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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 reprograming [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 *Xba*I and *Eco*RI 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 *Bbs*I site at their 5′ ends. After digestion with *Bbs*I, the fragments were ligated together to regenerate the complete sequence. The mutant ICR construct was generated by replacing the pXE *Nco*I-*Bam*HI fragment with the *Nco*I-*Bam*HI 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 *Nco*I and *Nhe*I sites. Finally, the 3-kb *Sac*I-*Bam*HI fragments from pXE and pXE (m-CTCF) were inserted into the blunted *Spe*I 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 *Pac*I (New England BioLabs). Methylation was confirmed by digestion with methylation-sensitive restriction enzyme *Hpa*II (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 *Pac*I 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% CO_2 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 *Dpn*I (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′-TGAACCGCATCGAGCTGAAGGG-3′ and 5′-TC-CAGCAGGACCATGTGATGCC-3′.

Bisulfite sequence methylation assay

Methylation of the genomic DNA of transgenic mice

and blastocysts was examined using 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′-GTTAATAGGGGGTGAGTTAATGGGT-3′, and 5′-ACTAACATAAACCCCTAACCTCATAA-3′ for CTCF1/2 1stPCR. 5′-AAAAGTGTTGTGATTATATAG-GAGG-3′, and 5′-CCCCTAACCTCATAAAACCCATA-AC-3′ for CTCF1/2 2ndPCR.

