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DNA Methylation Errors in Cloned Mouse Sperm by Germ Line Barrier Evasion¹

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ABSTRACT

The germ line reprogramming barrier resets parental epigenetic modifications according to sex, conferring totipotency to mammalian embryos upon fertilization. However, it is not known whether epigenetic errors are committed during germ line reprogramming that are then transmitted to germ cells, and consequently to offspring. We addressed this question in the present study by performing a genome-wide DNA methylation analysis using a target postbisulfite sequencing method in order to identify DNA methylation errors in cloned mouse sperm. The sperm genomes of two somatic cell-cloned mice (CL1 and CL7) contained significantly higher numbers of differentially methylated CpG sites ($P=0.0045$ and $P=0.0116$). As a result, they had higher numbers of differentially methylated CpG islands. However, there was no evidence that these sites were transmitted to the sperm genome of offspring. These results suggest that DNA methylation errors resulting from embryo cloning are transmitted to the sperm genome by evading the germ line reprogramming barrier.

cloning, DNA methylation, epigenetics, sperm, transgenerational effect

INTRODUCTION

The germ line reprogramming barrier resets parental epigenetic modifications according to sex. Reprogramming in the germ line results in epigenome modification of oocytes and sperm, enabling totipotency to be achieved by fertilization in mammals [1]. The complete and accurate DNA methylome of mice and humans has been determined by whole-genome sequencing [2–5], providing an opportunity to assess the accuracy and reliability of the germ line reprogramming

barrier. To survive through generations, organisms must remove epigenome errors caused by aging, DNA replication, and detrimental environmental stimuli from their germ cells [6, 7]; it is assumed that the germ line reprogramming barrier is essential for this purpose [8]. However, there is no concrete evidence to support this notion, and our understanding of this process is inadequate.

Transgenerational effects of nutritional defects, stress, and environmental factors on epigenome mutations have been extensively studied because these are implicated in various diseases [9, 10], and transmission of DNA methylation errors to descendants can have serious consequences. However, there is limited evidence for the transgenerational effects of epigenetic errors; one study showed that in utero undernourishment leads to sperm methylome errors associated with metabolic disease in offspring [11].

Somatic cell cloning technology and construction of nuclear-cytoplasmic cybrids confers totipotency to the genome of terminally differentiated cells [12–14]. However, these processes are frequently accompanied by dysplasia of the embryo and placenta [15–19]. Analyses of DNA methylation by bisulfite sequencing focusing on imprinted regions have revealed significant demethylation [20, 21]. An outstanding question is whether methylation errors are transmitted to germ cells beyond the germ line reprogramming barrier or whether they are corrected in the germ line. Somatic cell-cloned (CL) mice are considered as a useful model for addressing these questions.

To gain insight into the transmission of methylome errors through the germ line reprogramming barrier, we carried out a methylome analysis of sperm obtained from CL mice and their descendants. The postbisulfite adaptor tagging (PBAT) method combined with enrichment of target regions—known as SureSelect Methyl-Seq (SSM)—was used to identify DNA methylome errors in sperm derived from cloned mice [22]. We demonstrate that methylome errors persist in cloned mouse sperm as a result of germ line barrier evasion.

MATERIALS AND METHODS

Animals and Embryo Cloning

All animal experiments were approved by the Institutional Review Board of the University of Yamanashi (permit number A 24-50) and Tokyo University of Agriculture (permit number 100553). BDF1 mice (C57BL/6NJcl × DBA/2Jcl; CLEA Japan, Tokyo, Japan) were used in all experiments. Mature sperm was isolated from 10- to 12-wk-old mice.

Tail-tip fibroblast cell cultures were established at the time of sperm collection to obtain somatic donor nuclei for generating CL mice [17]. The donor nucleus was injected into enucleated oocytes from BDF1 mice according to our original protocol, in which reconstructed oocytes were treated with trichostatin A after oocyte activation. Four-cell-stage embryos were transferred into the oviducts of recipient females and 11 males in four litters developed to full term.

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Sperm Collection

Sperm were collected from CL mice exhibiting no phenotypic abnormalities, their descendants, and wild-type (WT) mice (10–12 wks old). After incubation in 200 μ l of TYH medium for 1 h, the sperm were collected by the swim-up method and used for DNA methylation analysis.

