RNA sequencing-based identification of aberrant imprinting in cloned mice

Hiroaki Okae1,3,†, Shogo Matoba4,†, Takeshi Nagashima2, Eiji Mizutani4,5, Kimiko Inoue4, Narumi Ogonuki4, Hatsune Chiba1,3, Ryo Funayama2, Satoshi Tanaka6, Nobuo Yaegashi7, Keiko Nakayama2, Hiroyuki Sasaki3,8, Atsuo Ogura4 and Takahiro Arima1,3,∗

1Department of Informative Genetics, Environment and Genome Research Center, 2Division of Cell Proliferation, United Centers for Advanced Research and Translational Medicine, Tohoku University Graduate School of Medicine, Sendai 980-8575, Japan 3JST, CREST, Saitama 332-0012, Japan 4RIKEN BioResource Center, Tsukuba, Ibaraki 305-0074, Japan 5Faculty of Life and Environmental Sciences, University of Yamanashi, Kofu 400-8510, Japan 6Laboratory of Cellular Biochemistry, Department of Animal Resource Sciences/Veterinary Medical Sciences, The University of Tokyo, Tokyo 113-8657, Japan 7Department of Obstetrics and Gynecology, Tohoku University Graduate School of Medicine, Sendai 980-8574, Japan and 8Division of Epigenomics, Department of Molecular Genetics, Medical Institute of Bioregulation, Kyushu University, Fukuoka 812-8582, Japan

Received May 8, 2013; Revised August 19, 2013; Accepted October 2, 2013

Animals cloned by somatic cell nuclear transfer (SCNT) provide a unique model for understanding the mechanisms of nuclear epigenetic reprogramming to a state of totipotency. Though many phenotypic abnormalities have been demonstrated in cloned animals, the underlying mechanisms are not well understood. In this study, we performed transcriptome-wide allelic expression analyses in brain and placental tissues of cloned mice. We found that Gab1, Sfmbt2 and Slc38a4 showed loss of imprinting in all cloned mice analyzed, which might be involved in placentomegaly of cloned mice. These three genes did not require de novo DNA methylation in growing oocytes for the establishment of imprinting, implying the involvement of a de novo DNA methylation-independent mechanism. Loss of Dlk1-Dio3 imprinting was also observed in nearly half of cloned mouse embryos and showed a strong correlation with embryonic lethality. Our findings are essential to understand the underlying mechanisms of developmental abnormalities of cloned animals. We also emphasize that particular attention should be paid to specific imprinted genes for therapeutic and agricultural applications of SCNT.

INTRODUCTION

Animals cloned by somatic cell nuclear transfer (SCNT) provide a unique model for understanding the mechanisms of nuclear epigenetic reprogramming to a state of totipotency (1,2). However, the cloning efficiency is very low regardless of the animal species or donor cell type. Though many phenotypic abnormalities have been demonstrated in cloned animals, including frequent embryonic and perinatal death and placentomegaly, the underlying mechanisms are not well understood (3).

Genomic imprinting is an epigenetic gene regulatory mechanism that leads to the preferential expression of the paternally or maternally inherited allele of a subset of genes. Most notably, DNA methylation, which occurs at discrete locations termed differentially methylated regions (DMRs) in the germline, initiates the imprinting process (4). The de novo methyltransferase Dnmt3a and the Dnmt3-related protein Dnmt3L are required for the establishment of germine DMRs (5,6). The majority of imprinted genes reside within imprinted domains, and these DMRs are known to control imprinted gene expression within the domain (7). Abnormal expression of some imprinted genes causes developmental defects (8), some of which are similar to those observed in cloned animals.

†H.O. and S.M. contributed equally to this work.