5′-CCCCAAAACCAACCAATATAACTCAC-3′, and 5′-TTTGTTAGGGATTGTGGGTTATGTG-3′ for CTCF3/4 1stPCR. 5′-AAAACCAACCAATATAACT-CACTATAA-3′, and 5′- CTTTGAGGAGTTTTAAGG-TAGAAGG-3′ for CTCF3/4 2ndPCR. 5′-GTA-ATATTTTTGGGGGTATAAGTTG-3′ and 5′-AAACTCATCAATATATCTTATCATATCTAA-3′ for GFP 1stPCR, and 5′-GTTGGAGTATAATTATAATAGT-TAT-3′ and 5′-CAATATATCTTATCATATCTAAC-CAACTAA-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-CAGCTATGAC-3′ and M4: 5′-GTTTTCCCAGTCAC-GAC-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 × l 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 on ice, 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 pronuclei 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 *Sac*I/*Bam*HI 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 *Pac*I 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 *Dpn*I 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
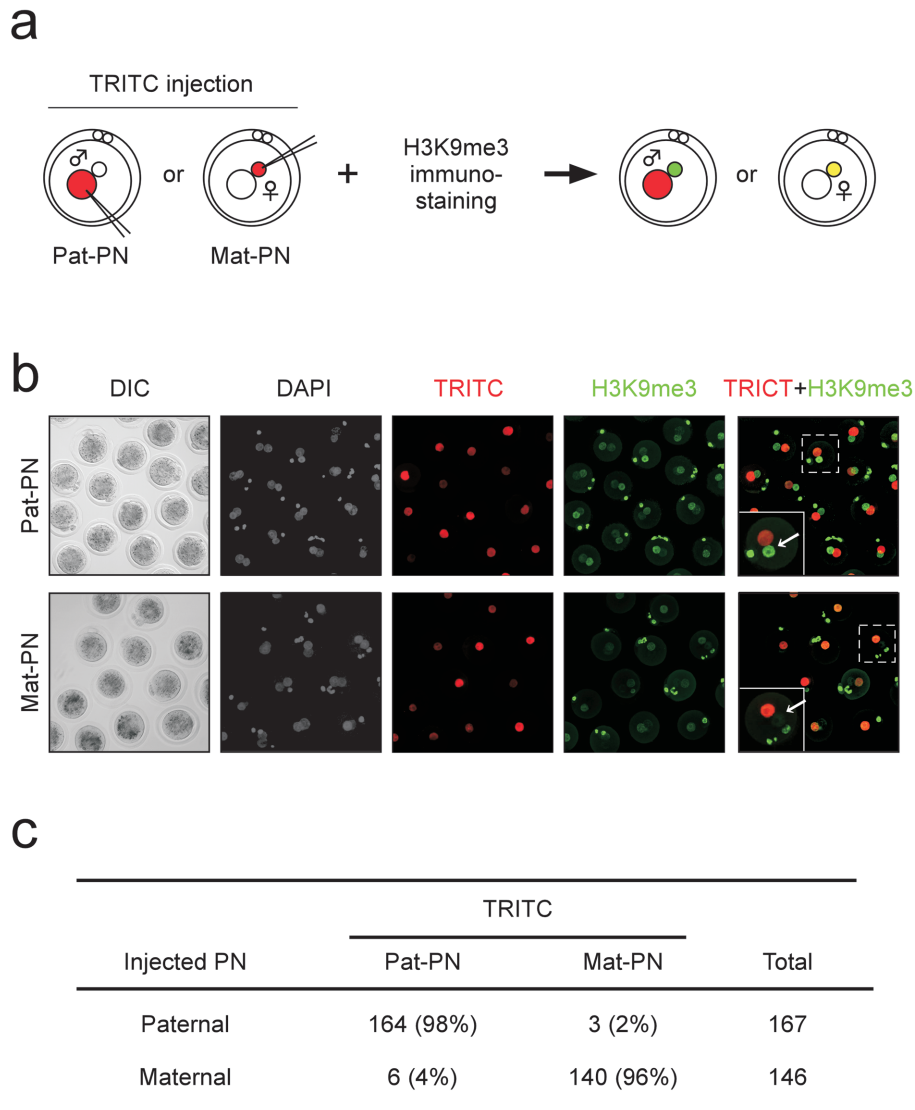
Fig. 1. Distinction of paternal and maternal pronuclei (PN) during pronuclear injection. (a) A schematic representation to show the discrimination between paternal (Pat-) and maternal (Mat-) PN. PN eggs were injected by TRITC-Dextran and immunostained for H3K9me3. TRITC-Dextran and H3K9me3 staining are shown in red and green, respectively. (b) A representative image of pronuclear eggs immunostained for H3K9me3 (green) after TRITC-Dextran (red) injection. White arrows indicate the maternal PN. The nuclei were stained with DAPI (gray). Inset shows an enlarged image of the dotted squares. (c) Efficiency of the distinction between paternal and maternal PN during pronuclear injection.

