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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.

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/6Jcl × 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 tricostatin 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.
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 μl 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 lithium chloride, and 1 μl of DNA was dissolved in 130 μl of 10 mM Tris-HCl (pH 8.0) and sheared with an SS20 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 μl) was mixed with 1.8× volume (234 μl) 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 μl of RNase-free water. The eluted DNA solution was dried under vacuum and dissolved in 7 μl 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 μl) that was fragmented and purified as described above was supplemented with 3 μl of formamide (biochemistry grade; Wako Pure Chemical Industries, Osaka, Japan) and overlaid with 80 μl 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 μl 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-μl 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 μl of binding buffer. The hybridization reaction was supplemented with 200 μl 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 μl 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 μl 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 μl) was mixed with 130 μl 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 μl 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://cres-t-ihec.jp/english/epigenome/index.html), except that the following primers were used. The primer used for first-strand synthesis was 5′-biotin ACA CTT TTC 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′-CGA GCA GAA GAC GGC ATCA CGA GAT XXX XXX GTA AAA CGA CGG CCA GCA GGA AAC AGC TAT GAC WWW 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 –pb. 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.bioinfomatics.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.bioreprod.org). Values for CGI and its shores (CGIs: ±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 2,454,646–2,840,983 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 CL7).

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

<table>
<thead>
<tr>
<th>Sample</th>
<th>Uniquely mapped reads</th>
<th>&gt;1 depth</th>
<th>&gt;5 depth</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT1</td>
<td>25,387,591</td>
<td>81.8</td>
<td>68.8</td>
</tr>
<tr>
<td>WT2</td>
<td>51,190,726</td>
<td>84.5</td>
<td>76.5</td>
</tr>
<tr>
<td>WT3</td>
<td>38,433,505</td>
<td>83.5</td>
<td>75.1</td>
</tr>
<tr>
<td>WT4</td>
<td>36,548,161</td>
<td>83.4</td>
<td>74.4</td>
</tr>
<tr>
<td>WT5</td>
<td>39,983,254</td>
<td>83.2</td>
<td>74.7</td>
</tr>
<tr>
<td>CL1</td>
<td>60,167,341</td>
<td>84.4</td>
<td>77.9</td>
</tr>
<tr>
<td>CL2</td>
<td>31,645,817</td>
<td>82.6</td>
<td>71.6</td>
</tr>
<tr>
<td>CL3</td>
<td>34,599,480</td>
<td>82.6</td>
<td>73.5</td>
</tr>
<tr>
<td>CL4</td>
<td>35,409,166</td>
<td>82.6</td>
<td>73.9</td>
</tr>
<tr>
<td>CL5</td>
<td>52,003,012</td>
<td>83.2</td>
<td>76.2</td>
</tr>
<tr>
<td>CL6</td>
<td>50,007,339</td>
<td>84.2</td>
<td>76.5</td>
</tr>
<tr>
<td>CL7</td>
<td>43,018,375</td>
<td>82.9</td>
<td>75.2</td>
</tr>
</tbody>
</table>
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.

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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 ±15% to ±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 CGIs in the sperm genome. CGI and CGIs 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 CGIs (DMIsh), respectively (Fig. 3, A and B, and Supplemental Table S3). We classified CGI and CGIs (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. CGIs 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.
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 α4. 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).

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 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 DMI 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/).

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 (*, △, and □). 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).
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 methylation dynamics in mice [2, 3]; DNA methylation is erased by E13.5 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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We thank Rumi Ohtake for assistance with next-generation sequencing data collection, and Hidehiko Ogawa and Yayoi Obata for helpful comments.

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7. Jenkins TG, Aston KI, Pflueger C, Cairns BR, Carrell DT. Age-associated 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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