∗To whom correspondence should be addressed at: Department of Informative Genetics, Environment and Genome Research Center, Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan. Tel: 81 227177844; Fax:+ 81 227177063; Email: tarima@med.tohoku.ac.jp

© The Author 2013. Published by Oxford University Press. All rights reserved.
For Permissions, please email: journals.permissions@oup.com
Abnormal imprinting of some imprinted genes has been reported in cloned animals (9–11). In particular, abnormal allelic expression or DMR methylation of H19, Igf2r, Peg3, Srrpm, Ascl2 and Xist has been reported in cloned mice (12–16). However, the vast majority of the abnormalities are only partial loss of imprinting or donor cell type-specific. For example, aberrant imprinting of H19 and Igf2r was frequently observed in embryonic stem cell-derived cloned mice (14), but not in somatic cell-derived ones (17). Furthermore, the relationships between developmental defects and imprinting abnormalities are poorly understood in cloned mice. The only exception is ectopic expression of Xist, which occurs in nearly all cloned mouse embryos, and accounts for embryonic loss at the postimplantation stage (16,18).

In the mouse, ~100 imprint genes have been identified, but the imprinting status of most known imprint genes has not been analyzed in cloned mice. Especially, we recently reported placenta-specific imprint genes, which show imprint expression in the placenta but are biallelically expressed in the fetus (19). It is possible that the nuclei of fetus-derived somatic cells do not acquire placenta-specific imprinting after SCNT. Therefore, we performed transcriptome-wide allelic expression analyses and found that three imprint genes, including two placenta-specific imprint genes, consistently showed loss of imprinting in all cloned mice analyzed (consistent loss of imprinting). We also verified the correlation between loss of Dlk1-Dio3 imprinting and embryonic lethality of cloned mice.

RESULTS

Transcriptome-wide analyses of imprinted gene expression in cloned mouse placentas and brains

For whole transcriptome sequencing analyses, we selected the placenta and brain because many imprint genes, including genes with tissue-specific imprint expression, are known to be expressed in these tissues (19,20). 129X1/SvJ (129) females were mated with Japanese fancy 1 (JF1) males to generate 129xJF1 male mice from which cumulus and Sertoli cells were isolated and used for SCNT. F1 embryos were also generated by in vitro fertilization (IVF) to provide a comparison. Placentas and brains were dissected from embryonic day (E) 13.5 embryos and analyzed. Forty-five known imprint genes had at least one single nucleotide polymorphism (SNP) site between 129 and JF1 and were expressed at a sufficient level to assess allelic expression (Supplementary Material, Table S1). For the analyses of allelic expression, the maternal read number divided by the paternal read number (M/P ratio) was calculated for each imprint gene. The maternal expression and paternal expression are defined as [M/P ratio] > 2 and [M/P ratio] < 0.5, respectively. There were 28 and 25 genes that showed imprinted expression in all IVF-derived placentas and brains, respectively (Fig. 1). Of these, eight genes showed abnormal allelic expression in at least two cloned placentas or brains (boxed in Fig. 1, note that Dlk1 showed aberrant expression in both tissues). Importantly, Gab1, Sfnmb2 and Slc38a4 showed biallelic expression in all cloned placentas analyzed (Fig. 1).

Imprinting status of placenta-specific imprinted genes in cloned mouse placentas

Two paternally expressed placenta-specific imprinted genes, Gab1 and Sfnmb2, were expressed biallelically in all cloned mouse placentas (Fig. 1). Gab1 is mainly expressed via a placenta-specific promoter in the normal placenta and has a novel DMR that is acquired after implantation (Fig 2 and Supplementary Material, Fig. S1). Gab1 was not detected in cumulus cells and showed biallelic expression in Sertoli cells (Fig. 2B). Gab1 showed biallelic expression in all cloned placentas analyzed (Fig. 2B) and was expressed at a level twice as high as in the IVF-derived placentas (Fig. 2C). Maternal allele-specific methylation was lost in the cloned mouse placentas (Fig. 2D).