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
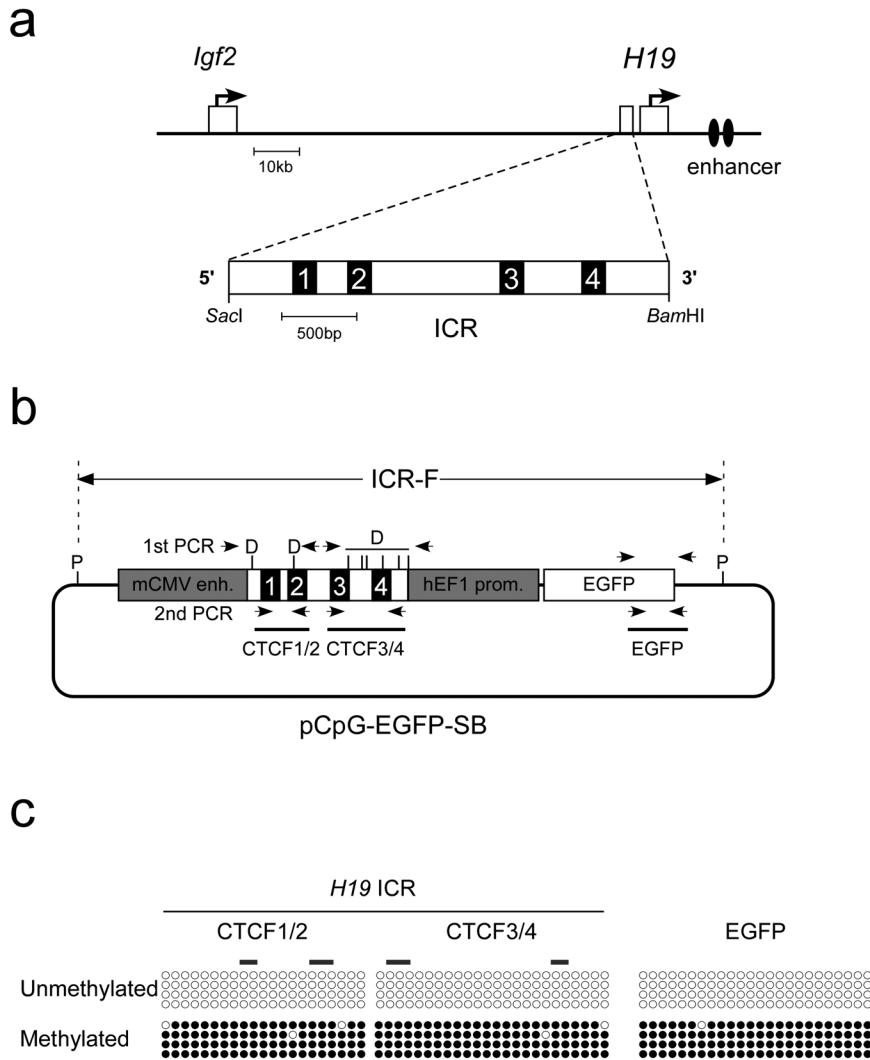
Fig. 2. Generation and *in vitro* methylation of *H19* ICR transgene. (a) Genomic structure of the mouse *Igf2/H19* locus. The *Igf2* and *H19* genes (open boxes) are −90 kb apart, and the expression of both genes depends on the shared 3′ enhancer (filled ovals). The *H19* ICR is located within a 2.9-kbp *Sac*I/*Bam*HI fragment. The black boxes in the enlarged map indicate the position of the CCCTC-binding factor (CTCF) binding sites. (b) A schematic map of the transgene containing *H19* ICR (ICR-F) used in this study. pCpG-EGFP-SB contains 2.9 kb of the mouse *H19* ICR (white box containing four black boxes), mCMV enhancer (gray box), hEF1 promoter (gray box), and EGFP (white box) sequences. CTCF1/2, CTCF3/4, and EGFP regions analyzed for methylation status are indicated in solid lines. Primers used for nested-PCR are shown by arrows. P, *Pac*I; D, *Dpn*I sites. (c) Confirmation of the methylation status in the *H19* ICR transgene fragment used for microinjection. Unmethylated and methylated transgenes were analyzed by bisulfite sequencing analysis using primers shown in B. Methylated and unmethylated CpG motifs are shown as filled and open circles, respectively. Each horizontal row represents a single DNA template molecule. Gray bars indicate the location of the CTCF-binding sites.

