Received: 7 August 2018 | Revised: 30 January 2019 | Accepted: 11 February 2019

DOI: 10.1002/mrd.23137

Molecular Reproduction & Development

RESEARCH ARTICLE

Ectopic expression of DNA methyltransferases DNMT3A2 and DNMT3L leads to aberrant hypermethylation and postnatal lethality in mice

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Funding information
Japan Society for the Promotion of Science, Grant/Award Numbers: 18H05547, 18J01481, 18H02355

Abstract

DNA methylation is generally known to inactivate gene expression. The DNA methyltransferases (DNMTs), DNMT3A and DNMT3B, catalyze somatic cell lineage-specific DNA methylation, while DNMT3A and DNMT3L catalyze germ cell lineage-specific DNA methylation. How such lineage- and gene-specific DNA methylation patterns are created remains to be elucidated. To better understand the regulatory mechanisms underlying DNA methylation, we generated transgenic mice that constitutively expressed DNMT3A and DNMT3L, and analyzed DNA methylation, gene expression, and their subsequent impact on ontogeny. All transgenic mice were born normally but died within 20 weeks accompanied with cardiac hypertrophy. Several genes were repressed in the hearts of transgenic mice compared with those in wild-type mice. CpG islands of these downregulated genes were highly methylated in the transgenic mice. This abnormal methylation occurred in the perinatal stage. Conversely, monoallelic DNA methylation at imprinted loci was faithfully maintained in all transgenic mice, except *H19*. Thus, the loci preferred by DNMT3A and DNMT3L differ between somatic and germ cell lineages.

KEYWORDS
DNA methylation, DNA methyltransferases, Down's syndrome, genomic imprinting, ontogeny

1 | INTRODUCTION

DNA methylation is an epigenetic modification indispensable for regulating gene expression, genomic imprinting, and genomic stability. It is well known that DNA methylation patterns are established by the DNA methyltransferases (DNMT), DNMT3A and DNMT3B, and their respective cofactor, DNMT3L, and are maintained by DNMT1 (Bourc'his, Xu, Lin, Bollman, & Bestor, 2001; Hata, Okano, Lei, & Li, 2002; Okano, Bell, Haber, & Li, 1999; Yoder, Soman, Verdine, & Bestor, 1997). *Dnmt1*-deficient mice die shortly after implantation owing to genome-wide DNA hypomethylation (Lei et al., 1996). Mice lacking *Dnmt3a* exhibit lethal phenotype at 4 weeks of age, while *Dnmt3b*-null embryos lose most of the DNA methylation in microsatellite DNA at chromosomal centromeres and die around the late gestational stage (Okano et al., 1999). Meanwhile, embryos with double-deficiency of *Dnmt3a* and *Dnmt3b* die immediately after implantation, indicating that in addition to their exclusive functions, DNMT3A and DNMT3B have some overlapping functions during embryonic development. It has also been reported that *Dnmt3a* and *Dnmt3b* double-knockout embryonic stem cells cannot form teratomas in nude mice (Chen, Ueda, Dodge, Wang, & Li, 2003; Pawlak & Jaenisch, 2011). Thus, DNMT3A and DNMT3B are responsible for triploblastic differentiation and somatic cell lineage-specific DNA methylation. On the contrary, conditional knockout approach showed that both *Dnmt3a* and *Dnmt3l* but not *Dnmt3b* are responsible for DNA methylation, including imprinted regions during gametogenesis (Kaneda et al., 2004, 2010). Fully grown oocytes lacking *Dnmt3a* lose methylation imprints in all 19 maternally imprinted differentially methylated regions (DMRs) and show hypomethylation of the whole genome (Shirane et al., 2013).