Sfnmb2 was also biallelically expressed in the cloned mouse placentas, and the expression level was twice as high as that of the IVF-derived placentas (Fig. 2E and F). Though there is no direct DNA methylation of the Sfnmb2 promoter region, we previously reported the enrichment of H3K9me2 and H3K27me3 on the maternal allele (19). This allele-specific accumulation of H3K9me2 and H3K27me3 was not observed in the cumulus-derived cloned mouse placenta and cumulus cells (Fig. 2G).

Unlike Gab1 and Sfnmb2, other placenta-specific imprint genes (Ppp1r9a, Ascl2, Tssc4 and Slc22a3) showed normal maternal allele-specific expression in the cloned mouse placentas (Supplementary Material, Fig. S2A). Brain-specific imprint genes also showed normal allelic expression in the cloned mouse brains (Bcap in Fig. 1, Grb10 and Ube3a in Supplementary Material, Fig. S2B).

Consistent loss of Slc38a4 imprinting in cloned mice

Slc38a4 shows paternal allele-specific expression both in embryonic and extraembryonic tissues and has a germline DMR (21,22). Slc38a4 also showed paternal expression in donor cells but was biallelically expressed in the cloned mouse brains and placentas (Fig. 3A). Increased expression of Slc38a4 was observed both in the cloned mouse brains and, to a lesser extent, in placentas (Fig. 3B). Maternal allele-specific methylation of the Slc38a4 DMR was observed in cumulus cells but not in the cumulus-derived cloned mouse brain (Fig. 3C). Demethylation of the Slc38a4 DMR was already evident in the cloned blastocysts (Fig. 3D) and also observed in adult cloned mice (Supplementary Material, Fig. S3A). Interestingly, the paternally biased expression was not lost in embryos obtained from Dnmt3L-deficient and oocyte-specific Dnmt3a/3b-deficient female mice (Fig. 3E). It is already reported that the Slc38a4 DMR is not methylated at all in Dnmt3L-deficient oocytes (23). These data strongly suggested that during oocyte growth, de novo DNA methylation was dispensable for imprint expression of Slc38a4.

Correlation between loss of Dlk1-Dio3 imprinting and embryonic lethality of cloned mice

Five imprinted genes showed abnormal allelic expression in some cloned placentas or brains (Fig. 1). Among them, we focused on Dlk1 because the Dlk1-Dio3 domain (Fig. 4A) is important for normal mouse development (24). Dlk1 showed normal paternal expression in the donor cells (Fig. 4B). Biallelic expression of Dlk1 was observed in the placentas and fetuses.
derived from cumulus clones #3 and #4 and Sertoli clone #2 (Fig. 4B and Supplementary Material, Fig. S4A). In the brains derived from cumulus clone #3 and Sertoli clone #2, Dlk1 also showed biallelic expression (Fig. 4B). In the cloned mouse brains, biallelic expression of Dlk1 correlated with increased expression of Dlk1 (Fig. 4C). In the brains derived from cumulus clone #3 and Sertoli clone #2, both alleles were methylated (Fig. 4D). In the whole fetus of cumulus clone #4, gain of maternal methylation of the IG-DMR was also observed (Fig. 4D). Gain of maternal methylation of the IG-DMR was also found in the cloned placentas (Supplementary Material, Fig. S4E). Similar data were obtained for the Gtl2-DMR (Supplementary Material, Fig. S5).

Placental development without a viable fetus is frequently observed in SCNT (27). We compared the imprinting status of the Dlk1-Dio3 domain in term placentas with and without viable fetuses, which were produced by a series of experiments using Xist-siRNA (Supplementary Material, Table S2). The use