DNA preparation

Sperm DNA was isolated by a standard phenol-chloroform extraction procedure with dithiothreitol, and 1 μ g of DNA was dissolved in 130 μ l of 10 mM Tris-HCl (pH 8.0) and sheared with an S220 focused ultrasonicator (Covaris, Woburn, MA), yielding 500-bp fragments. The AMPure XP system (Agilent Technologies, Santa Clara, CA) was used to purify the fragmented DNA as follows. Sheared DNA (130 μ l) was mixed with 1.8 \times volume (234 μ l) of AMPure XP reagent and allowed to stand for 15 min at room temperature. The beads were collected using a magnetic stand, the supernatant was removed, and pelleted beads were rinsed with 70% ethanol and dried by incubation at 37°C for 5 min. DNA was then eluted from the beads with 20 μ l of RNase-free water. The eluted DNA solution was dried under vacuum and dissolved in 7 μ l of RNase-free water.

Target Enrichment

Targets' enrichment by liquid-phase hybridization capture was performed using the SureSelect Mouse Methyl-Seq kit (Agilent Technologies) [22]. Genomic DNA (7 μ l) that was fragmented and purified as described above was supplemented with 3 μ l of formamide (biochemistry grade; Wako Pure Chemical Industries, Osaka, Japan) and overlaid with 80 μ l of mineral oil (Sigma-Aldrich, St. Louis, MO). The DNA was completely denatured by incubating the tube at 99°C for 10 min; the tube was then cooled to and maintained at 65°C for at least 5 min before adding the following reagents. Hybridization buffer and capture probe mix were prepared according to the manufacturer's protocol, and they were each overlaid with 80 μ l of mineral oil and incubated at 65°C for 10 min. The two solutions were then combined and mixed thoroughly by pipetting. The combined solution was transferred to a tube containing the denatured input DNA (maintained at 65°C as described above), and the solution was thoroughly mixed by pipetting. The tube was incubated at 65°C for 24 h to allow hybridization between probes and targets. A 50- μ l volume of well-suspended DynaBeads MyOne Streptavidin T1 solution (Life Technologies, Carlsbad, CA) was placed in a 1.5-ml tube, and the beads were washed twice with 200 μ l of binding buffer. The hybridization reaction supplemented with 200 μ l of binding buffer was added to the pelleted beads with thorough mixing. After incubation at room temperature for 30 min with agitation, the beads were collected using a magnetic stand and were washed with 500 μ l of wash buffer 1, and then subjected to three rounds of washing and resuspension in prewarmed buffer 2, followed by incubation at 65°C for 10 min. After removing the washing solution from the tube, enriched DNA was eluted by incubating the beads in 20 μ l of elution buffer at room temperature for 20 min. The eluate was immediately subjected to bisulfite treatment.

Bisulfite Treatment

The EZ DNA Methylation-Gold kit (Zymo Research, Irvine, CA) was used for bisulfite treatment of target-enriched DNA according to the manufacturer's instructions. The enriched DNA solution (20 μ l) was mixed with 130 μ l of freshly prepared CT conversion reagent, and the mixture was incubated at 64°C for 2.5 h. The 10-min incubation step at 98°C was omitted because the target-enriched DNA was already denatured. After purification and desulfonation, bisulfite-treated DNA was eluted with 20 μ l of M-elution buffer.

PBAT Library Construction and Illumina Sequencing

We used bisulfite-treated DNA for library preparation according to the PBAT protocol [22] (also available from <http://crest-ihc.jp/english/epigenome/index.html>), except that the following primers were used. The primer used for first-strand synthesis was 5'-biotin ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT WWW WNN NN-3' (W = A or T). The indexed primer used for second-strand synthesis was 5'-CAA GCA GAA GAC GGC ATA CGA GAT XXX XXX GTA AAA CGA CGG CCA GCA GGA AAC AGC TAT GAC WNN WNN NN-3', where XXX XXX represents the index sequence of each primer. The constructed SSM-PBAT libraries were sequenced as previously described [2–5] using the Illumina HiSeq2500 system (San Diego, CA).

Target Methylome Sequence Alignment and Statistical Analysis

SSM-PBAT reads were aligned to the mouse genome (mm10; Genome Reference Consortium Mouse Build 38) using the Bismark tool (v.0.10.0; <http://www.bioinformatics.babraham.ac.uk/projects/bismark/>), with the specific options: `-q -n 2 -l 93 -pbat`. Statistical significance of DNA methylation at each CpG site and CGI (CpG islands) was evaluated by the Fisher exact test and the Mann-Whitney *U* test. Composite profiles were generated using SeqMonk (<http://www.bioinformatics.babraham.ac.uk/projects/seqmonk/>).

