TRITC-Dextran microinjection indicated that transgene fragments are correctly microinjected into the paternal or maternal pronuclei. Integration of the foreign DNA usually occurs at one site on one chromosome by microinjection into the pronuclei such that all cells are hemizygous for the foreign DNA in transgenic mice. Indeed, Wilkie et al. showed that about 70% of transgenic mice produced by microinjection transmitted the foreign DNA to approximately half of their offspring [30]. We also reported that incorporation of the transgene was observed at a single location in 82.4% of the transgenic mouse lines [21]. These data imply that the integration event most likely occurs before or during DNA replication of the first cell cycle, leading to the conclusion that the transgenes microinjected into the paternal and maternal pronucleus were integrated into the paternal and the maternal genome, respectively. However, we still need to confirm whether DNA fragments are integrated into the paternal or maternal genome before syngamy. Moreover, the epigenetic marks affected by the chromosomal position effect may also nonspecifically influence the methylation level of the transgene. A usage of locus-specific integration systems, such as Cre/loxP [1, 22] and CRISPR/Cas9 systems [29], would provide a reliable result by enabling introduction of the ICR-F into an identical chromosome region, and also by targeting of the ICR-F to the paternal or maternal genome.

When we used an artificially methylated ICR-F including H19 ICR, approximately half the CpGs in the H19 ICR were demethylated at the blastocyst stage, as well as EGFP region in the same fragment, in both paternal and maternal injections. This suggests that the methylated ICR-F was exposed to genome-wide active and/or replication-dependent DNA demethylation, which occur during early embryogenesis in the paternal and maternal genomes, respectively. The fact that in vitro methylated DNA fragments were demethylated during pre-implantation stage as well as the endogenous genome could open the possibility of elucidating the mechanism of how imprinting regions are protected against genome-wide demethylation. The paternally imprinted H19 is known to be protected from active demethylation in zygotoby binding of PGC7/Stella/Dppa3 to dimethylated histone H3 lysine 9 (H3K9me2) localized at the loci, thereby blocking the activity of TET3 methylcytosine oxidase [19, 20]. Moreover, maternal and zygotic DNMT1, and zinc finger protein ZFP57 are also required to maintain DNA methylation imprints during pre-implantation development [10, 14]. However, the mechanism maintaining parental- and sequence-specific methylation patterns is largely unknown. Taking advantage of our system by tracing the methylation status of the DNA integrated into genome after fertilization, microinjection of in vitro-methylated DNA fragments or -assembled nucleosome with modified histones into paternal or maternal pronucleus may provide a new approach for elucidating the mechanisms regulating DNA methylation and/or demethylation during embryogenesis.

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