---

**Figure 1.** Transcriptome-wide analyses of imprinted gene expression in cloned mouse placentas and brains. The maternal read number divided by the paternal read number (M/P ratio) is color-coded. The maternal expression (red) and paternal expression (blue) are defined as [M/P ratio] ≥ 2 and [M/P ratio] < 0.5, respectively. The biallelic expression (green) is defined as 2 ≥ [M/P ratio] ≥ 0.5. Imprinted genes that showed abnormal allelic expression in at least two cloned placentas or brains are indicated by red boxes. Placenta-specific imprinted genes are marked with asterisks.
of *Xist*-siRNA increases cloning efficiency (18) and enables the comparison of placentas with and without viable fetuses within the same experiment. Biallelic expression of *Dlk1* was consistently observed in placentas, brains and fetuses (Fig.4B and Supplementary Material, Fig. S4A). Therefore, if *Dlk1* shows biallelic expression in the placenta, it is predicted that *Dlk1* would also show biallelic expression in the brain and fetus. A clear increase of abnormal maternal expression of *Dlk1* was observed in the placentas without viable fetuses compared with the placentas with viable fetuses (P < 10⁻⁹) (Fig. 4E), alongside hypermethylation of the IG-DMR (Supplementary Material, Fig. S6). On the other hand, allelic expression profiles of *Sgce* and *Zim1* were comparable between placentas with viable fetuses and without viable fetuses (P > 0.05, Supplementary Material, Fig. S4F).

**Confirmation of loss of imprinting in cloned mouse placentas produced without trichostatin A (TSA) treatment**

Figures 1–4 present data from cloned mouse embryos produced using TSA to increase cloning efficiency. TSA treatment might affect allelic expression of some imprinted genes. Therefore, we confirmed biallelic expression of *Gab1*, *Sfmbt2* and *Sle38a4* in four cloned mouse placentas produced without TSA treatment (Supplementary Material, Fig. S3B). Similarly, biallelic
expression of Dlk1 was observed in two of four cloned mouse placentas produced without TSA treatment (Supplementary Material, Fig. S4G). These data indicated that loss of Gab1, Sfmbt2, Slc38a4 and Dlk1 imprinting was caused by SCNT, not by TSA treatment.

**Characteristics of genes with consistent loss of imprinting in cloned mice**

As described earlier, Gab1, Sfmbt2 and Slc38a4 showed consistent loss of imprinting in cloned mice. These three genes were all maternally repressed and did not require de novo DNA methylation in growing oocytes for the establishment of imprinting (Table 1). No paternally methylated germline DMR has been found near these genes in the recent whole-genome DNA methylation analysis of germ cells (23). Importantly, these genes showed normal expression levels in the placentas of bi-maternal embryos that contained genomes from non-growing and fully grown oocytes (Table 1 and (28)). If a paternally expressed gene is not regulated by maternal imprinting, it is repressed in bi-maternal embryos (Supplementary Material, Fig. S7A). For example, Igf2 and Dlk1, which are paternally expressed and regulated by paternal imprinting, showed greatly reduced expression in bi-maternal embryos compared with wild-type embryos (Table 1). These data suggest that imprinting of Gab1, Sfmbt2 and Slc38a4 may be
Figure 4. Loss of Dlk1-Dio3 imprinting and embryonic lethality in cloned mice. (A) Genomic structure of the Dlk1-Dio3 domain. Maternally expressed genes and paternally expressed genes are in red and blue, respectively. Open circles and filled circles indicate unmethylated and methylated DMRs, respectively. M, maternal allele; P, paternal allele. (B) Allelic expression of Dlk1. Biallelic expression is marked with an asterisk. For cumulus clone #4, the brain sample was not analyzed because of severe growth retardation of this clone. (C) Real-time RT–PCR analysis of Dlk1, Gtl2, Rian and Mirg in the brain. The mean expression level of the
Table 1. Unique characteristics of Gab1, Sfmbt2 and Slc38a4