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
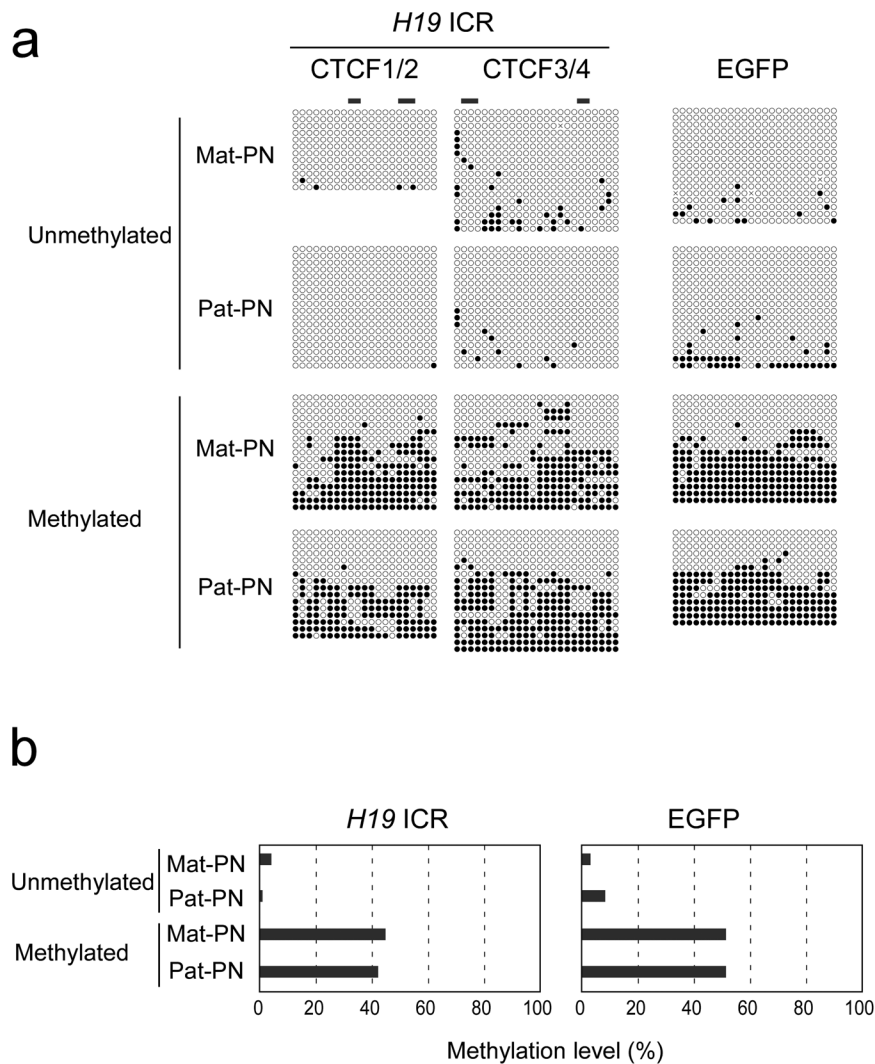
Fig. 3. Methylation analysis of transgenic *H19* ICRs at blastocyst stage. (a) Bisulfite sequencing analysis of embryos that developed to blastocyst stage after microinjection of the ICR-F into the paternal or maternal PN. The methylation status of the ICR-F was indicated as described in Fig. 2c. Genomic DNA of 5–10 blastocysts was extracted as a pool and subjected to *Dpn*I digestion before bisulfite treatment to eliminate the transgene fragments that were unintegrated into the genome. The result is composed of data from three pools (for the CTCF1/2 region by maternal injection using unmethylated transgene, two pools were analyzed). At least six clones were sequenced from each pool. The data from three pools were combined, because the methylation level of each pool was almost the same. (b) Ratio of the DNA methylation levels at *H19* ICR (CTCF1/2 and CTCF3/4) and EGFP regions are indicated.