RESULTS

Summary of SSM-PBAT Library

Somatic cell nuclear transfer (SCNT) clones used in these experiments had normal phenotype, and their sperm were active and were obtained at high concentrations. Sequence analysis of sperm samples from five wild-type (WT) and eight SCNT mice yielded 25.4 million to 60.7 million reads (29.1%–47.5% of total reads) that mapped to the mm10 reference (Table 1). The average depth of each sample was 15.2–37.6, which covered 68.8%–77.9% of target regions at five depths. This indicated that obtained sequencing data were sufficient for further analysis of clone-specific methylation errors.

We also determined the correlation coefficient of DNA methylation data between individuals. The correlation coefficients showed similarly high values (i.e., 0.97–0.99; Supplemental Fig. S1A; Supplemental Data are available online at www.biolreprod.org). Values for CGI and its shores (CGIsh: ± 2 kb of CGI), and germ line differentially methylated regions (DMRs; Supplemental Fig. S1, B–D) were also similar. A violin plot analysis showed that all samples analyzed belonged to the same group (Supplemental Fig. S2). These results demonstrate that overall methylation profiling does not detect latent methylation errors in the sperm of CL mice, although they may be present, albeit at a low rate and with a high degree of variability.

Profiling of Differentially Methylated Sites and Islands

To identify differentially methylated sites (DMS) in individual sperm genome libraries, the methylation level of each CpG site was compared to the average level in WT, and significance was assessed with Fisher exact test ($P < 0.01$). Both hypermethylated and hypomethylated DMS were detected, ranging from 154 to 875 and 106 to 1874, respectively (Fig. 1A), out of a total of 2454646–2840983 tested CpG sites (Supplemental Table S1). Remarkably, two of eight CL mice (CL1 and CL7) had significantly higher numbers of DMS (Grubbs test for outliers; $P = 0.0045$ in CL1, $P = 0.0116$ in

TABLE 1. Summary of SSM-PBAT libraries in cloned mouse sperm.

Sample	Uniquely mapped reads	Coverage, %	
		≥ 1 depth	≥ 5 depth
WT1	25 387 591	81.8	68.8
WT2	51 190 726	84.5	76.5
WT3	38 433 505	83.5	75.1
WT4	36 548 161	83.4	74.4
WT5	38 983 254	83.2	74.7
CL1	60 617 341	84.4	77.9
CL2	31 645 817	82.6	71.6
CL3	34 599 480	82.6	73.5
CL4	35 409 166	82.6	73.9
CL5	52 003 012	83.2	76.2
CL6	50 007 339	84.2	76.5
CL7	43 018 375	82.9	75.2

A

No. DMS

	WT1	WT2	WT3	WT4	WT5	CL1	CL2	CL3	CL4	CL5	CL6	CL7
Hypermethylated	504	154	303	344	155	223	757	461	483	459	344	875
Hypomethylated	356	686	106	164	243	1551	300	379	343	491	220	1874