<table>
<thead>
<tr>
<th>Gene</th>
<th>Frequency of loss of imprinted expression</th>
<th>de novo DNA methylation in growing oocytes</th>
<th>Expression in bi-maternal placentas (fold decrease)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gab1</td>
<td>14/14a</td>
<td>Not required (19)</td>
<td>1.38 ± 0.12</td>
</tr>
<tr>
<td>Sfmbt2</td>
<td>14/14a</td>
<td>Not required (19)</td>
<td>1.31 ± 0.39</td>
</tr>
<tr>
<td>Slc38a4</td>
<td>14/14a</td>
<td>Required</td>
<td>1.07 ± 0.15</td>
</tr>
<tr>
<td>Igf2</td>
<td>0/8</td>
<td>Not required (9)</td>
<td>37.9 ± 14.8c</td>
</tr>
<tr>
<td>Dlk1</td>
<td>5/12</td>
<td>Required</td>
<td>24.5 ± 5.4c</td>
</tr>
<tr>
<td>Peg10</td>
<td>0/8</td>
<td>Required</td>
<td>1.03 ± 0.22</td>
</tr>
<tr>
<td>Mest</td>
<td>1/8</td>
<td>Required</td>
<td>1.38 ± 0.47</td>
</tr>
<tr>
<td>Peg3</td>
<td>0/8</td>
<td>Required</td>
<td>1.13 ± 0.15</td>
</tr>
<tr>
<td>Dlk1</td>
<td>14/14a</td>
<td>Required</td>
<td>1.38 ± 0.12</td>
</tr>
</tbody>
</table>

Paternally expressed genes known to be regulated by DNA methylation during spermatogenesis (Igf2 and Dlk1) and oogenesis (Peg10, Mest and Peg3) are included for comparison. The frequency of loss of imprinted expression is based on results obtained using E13.5 cloned mouse placentas. Microarray data of bi-maternal placentas (28) are used for the calculation of the fold decrease (mean ± SD from three replicates).

aStatistically significant compared with IVF embryos (P < 0.05, Fisher’s exact test).

bStatistically significant compared with control placentas (P < 0.05, Student’s t-test).

Established during oocyte growth in a de novo DNA methylation-independent manner.

**DISCUSSION**

Relationships between developmental abnormalities and imprinting defects in cloned mice

We found a correlation between embryonic lethality and loss of Dlk1-Dio3 imprinting. Loss of Dlk1-Dio3 imprinting in cloned mice was caused by the paternalization of the maternal allele, which is very similar to the paternal transmission of IG-DMR mice was caused by the paternalization of the maternal allele, in term placentas from a series of experiments using E13.5 cloned mouse placentas. Microarray data of bi-maternal placentas (28) are used for the calculation of the fold decrease (mean ± SD from three replicates).

Cloned mouse embryos frequently show reduced or ectopic expression of Oct4 during the preimplantation stage (31), and it is possible that abnormal expression of Oct4 induces loss of Dlk1-Dio3 imprinting in cloned mice.

Placentomegaly is observed in all cloned mouse concepti (3). Sfmbt2 is the only known protein-coding gene in proximal chromosome 2. The paternal duplication of proximal chromosome 2, which causes overexpression of Sfmbt2, is known to result in placentomegaly (32). In contrast, deficiency of Gab1 is known to result in the reduction of placental size (33). The overexpression of Sfmbt2 and Gab1 observed in cloned placentas might be involved in placentomegaly of cloned mice. It is also proposed that imprinted expression of Slc38a4 in the placenta may be important for the normal supply of neutral amino acids to the fetus (34). Thus Slc38a4-knockout or -transgenic mice, which have not been reported, merit further investigation to understand the phenotypic abnormalities of cloned animals.

While we focused on imprinted genes in this study, many non-imprinted genes were also reported to show aberrant expression in cloned mice, especially in the placenta (35). Ablation expression of both imprinted genes and non-imprinted genes may impact the size and viability of cloned mouse embryos.