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
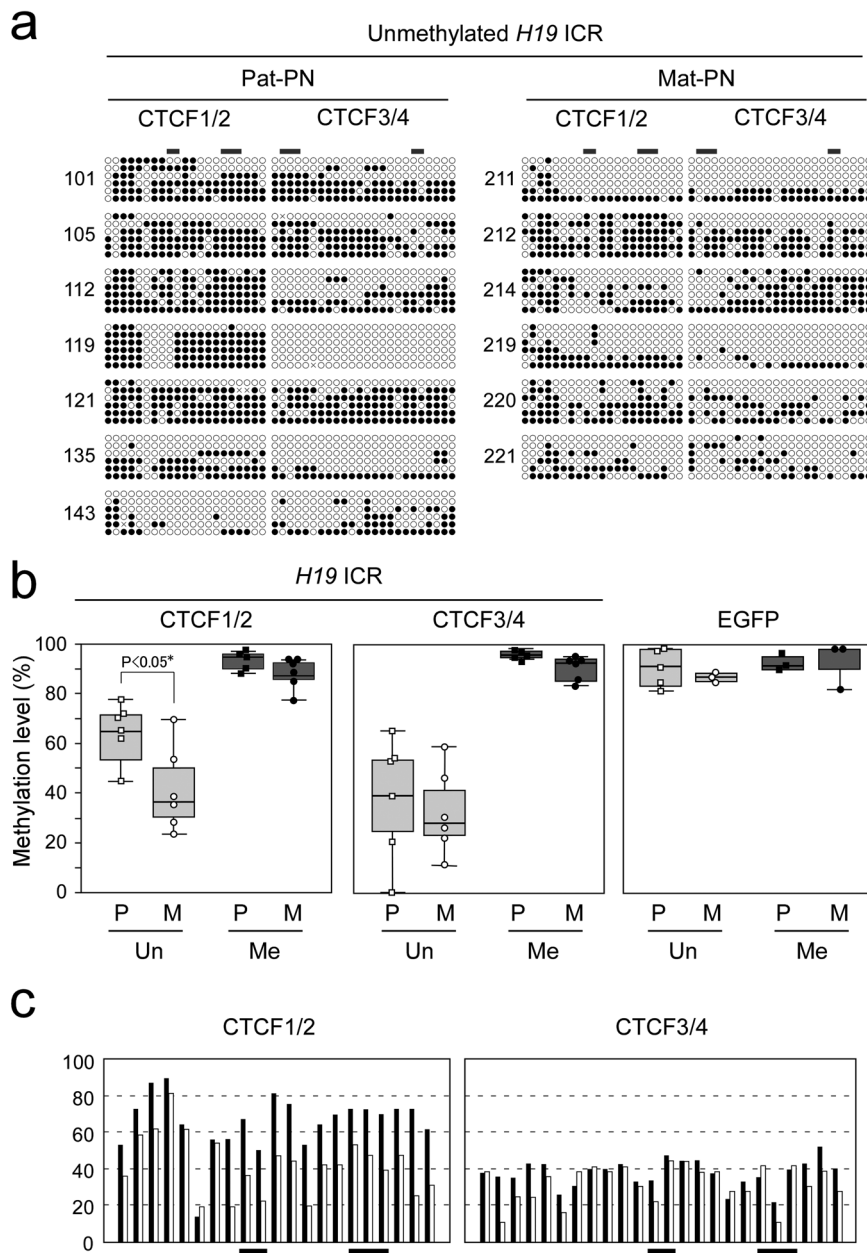
Fig. 4. Methylation analysis of the transgenic *H19* ICR in somatic cells of the founder transgenic mice. (a) Bisulfite sequencing analysis of the ICR-F in transgenic founder mice that were obtained by injecting unmethylated DNA fragments into the paternal or maternal PN. The methylation status of the 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.
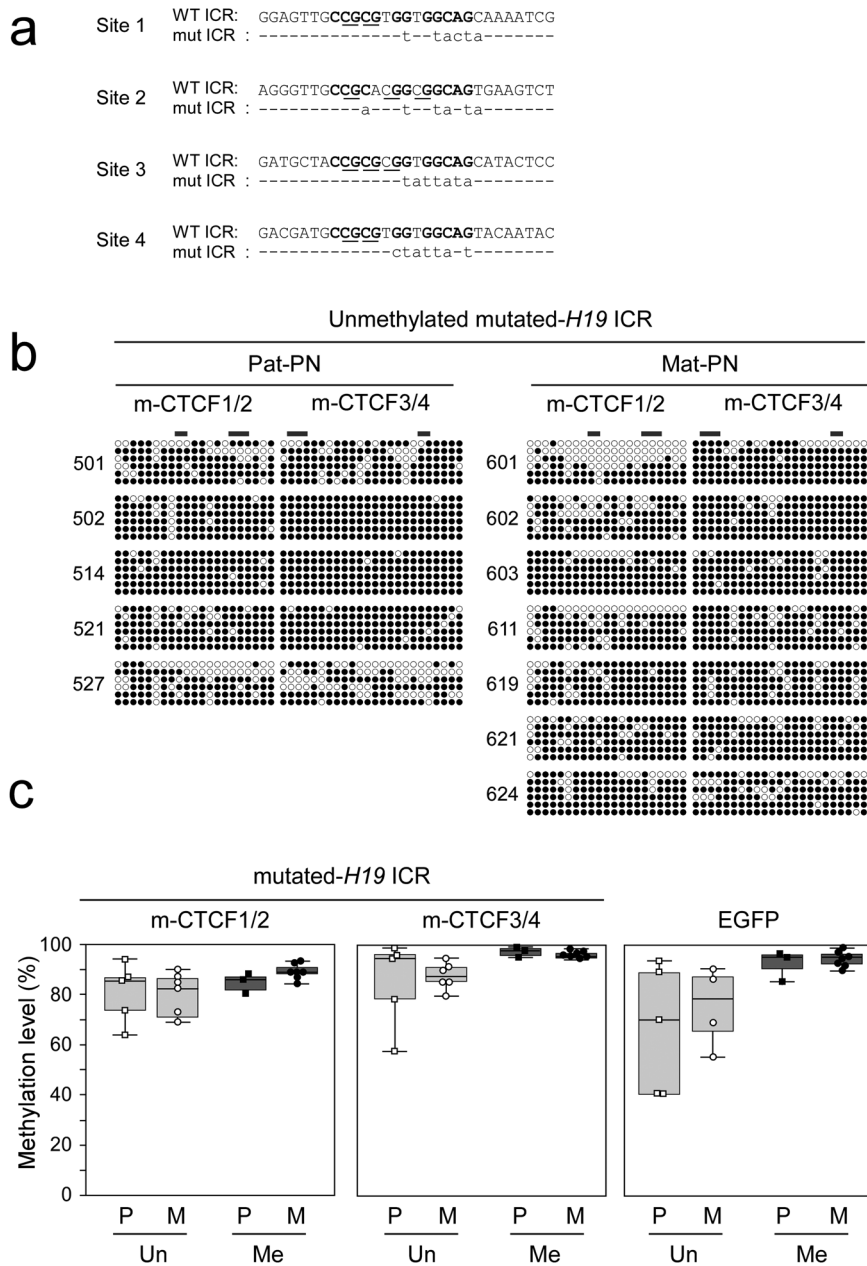
Fig. 5. Methylation analysis of mutated transgenic *H19* ICR in somatic cells of the transgenic mouse lines. (a) Sequences of CTCF-binding sites in mutant (mut)*H19* ICR compared with the wild-type (WT) ICR. CTCF-binding motifs and CpG dinucleotides are indicated in bold and underlined, respectively. Mutated nucleotides in the mutant ICR are shown in lowercase. (b) Bisulfite sequencing analysis of the mutated ICR-F in transgenic founder micethat 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 im-