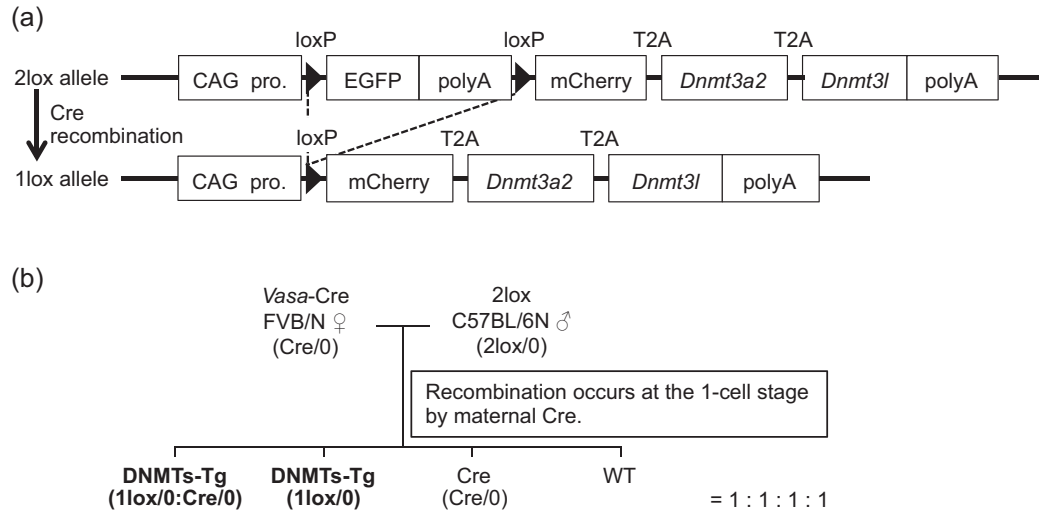

FIGURE 1 Schematic representation for generation of transgenic mice. (a) Floxed 2lox allele expresses only EGFP driven by chicken β-actin promoter (CAG pro.). Cre recombinase deletes EGFP-polyA cassette and induces the expressions of mCherry, *Dnmt3a2*, and *Dnmt3l* (1lox allele). All coding sequences are interlinked by T2A peptides. (b) Gamete-specific *Vasa*-Cre heterozygous transgenic female mouse (Cre/0) is crossed with a heterozygous flox male (2lox/0). Maternally inherited Cre activates recombination in zygotes to produce four types of progeny in equal ratio: DNMTs-Tg (1lox/0:Cre/0), DNMTs-Tg (1lox/0), Cre (Cre/0), and wild type (WT). DNMT: DNA methyltransferase; EGFP: enhanced green fluorescent protein; Tg: transgenic

Conditional deletion of *Dnmt3a* in prospermatogonia abolishes DNA methylation in three paternal imprinted regions and leads to azoospermia (Kaneda et al., 2004). However, germ cell-specific *Dnmt3b* knockout mice produce functional gametes and maintain their fertility (Kaneda et al., 2004, 2010).

DNMT3L itself does not have enzymatic activity but acts as a coenzyme for other DNMT3 enzymes, thereby promoting de novo methylation activity (Suetake, Shinozaki, Miyagawa, Takeshima, & Tajima, 2004). Although normal embryonic development occurs in *Dnmt3l*-deficient mice, males and females are both infertile owing to abnormal DNA methylation in the gametes, similar to the phenotype of the *Dnmt3a* conditional knockout mice (Bourc'his et al., 2001; Hata et al., 2002). It has also been found that N-terminal-truncated DNMT3A (DNMT3A2) is dominantly expressed in germ cells, indicating that DNMT3A2 and DNMT3L are responsible for germ cell-specific DNA methylation in almost the entire genome including the imprinted regions. Moreover, their coexpression is induced only in germ cell lineage in vivo (Hara, Takano, Fujikawa, et al., 2014; Lees-Murdock, Shovlin, Gardiner, De Felici, & Walsh, 2005; Sakai, Suetake, Shinozaki, Yamashina, & Tajima, 2004).

Because of the necessity and the unique function of each DNMT for normal embryo development, disruption of any DNMT activity affects developmental stage- and tissue-specific DNA methylation patterns. However, it is unclear whether the innate DNA methylation profile and normality of embryo development is still maintained when cell lineage-specific expression of DNA methyltransferases is altered. In this study, to address the question, we generated transgenic (Tg) mice coexpressing both DNMT3A2 and DNMT3L ectopically after fertilization and investigated the phenotypes and DNA methylation profiles of these mice. Our approach will help in understanding the mechanisms underlying the maintenance and/or disruption of normal DNA methylation patterns essential for mammalian ontogeny.

2 | RESULTS

2.1 | Generation of Tg mice ectopically expressing DNMT3A2 and DNMT3L (DNMTs-Tg mice)

We previously generated Tg mice harboring *Dnmt3a2* and *Dnmt3l* downstream of the floxed-GFP cassette (2lox; Hara, Takano, Fujikawa, et al., 2014). DNMTs-Tg (1lox) mice were generated as shown in Figure 1. As expected, *Vasa*-driven Cre recombination induced the constitutive expression of both DNMT3A2 and DNMT3L from the preimplantation stage throughout life (Figure 2a,b). Although DNMTs-Tg mice developed normally to term in the expected Mendelian ratios and exhibited normal weights (DNMTs-Tg; 4.77 ± 0.24 g [$N = 7$], wild type [WT]; 4.32 ± 0.25 g [$N = 13$], $p = 0.26$, at 1 week), all mice showed delayed postnatal growth and finally died within 20 weeks after birth in two DNMTs-Tg lines, namely ak and p (Figure 2c–e and Figure S1). We previously reported that lines ak and p harbored 8–10 and 3–5 copies of Tg, respectively. Methylation catalytic activity increased depending on the copy numbers of Tg (Hara, Takano, Fujikawa, et al., 2014). Since line ak exhibited more severe phenotypes than line p, line ak was used for phenotypic analysis and following genetic analyses. Some DNMTs-Tg mice also developed cardiac hypertrophy (Figure 2f,g and Table S1). These results indicate that ectopic expression of DNMT3A2 and DNMT3L induces postnatal lethality, which presumably resulted from abnormal DNA methylation.