Consistent loss of imprinting caused by incomplete imprinting memory in somatic cells

Two placenta-specific imprinted genes, Gab1 and Sfmbt2, showed complete loss of imprinting in all cloned placentas analyzed. In the donor cells, neither gene showed imprinted expression. In the promoter region, allele-specific DNA methylation of Gab1 and allelic enrichment of histone modifications of Sfmbt2 were not observed in donor cells or cloned placentas. These data suggested that the imprinting of Gab1 and Sfmbt2 was not retained in somatic cells and not reinstated in the placental lineages derived from cloned somatic cells.

In addition to Gab1 and Sfmbt2, there are several additional genes that show tissue-specific imprinted expression. We found that four placenta-specific and three brain-specific imprinted genes showed normal monoallelic expression in the cloned mice. These seven genes are all present within imprinted regions. These seven tissue-specific imprinted genes was reestablished after SCNT, using imprinting marks retained in the clusters.

Slc38a4 shows imprinting expression both in the normal fetus and placenta (22), and imprinting methylation at the Slc38a4 DMR was maintained in the donor cells. However, the Slc38a4 DMR of somatic cells was not protected from demethylation during preimplantation development. Interestingly, paternal expression of Slc38a4 was not lost in embryos obtained from Dnmt3L-deficient and oocyte-specific Dnmt3a/3b-deficient female mice. Therefore, we hypothesize that, during oogenesis,
Slc38a4 acquires an imprinting mark[s] other than DNA methylation, which is required for the protection of the Slc38a4 DMR from demethylation during preimplantation development but which is lost in somatic cells. Gab1, Sfmbt2 and Slc38a4 may not require de novo DNA methylation for the establishment of imprinting (Table 1). De novo DNA methylation has already been shown to be dispensable for imprinting of the maternal X-chromosome (36), and Xist shows ectopic expression in nearly all cloned mouse embryos (16). It is interesting to speculate that the de novo DNA methylation-independent establishment of imprinting may be a common feature of imprinted genes showing consistent imprinting defects in cloned mice.

In conclusion, we found that Gab1, Sfmbt2 and Slc38a4 showed consistent loss of imprinting in cloned mice. It is likely that an imprinting mark[s] other than DNA methylation may be required for the establishment of imprinting of these genes. We also verified the correlation between loss of Dlk1-Dio3 imprinting and embryonic lethality of cloned mice. These findings will have value both for elucidating the mechanisms involved in reprogramming and also determining the potential risks of clinical applications of nuclear reprogramming.

MATERIALS AND METHODS

Ethics statement

All animal experiments were performed at RIKEN Tsukuba Institute in accordance with the Animal Experimentation Committee’s guiding principles.

SCNT

To generate [129xJF1]F1 mice, 129 females were mated with JF1 males. B6 females were mated with DBA males to generate [B6xDBA]F1 mice. Nuclear transfer was carried out with a Piezo-driven micromanipulator (PMM-150FU, Prime Tech, Ibaraki, Japan) as described in Wakayama et al. (37) and Ogura et al. (38). For the preparation of nuclear donor cells, cumulus cells were collected from female mice at 8–12 weeks of age, and Sertoli cells were collected from male mice at 1–9 days of age as described in Ogura et al. (38). Donor nuclei were injected into enucleated recipient oocytes collected from [B6xDBA]F1 female mice, and the reconstructed embryos were treated with 50 nM TSA (Sigma–Aldrich, St. Louis, MO, USA) for 8 h in total. To obtain the data shown in Supplementary Material, Figures S3B and S4G, the reconstructed embryos were not treated with TSA. For the analyses of term placentas, microinjection of Xist-siRNA was performed as described in Matoba et al. (18). After nuclear transfer, some embryos in the two-cell stage were transferred to pseudopregnant ICR mice, and others were collected at the blastocyst stage. [129xJF1]F1 embryos generated by conventional IVF (39) were used as controls for allelic expression and epigenetic analyses. Cloned embryos were delivered by cesarean section, and only placentas were collected and used for the analyses, shown in Figure 4E and Supplementary Material, Figure S4F. In the case that neonates at birth were alive, placentas were classified as ‘placentas with viable fetuses’. In the case that fetuses were dead or absorbed, placentas were classified as ‘placentas without viable fetuses’.