portant 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 zygotes by 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.

References

1. Araki, K., Araki, M., Miyazaki, J., and Vassalli, P. 1995. Site-specific recombination of a transgene in fertilized eggs by transient expression of Cre recombinase. *Proc. Natl. Acad. Sci. USA* 92: 160–164. [Medline] [CrossRef]
2. Bartolomei, M.S., Zemel, S., and Tilghman, S.M. 1991. Parental imprinting of the mouse H19 gene. *Nature* 351: 153–155. [Medline] [CrossRef]
3. Behringer, R.G.M., Nagy, K.V., and Nagy, A.2014. Manipulating the Mouse Embryo: A Laboratory Manual, Fourth edition, Cold Spring Harbor Laboratory Press, New York.
4. Bell, A.C. and Felsenfeld, G. 2000. Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene. *Nature* 405: 482–485. [Medline] [CrossRef]
5. Biliya, S. and Bulla, L.A. Jr. 2010. Genomic imprinting: the influence of differential methylation in the two sexes. *Exp. Biol. Med. (Maywood)* 235: 139–147. [Medline] [CrossRef]
6. DeChiara, T.M., Robertson, E.J., and Efstratiadis, A. 1991.

Parental imprinting of the mouse insulin-like growth factor II gene. *Cell* 64: 849–859. [Medline] [CrossRef]

7. Ferguson-Smith, A.C. 2011. Genomic imprinting: the emergence of an epigenetic paradigm. *Nat. Rev. Genet.* 12: 565–575. [Medline] [CrossRef]

8. Gebert, C., Kunkel, D., Grinberg, A., and Pfeifer, K. 2010. H19 imprinting control region methylation requires an imprinted environment only in the male germ line. *Mol. Cell. Biol.* 30: 1108–1115. [Medline] [CrossRef]

9. Hark, A.T., Schoenherr, C.J., Katz, D.J., Ingram, R.S., Levorse, J.M., and Tilghman, S.M. 2000. CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus. *Nature* 405: 486–489. [Medline] [CrossRef]

10. Hirasawa, R., Chiba, H., Kaneda, M., Tajima, S., Li, E., Jaenisch, R., and Sasaki, H. 2008. Maternal and zygotic Dnmt1 are necessary and sufficient for the maintenance of DNA methylation imprints during preimplantation development. *Genes Dev.* 22: 1607–1616. [Medline] [CrossRef]

11. Hori, N., Nakano, H., Takeuchi, T., Kato, H., Hamaguchi, S., Oshimura, M., and Sato, K. 2002. A dyad oct-binding sequence functions as a maintenance sequence for the unmethylated state within the H19/Igf2-imprinted control region. *J. Biol. Chem.* 277: 27960–27967. [Medline] [CrossRef]

12. Kaneda, M., Okano, M., Hata, K., Sado, T., Tsujimoto, N., Li, E., and Sasaki, H. 2004. Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. *Nature* 429: 900–903. [Medline] [CrossRef]

13. Lawson, H.A., Cheverud, J.M., and Wolf, J.B. 2013. Genomic imprinting and parent-of-origin effects on complex traits. *Nat. Rev. Genet.* 14: 609–617. [Medline] [CrossRef]

14. Li, X., Ito, M., Zhou, F., Youngson, N., Zuo, X., Leder, P., and Ferguson-Smith, A.C. 2008. A maternal-zygotic effect gene, Zfp57, maintains both maternal and paternal imprints. *Dev. Cell* 15: 547–557. [Medline] [CrossRef]

15. Li, Y. and Sasaki, H. 2011. Genomic imprinting in mammals: its life cycle, molecular mechanisms and reprogramming. *Cell Res.* 21: 466–473. [Medline] [CrossRef]

16. Matsuzaki, H., Okamura, E., Fukamizu, A., and Tanimoto, K. 2010. CTCF binding is not the epigenetic mark that establishes post-fertilization methylation imprinting in the transgenic H19 ICR. *Hum. Mol. Genet.* 19: 1190–1198. [Medline] [CrossRef]

17. Matsuzaki, H., Okamura, E., Shimotsuma, M., Fukamizu, A., and Tanimoto, K. 2009. A randomly integrated transgenic H19 imprinting control region acquires methylation imprinting independently of its establishment in germ cells. *Mol. Cell. Biol.* 29: 4595–4603. [Medline] [CrossRef]