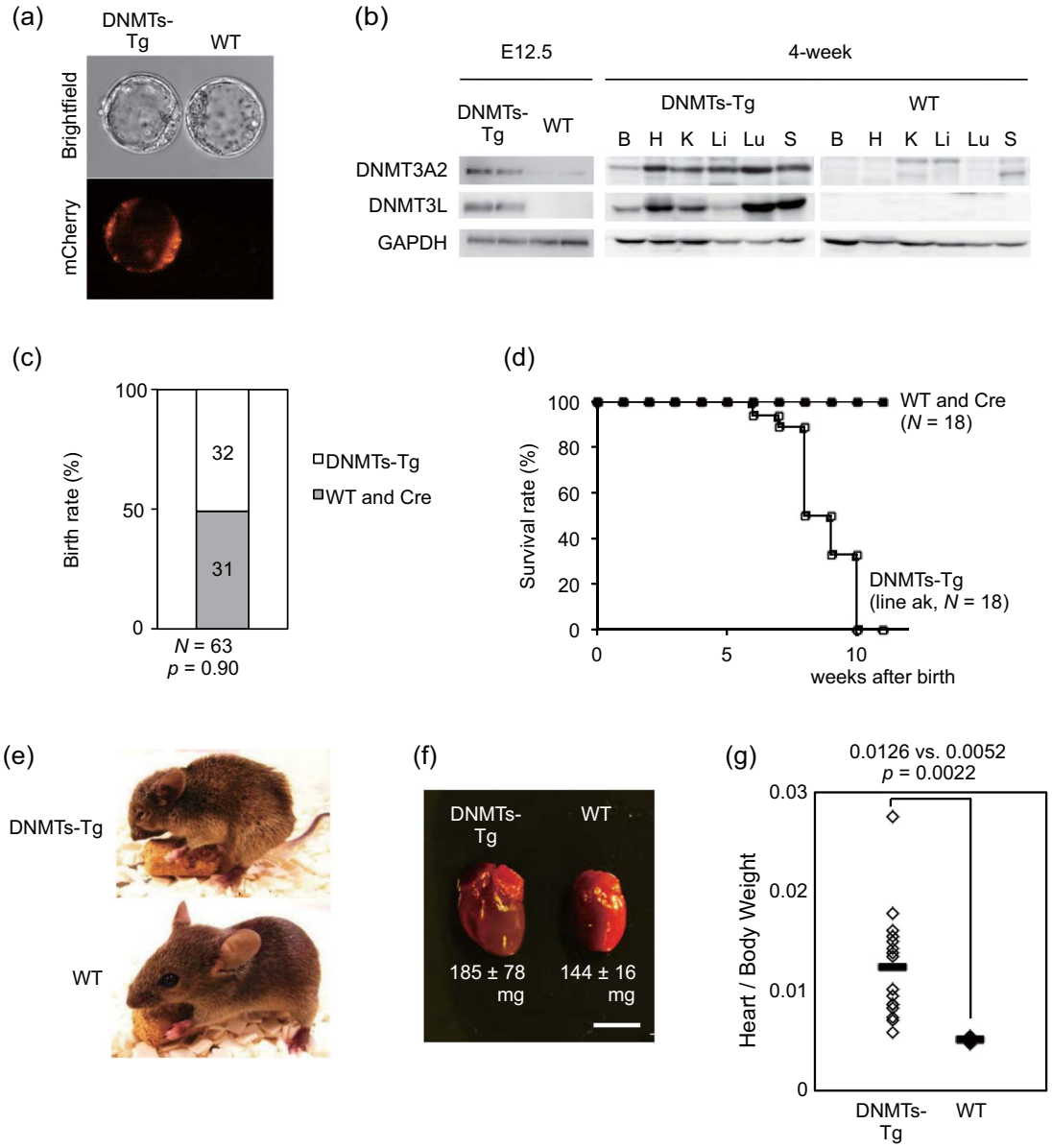

FIGURE 2 Phenotypes of Tg mice expressing DNMT3A2 and DNMT3L ectopically. (a) mCherry fluorescence in DNMTs-Tg embryos at the blastocyst stage. (b) Western blot analysis for DNMT3A2 and DNMT3L in embryos at E12.5 and somatic tissues at 4-week of age. B, Brain; H, heart; K, kidney; Li, liver; Lu, lung; and S, spleen. Birth rate (c) and survival rate (d) of 1lox-positive DNMTs-Tg ($N = 18$) and -negative WT and Cre mice ($N = 18$). DNMTs-Tg mice were small in size and showed poor growth (e) and cardiac hypertrophy (f). (g) Ratio of the weight of the heart per body in DNMTs-Tg mice. Open and closed figures indicate DNMTs-Tg and WT mice, respectively. All the represented data are derived from a DNMTs-Tg line (ak). DNMT: DNA methyltransferase; Tg: transgenic; WT: wild type

2.2 | Screening of candidate DNMT3A2 and DNMT3L-target genes

To identify genes that were abnormally hypermethylated by exogenous DNMT3A2 and DNMT3L, we first conducted comprehensive gene expression analyses in heart tissues of mice. *Dnmt3l* and *Dnmt3a* were upregulated in the DNMTs-Tg mice (Table S2). Hierarchical clustering analysis of the microarray data showed the differential gene expression