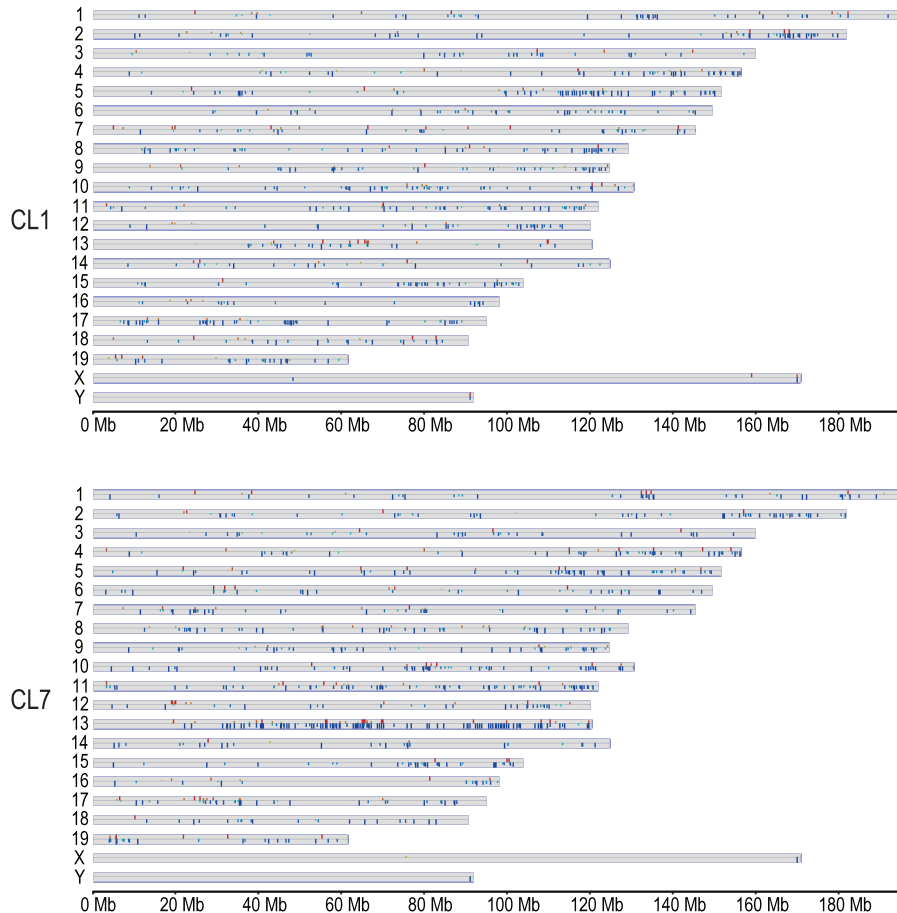
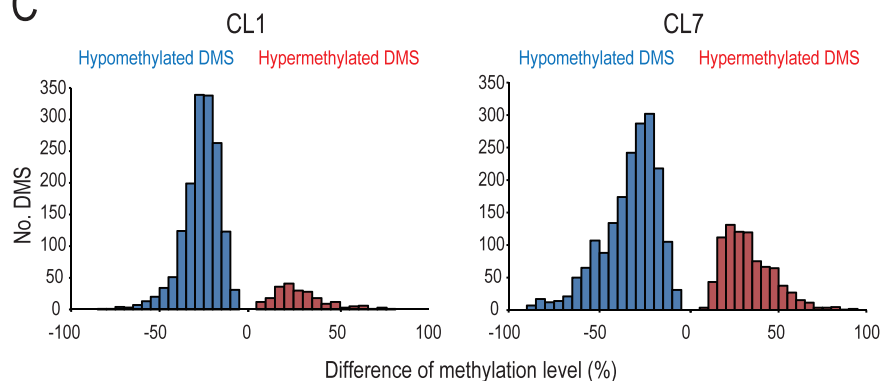
B**C**

FIG. 1. Differentially methylated CpG sites in CL mouse sperm. **A**) Number of hypermethylated and hypomethylated DMS in the sperm of WT ($n = 5$) and CL ($n = 7$) mice. DNA methylation levels at each CpG site across targeted regions (2 881 255 CpG sites) were compared between each individual and the average of WT mice, which served as the normal sperm methylation control. Fisher exact test was used to assess DMS ($P < 0.01$). **B**) DMS distribution on each chromosome of CL1 and CL7 mouse sperm. Hypermethylated (red) and hypomethylated (blue) DMS are shown as a chromosome-scale distribution. **C**) Differential methylation levels at DMS in CL1 and CL7 mouse sperm. Histograms of differences between CL and the average WT methylation level (AWT) were constructed based on hypermethylated (CL – AWT) and hypomethylated (AWT – CL) DMS.

CL7)—which were predominantly hypomethylated (Grubbs test for outliers; $P = 0.0393$ in CL1, $P = 0.0004$ in CL7)—than the others. The DMS were widespread in the sperm genome and were potentially associated with genome-wide DNA methylation and/or demethylation events through germ line reprogramming (Fig. 1B and Supplemental Table S2). Interestingly, DMS were frequently located on chromosome 13 in CL7 (hyper: 0.089%; hypo: 0.419%). Differences at hypermethylated and hypomethylated DMS in CL1 and CL7 were mostly in the $\pm 15\%$ to $\pm 50\%$ range (Fig. 1C). Differentially methylated sites were detected in promoter and exon regions, although many were located in intergenic and intronic regions (Fig. 2). These results demonstrate that aberrant CpG loci are present in the CL mouse sperm genome, consistent with the error-prone signature of epigenetic reprogramming in somatic cell cloning.