Whole transcriptome sequencing and data analysis

Total RNA was extracted from brains and placentas of E13.5 embryos. Four micrograms of total RNA was used for library construction using TruSeq RNA Sample Prep Kit v2 (Illumina, CA, USA) according to the manufacturer’s protocol. Briefly, poly-A-containing mRNAs were purified using poly-T oligo-attached magnetic beads. The purified mRNAs were fragmented using divalent cations under elevated temperatures and then converted to dsDNA by two rounds of cDNA synthesis using reverse transcriptase and DNA polymerase I. After an end repair process, DNA fragments were ligated with adaptor oligos. The ligated products were amplified using 15 cycles of PCR to generate an RNA-seq library. Library integrity was verified by Bioanalyzer DNA1000 assay (Agilent Technologies, CA, USA). Sequencing was performed in 39-bp paired-end mode using a Genome Analyzer Ix (Illumina).

A total of 2 012 365 798 reads were obtained for 25 samples. Sequenced reads were all filtered by the sequence quality score. If the average quality score of at least one of a paired read was <20, the paired read was discarded. Filtered reads were mapped to the reference mouse genome (UCSC mm9) by using Novoalign (V.2.07.13) (http://novocraft.com/) with the parameter ‘-r Random’. Mapping results were further processed using the Picard MarkDuplicate program (version 1.67) (http://picard.sourceforge.net/) to remove duplicate reads. Samples obtained from 129 and JF1 inbred mice were used for the identification of SNPs. SNP candidates were searched for with the The Genome Analysis Toolkit (GATK) (version 1.6–5) (40) according to its recommended procedure, and a total of 1 756 512 SNPs were identified. SNPs meeting the following criteria were used for further analysis: ≥90% reads of 129-derived samples and <10% reads of JF1-derived samples identical to the reference sequence, or the opposite case.

Known imprinted genes were selected based on imprinted gene databases (http://igc.otago.ac.nz/ and http://www.har.mrc.ac.uk/). Imprinted genes annotated by RefSeq, except miRNAs and snoRNAs, were analyzed. Placenta-specific imprinted genes Tfp12, Tspy332, Cd81 and Ano1 were excluded because these genes were highly expressed by maternal cells in the placenta. Only genes that had at least one SNP site with ≥10 reads in all brain or placental samples were used for the allelic expression analysis. For the analyses of allelic expression of imprinted genes, we used all mapped reads for all SNPs identified (including SNPs with <10 reads), and the numbers of paternal and maternal reads were summed for each gene. The maternal read number divided by the paternal read number (M/P ratio) was calculated. The maternal expression and paternal expression are defined as [M/P ratio] >2 and [M/P ratio] <0.5, respectively.

Preparation of embryos from Dnmt3L-deficient and oocyte-specific Dnmt3a/3b-deficient females

Production of mice with the conditional alleles, referred to as Dnmt3a2lox/2lox and Dnmt3b2lox/2lox, was described previously (5,41). To disrupt the conditional alleles in growing oocytes, the mice were crossed with those carrying a Zp3-Cre gene (42). The precise timing of conditional deletion of Dnmt3a and Dnmt3b by Zp3-Cre is described elsewhere (43). By crossing Dnmt3a2lox/2lox, Dnmt3b2lox/2lox, Zp3-Cre females with wild-type
JF1 male mice, we obtained \([Dmnt3a^{-/-}, Dmnt3b^{-/-}]\) E9.5 embryos. \(Dmnt3L\) knockout female mice (44) were also crossed with wild-type JF1 male mice to obtain \(Dmnt3L^{-/-}\) E9.5 embryos.