18. Matsuzaki, H., Okamura, E., Takahashi, T., Ushiki, A., Nakamura, T., Nakano, T., Hata, K., Fukamizu, A., and Tanimoto, K. 2015. De novo DNA methylation through the 5′-segment of the H19 ICR maintains its imprint during early embryogenesis. *Development* 142: 3833–3844. [Medline] [CrossRef]

19. Nakamura, T., Arai, Y., Umehara, H., Masuhara, M., Kimura, T., Taniguchi, H., Sekimoto, T., Ikawa, M., Yoneda, Y., Okabe, M., Tanaka, S., Shiota, K., and Nakano, T. 2007. PGC7/Stella protects against DNA demethylation in early embryogenesis. *Nat. Cell Biol.* 9: 64–71. [Medline] [CrossRef]

20. Nakamura, T., Liu, Y.J., Nakashima, H., Umehara, H., Inoue, K., Matoba, S., Tachibana, M., Ogura, A., Shinkai, Y., and Nakano, T. 2012. PGC7 binds histone H3K9me2 to protect against conversion of 5mC to 5hmC in early embryos. *Nature* 486: 415–419. [Medline]

21. Nakanishi, T., Kuroiwa, A., Yamada, S., Isotani, A., Yamashita, A., Tairaka, A., Hayashi, T., Takagi, T., Ikawa, M., Matsuda, Y., and Okabe, M. 2002. FISH analysis of 142 EGFP transgene integration sites into the mouse genome. *Genomics* 80: 564–574. [Medline] [CrossRef]

22. Ohtsuka, M., Miura, H., Sato, M., Kimura, M., Inoko, H., and Gurumurthy, C.B. 2012. PITT: pronuclear injection-based targeted transgenesis, a reliable transgene expression method in mice. *Exp. Anim.* 61: 489–502. [Medline] [CrossRef]

23. Okamura, E., Matsuzaki, H., Sakaguchi, R., Takahashi, T., Fukamizu, A., and Tanimoto, K. 2013. The H19 imprinting control region mediates preimplantation imprinted methylation of nearby sequences in yeast artificial chromosome transgenic mice. *Mol. Cell. Biol.* 33: 858–871. [Medline] [CrossRef]

24. Olek, A. and Walter, J. 1997. The pre-implantation ontogeny of the H19 methylation imprint. *Nat. Genet.* 17: 275–276. [Medline] [CrossRef]

25. Reik, W. and Walter, J. 2001. Genomic imprinting: parental influence on the genome. *Nat. Rev. Genet.* 2: 21–32. [Medline] [CrossRef]

26. Schoenherr, C.J., Levorse, J.M., and Tilghman, S.M. 2003. CTCF maintains differential methylation at the Igf2/H19 locus. *Nat. Genet.* 33: 66–69. [Medline] [CrossRef]

27. Tanimoto, K., Shimotsuma, M., Matsuzaki, H., Omori, A., Bungert, J., Engel, J.D., and Fukamizu, A. 2005. Genomic imprinting recapitulated in the human beta-globin locus. *Proc. Natl. Acad. Sci. USA* 102: 10250–10255. [Medline] [CrossRef]

28. Tucker, K.L., Beard, C., Dausmann, J., Jackson-Grusby, L., Laird, P.W., Lei, H., Li, E., and Jaenisch, R. 1996. Germline passage is required for establishment of methylation and expression patterns of imprinted but not of nonimprinted genes. *Genes Dev.* 10: 1008–1020. [Medline] [CrossRef]

29. Wang, H., Yang, H., Shivalila, C.S., Dawlaty, M.M., Cheng, A.W., Zhang, F., and Jaenisch, R. 2013. One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell* 153: 910–918. [Medline] [CrossRef]

30. Wilkie, T.M., Brinster, R.L., and Palmiter, R.D. 1986. Germline and somatic mosaicism in transgenic mice. *Dev. Biol.* 118: 9–18. [Medline] [CrossRef]