We then assessed the contribution of clustered DMS to CGI and CGIsh in the sperm genome. CGI and CGIsh that included $>15\%$ DMS of total CpGs and were significantly different by the Mann-Whitney U test ($P < 0.01$) were defined as differentially methylated CGIs (DMI) and CGIsh (DMIsh),

respectively (Fig. 3, A and B, and Supplemental Table S3). We classified CGI and CGIsh (23 021 both upstream and downstream) in each CL mouse as DMI or DMIsh. In total, 27 DMI (hyper: 11; hypo: 16) and 9 DMIsh (hyper: 6; hypo: 3) were identified; consistent with the DMS analysis, many DMI were observed in the sperm libraries of CL1 and CL7. Although further studies are required to discriminate between cloning-associated errors and occurring variations, these data provide experimental evidence that CGI methylation in CL mice is distinct from that observed in WT mice.

Interestingly, some DMI and DMIsh were common to multiple CL mouse sperm libraries. CGIsh reportedly discriminate between different cell types, including somatic [23] and reprogrammed [24] cells, and are therefore potential targets for DNA methylation errors. For example, a hypermethylated DMI (CGI4464) was observed in three of the seven CL mice (Fig. 3B and Supplemental Fig. S3), and two of the mice had the same hypomethylated DMI (CGI11496). These DMI may be loci that are susceptible to changes in methylation caused by erroneous germ line reprogramming. Similarly, some DMIsh were consistently hypermethylated and hypomethylated in the