**Real-time RT–PCR**

Total RNA was prepared using an RNeasy mini Kit and RNase-free DNase (Qiagen, CA, USA). First-strand cDNA was synthesized from total RNA using PrimeScript II (Takara Bio, Shiga, Japan). Real-time PCR reaction was done with SYBR Premix Ex Taq II (Takara Bio). The amount of target mRNA was determined from the appropriate standard curve and normalized to the amount of \(\beta\)-actin mRNA. The primer sets are shown in Supplementary Material, Table S3.

**Analysis of allelic expression**

PCR amplification was performed using KOD FX (TOYOBO, Osaka, Japan). PCR products were Sanger-sequenced, and the sequence chromatograms were analyzed with Sequencing Analysis Software v5.4 (Applied Biosystems, CA, USA). Multiple sequence alignments were done using GENETYX version 10.0.3 (GENETYX, Tokyo, Japan). For the quantification of expression alleles in Figure 4E, the peak heights of sequence chromatograms were used as expression levels. The allelic expression of \(Rtl1\) was analyzed using 3′ rapid amplification of cDNA ends (RACE) as previously reported (45). The primer sets and SNP positions are shown in Supplementary Material, Table S3.

**Bisulfite sequencing**

DNA samples were treated with sodium bisulfite using an EZ DNA Methylation Kit (Zymo Research, Orange, CA) and PCR-amplified using TaKaRa EpiTaq™ HS (Takara Bio). The PCR products were cloned into the pGEM-T Easy vector (Promega, Wisconsin, USA), and individual clones were sequenced. Primers used are listed in Supplementary Material, Table S3.

**ChIP and SNuPE**

ChIP analysis was performed using a Magna ChIP G Chromatin Immunoprecipitation Kit (Millipore, Temecula, CA) according to the manufacturer’s protocol. The following antibodies were used: dimethylated H3-Lys9 and trimethylated H3-Lys27 (Millipore). The precipitated DNA was PCR-amplified, and the allelic histone modifications were analyzed using SNuPE. SNuPE analysis was performed using a SNaPshot Multiplex Kit (Applied Biosystems) according to the manufacturer’s protocol. The peak height was determined using GeneMapper v4.1 (Applied Biosystems). Primers used are listed in Supplementary Material, Table S3.

**Imprinted gene expression in bi-maternal placentas**

Microarray data for three types of bi-maternal placentas (28) were used for the expression analysis presented in Table 1. For \(Dlk1\), we used the data for embryos in which the \(Igf2-H19\) domain in the distal region on chromosome 7 was switched from the maternal to the paternal epigenotype. For \(Igf2\), we used the data for embryos in which the \(Dlk1-Dio3\) domain in the distal region on chromosome 12 was switched from the maternal to the paternal epigenotype. For other imprinted genes, we used the data for embryos in which the both domains in the distal regions on chromosomes 7 and 12 were switched from the maternal to the paternal epigenotype.

**Accession numbers**

All sequencing data are deposited in DDBJ Sequence Read Archive (DRA) under the accession number DRA000627.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

**ACKNOWLEDGEMENTS**

We thank Ms N. Miyouchi, Ms F. Sato, Ms M. Tsuda, Ms N. Koshita, Ms M. Kikuchi and Mr K. Kuroda for technical assistance and Dr Rosalind M. John for support and valuable suggestions. The microarray data of bi-maternal placentas were kindly provided by Dr T. Kono and Mr F. Cao. We also thank the Biomedical Research Core of Tohoku University Graduate School of Medicine for technical support.

**Conflict of Interest statement.** None declared.

**FUNDING**

This work was supported by Grants-in-Aid for Scientific Research (KAKENHI) from Japan Society for the Promotion of Science (22800084, 23220011, 23390385, 24613001 and 25670691), KAKENHI from the Ministry of Education, Culture, Sports, Science and Technology (20062010, 23013003 and 25112009) and the Takeda Science Foundation.

**REFERENCES**