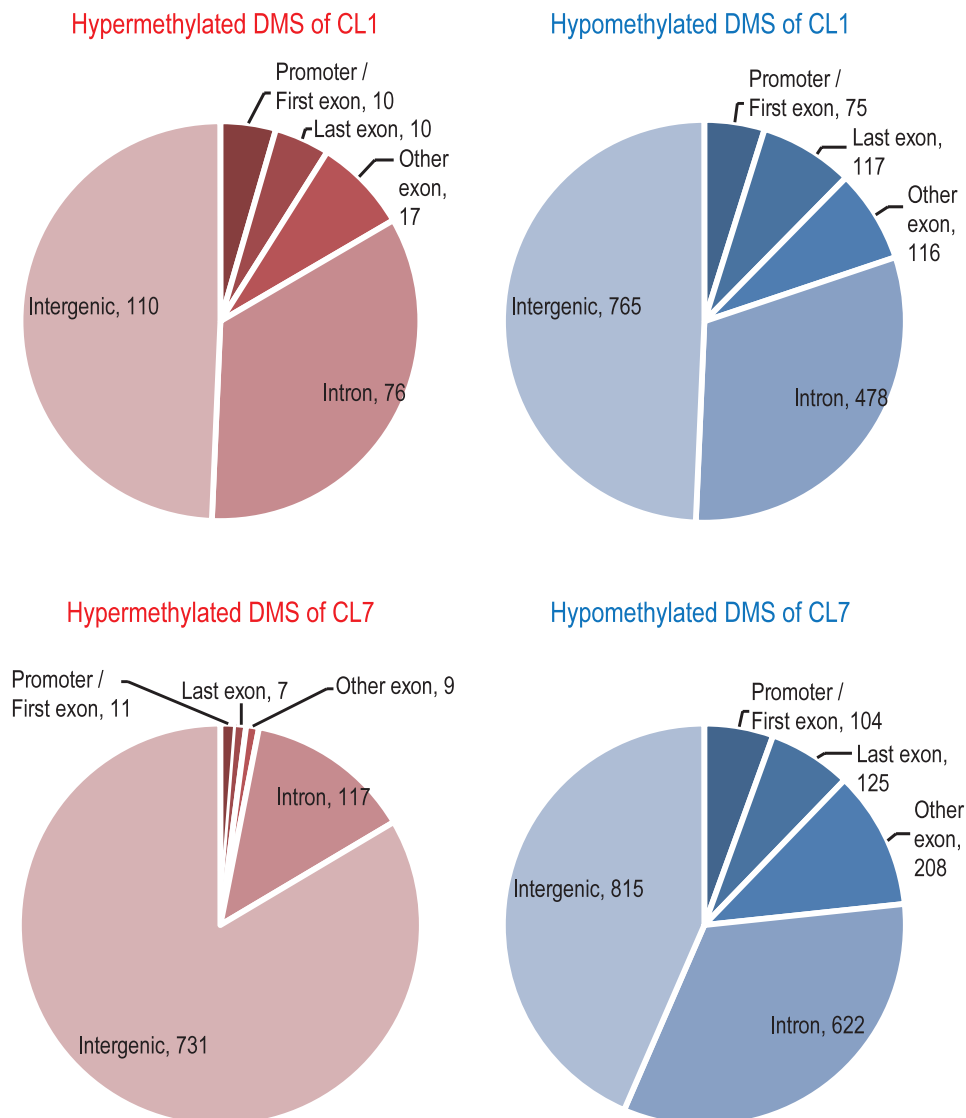


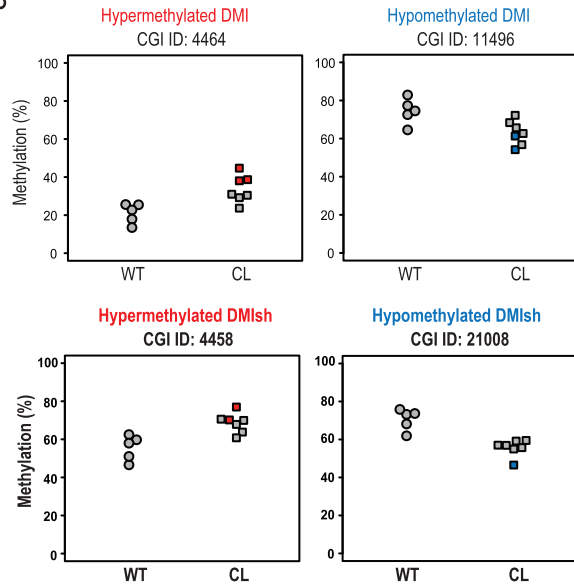
FIG. 2. Distribution of DMS in specific genomic regions in CL1 and CL7. Pie charts show the frequency of DMS in genic, intronic, and intergenic regions.

A

No. DMI	CL1	CL2	CL3	CL4	CL5	CL6	CL7
Hypermethylated	0	3	1	2	2	0	3
Hypomethylated	10	0	0	0	1	0	5

No. DMish	CL1	CL2	CL3	CL4	CL5	CL6	CL7
Hypermethylated	0	2	0	0	1	0	3
Hypomethylated	0	0	0	0	0	0	3

B



C



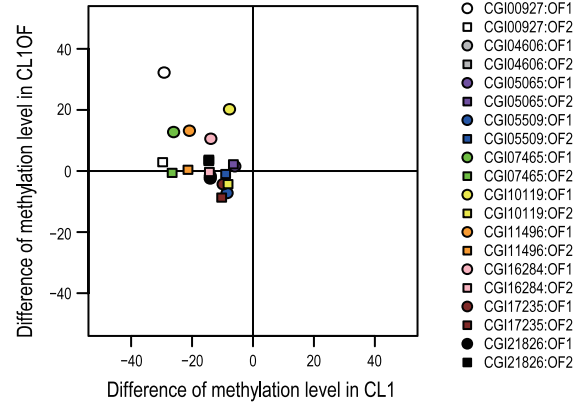
FIG. 3. Differentially methylated CGI in CL mouse sperm. **A**) Number of hypermethylated and hypomethylated DMI and DMish in the sperm of individual CL mice. **B**) Methylation level of common DMI (CGI IDs: 4464 and 11496) and DMish (CGI IDs: 4458 and 21008) in individual WT and CL mice. Red and blue squares represent individuals exhibiting significant differences ($P < 0.01$). **C**) A motif of DMish that binds to the reprogramming factors POU domain/class 5/transcription factor 1 (OCT4) and sex-determining region Y-box 17 (SOX17). The motif that shows 60% homology to target sequences was detected at a significant frequency ($P < 0.01$) by HOMER (<http://homer.salk.edu/homer/>).

cloned mice (Fig. 3B). To investigate the possible effect of DMI and DMish on gene expression, we investigated the neighboring genes (± 5 kb from DMI or DMish; Table 2). The DMI existed in the promoter of small *G-protein-signaling modulator 1*, *complexin 3*, *WNK lysine-deficient protein kinase 4*, *Arhgap*, *PC esterase domain-containing 1B*, and *histocompatibility 2 O region α* genes, whereas DMish were also located in the promoter of *gap junction protein $\alpha 4$* . Interestingly, a motif (CCATTGTATGCAAAT) that binds to the reprogramming factors POU domain/class 5/transcription factor 1 and sex-determining region Y-box 17 (also known as OCT4 and SOX17, respectively) was detected in the DMIs (Fig. 3C).

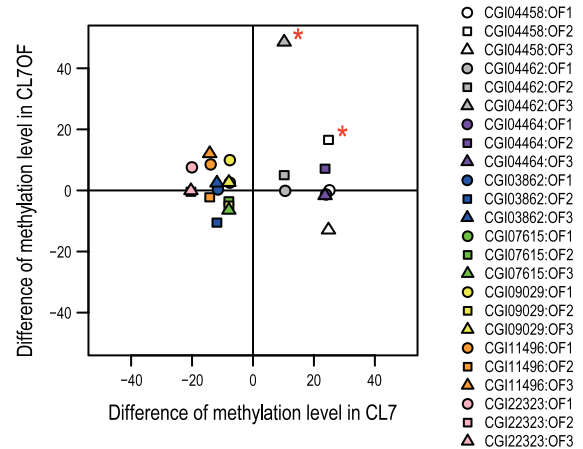
Transmission of CL DMI to Offspring

To determine whether DNA methylation errors in the sperm of SCNT cloned mice are transmitted to the genome of descendants' sperm, we analyzed sperm derived from CL1 and CL7 offspring (Supplemental Table S4). There was no evidence that DNA methylation errors in the sperm of CL mice were present in the sperm of their offspring (Fig. 4 and

A



B



C

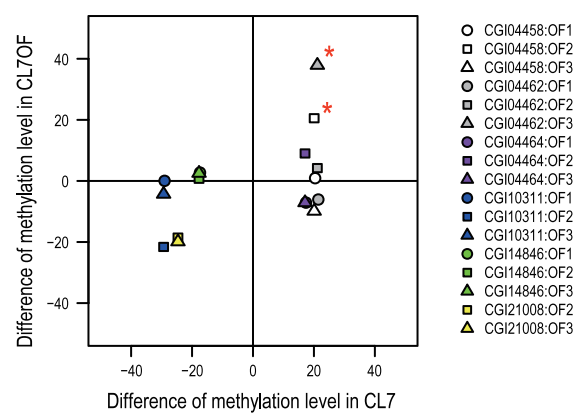


FIG. 4. DNA methylation status in sperm from offspring of CL1 and CL7. Results are shown as methylation change breadth in a two-dimensional coordinate graph. Methylation levels of each DMI (**A**: CL1/CL1OF; **B**: CL7/CL7OF) and DMish (**C**: CL7/CL7OF) are represented by different colors, and each offspring is represented by a different symbol (\circ , Δ , and \square). DMI and DMish marked by asterisks are significantly different ($P < 0.01$). Statistical significance was evaluated in the same way as for CL mouse data sets (see text).

TABLE 2. The neighboring genes of DMI and DMIs in sperm of cloned mice.

CGI	Status	Clone no.	Chromosome	Position	Genes in promoter	Genes in gene body
DMI						
CGI00927	Hypo	1	chr1	Intergenic		
CGI11496	Hypo	1, 7	chr2	Gene body		Ppapdc3 (NM_145521)
CGI16284	Hypo	1	chr5	Promoter & gene body	Sgsm1(NM_001162965)	Sgsm1(NM_172718)
CGI17235	Hypo	1	chr6	Gene body		Kcp (NM_001029985)
CGI21826	Hypo	1, 5	chr9	Promoter & gene body	Cplx3 (NM_146222)	Cplx3 (NM_146223)
CGI22323	Hypo	7	chr9	Gene body		Bsn (NM_007567)
CGI03862	Hypo	7	chr11	Promoter & gene body	Wnk4 (NM_175638)	Wnk4 (NM_175638)
CGI04458	Hyper	2, 3, 4, 5	chr12	Intergenic		
CGI04464	Hyper	2, 5, 7	chr12	Intergenic		
CGI04606	Hypo	1	chr12	Gene body		Egln3 (NM_028133)
CGI05065	Hypo	1	chr12	Intergenic		
CGI05509	Hypo	1	chr13	Intergenic		
CGI07465	Hypo	1	chr15	Promoter & gene body	Arhgap (8NM_028455)	Gm20556 (NR_040347)
CGI07615	Hypo	7	chr15	Promoter & gene body	Pced1b (NM_172293)	Amigo2 (NM_178114)
CGI09029	Hypo	7	chr17	Promoter & gene body	H2-Oa (NM_008206)	BC051537 (NR_046183)
CGI10119	Hypo	1	chr18	Gene body		Afap111 (NM_178928)
DMIs						
CGI14846 – 2kb	Hypo	7	chr4	Promoter & gene body	Gja4 (NM_008120)	Gja4 (NM_008120)
CGI21008 – 5kb	Hypo	7	chr8	Gene body		Zfx3 (NM_007496)
CGI04458 + 5kb	Hyper	2, 7	chr12	Intergenic		
CGI04462 + 5kb	Hyper	7	chr12	Intergenic		
CGI04464 + 5kb	Hyper	2, 5, 7	chr12	Intergenic		
CGI10311 – 5kb	Hypo	7	chr18	Gene body		Sall3 (NM_178280)

Supplemental Tables S5–S7). Although the DMI (CGI ID 4462) of CL7 was, in fact, transmitted to offspring No. 3 (Fig. 4, B and C, and Supplemental Tables S6 and S7), this likely arose by stochastic occurrence of DMS and DMI.

DISCUSSION

Somatic cell cloned embryos are produced by transplantation of a terminally differentiated cell to enucleated oocytes [17]. The nucleus-cytoplasmic cybrid environment induces reprogramming of the donor nucleus to achieve totipotency; however, this can lead to the occurrence of epigenetic errors.

Because of developmental failure caused by inappropriate epigenetic reprogramming, the majority of CL embryos die during postimplantation development [15, 20, 25], whereas CL animals that develop to full term have relatively minor epigenetic errors that are not life-threatening. Furthermore, aberrations in DNA methylation in individual CL animals occur randomly at different sites and to varying degrees [26]. The stochastic nature of clone-associated reprogramming errors [27, 28] raises the possibility that any DMS are randomly distributed throughout the genome at multiple loci. The present study examined whether epigenetic reprogramming errors penetrate the germ line reprogramming barrier and are transmitted to sperm. Target DNA methylome data demonstrated that such errors occurred above the stochastic rate in the sperm genome of two of seven CL mice, suggesting that the germ line reprogramming machinery—which is responsible for transmitting correct genetic and epigenetic information to the descendant—is not flawless.

In mouse embryonic stem cell cloning, malformations observed at birth, such as fetal overgrowth, hypertrophic placenta, and undeveloped palpebral, among others, are not transmitted to descendants. For instance, in a previous study we produced male and female cloned mice derived from XY and XO embryonic stem cells of the cell line, and there were no malformations in later generations obtained by mating them [29]. However, the fact that a normal phenotype has been restored in descendants is not evidence that all epigenetic errors have been reprogrammed. In fact, the present study detected

some regions that deviated markedly from the normal rate of DNA methylation. However, it is difficult to suggest that DMRs affect the expression of neighboring genes. On the other hand, even in the absence of phenotypic anomalies, DMRs that potentially regulate gene expression may be concealed in the sperm of CL mice. Even if methylation errors are generated stochastically, they are likely to cluster at specific sites or in specific regions. DNA-binding factors are known to locally influence DNA methylation state [30], suggesting that the spatial organization of chromatin determines fluctuations in DNA methylation. Therefore, information regarding chromatin landscapes may help to identify epimutation hot spots in sperm.

An open question is how methylation errors occur in the CL germ line. It can be supposed that germ line reprogramming machinery regulates DMRs, which directly affects embryonic development. However, this regulation may not extend to other nonessential DMRs. This is supported by the observation that considerable variation was detected in the control sperm genome. We have previously described germ line DNA methylome dynamics in mice [2, 3]; DNA methylation is erased by E 13.5 in both female and male primordial germ cells (PGCs), and sex-specific methylation patterns are established in each gamete. Thus, DNA methylation errors can result from errors in PGCs and during de novo methylation. It has been also reported that sperm DNA methylation changes of aged mice affect offspring behavior [31]. Moreover, it was found that histone modifications represent epigenetic features of PGCs after DNA methylation erasure, because H3K4me3 and H3K427me3 were enriched in specific genomic regions [32, 33]. These histone modification errors could potentially lead to DNA methylation errors in CL sperm. Furthermore, it remains unclear whether parental epigenetic errors are transmitted to the next generation [34, 35]. Our study showed that DNA methylation errors in the CL sperm genome were erased by their descendants' germ line reprogramming machinery. Thus, this machinery has the ability to correct errors at functionally important genomic regions, but it may be less sensitive to trivial errors.

To date, a particular correction of epigenetic modification has been shown to improve the development of SCNT

embryos: for example, trichostatin A, a histone deacetylase inhibitor, opens the closed chromatin structure and induces gene expression [36–38]. A recent study also showed that the acceleration of DNA demethylation with PP242, a specific inhibitor for mammalian target of rapamycin, improved the development of mouse SCNT embryos [39]. These results revealed that despite the fact that it exerts a restrictive effect, appropriate epigenetic modification is a crucial element for the development of SCNT embryos. However, errors in the epigenetic modification in cloned embryos would be expanded to the entire genome. Despite this, the direct relationships between mutations in a domain and the development of the embryos remained difficult to understand.

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