profiles between DNMTs-Tg and WT mice (Figure 3a). In total, 1,313 probe entities (1,070 genes) were identified as differentially expressed genes at statistically significant levels ($p < 0.05$) when comparing the expression in DNMTs-Tg mice with that in WT mice (Figure 3b and Table S2 and S3). Then, we focused on significantly downregulated genes in the DNMTs-Tg mice. These genes were further filtered by using following criteria: Genes showing over a five-fold decrease in all the DNMTs-Tg mice, and the presence of CpG-rich sequences in their transcription start sites (TSS). Among the 549 genes (666 probes), *Apeh*, *Bdh1*, *Bex1*, *Dcaf12l1*, and *Rps4l* were selected as candidate genes for methylation analysis. Expression levels of the candidate genes were validated by quantitative reverse-transcription polymerase chain reaction (RT-PCR) (Figure S2). Additionally, our microarray analysis showed that the expression of genes encoding collagen was increased only in DNMTs-Tg #1, which displayed cardiac hypertrophy (Figure S3).

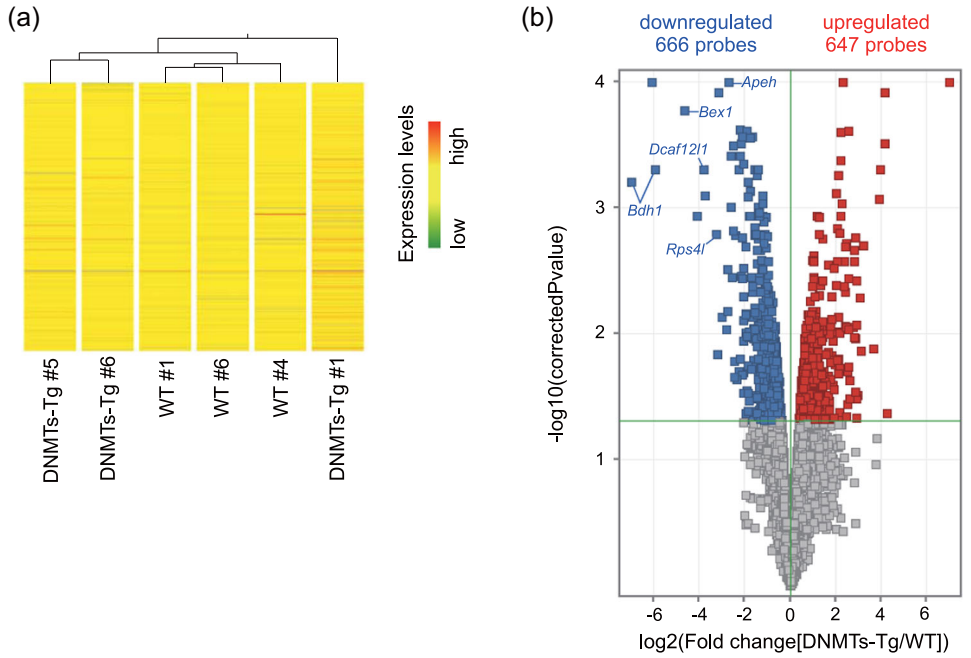

FIGURE 3 Gene expression profiles in heart tissues of adult DNMTs-Tg mice by microarray. (a) Hierarchical clustering indicates differences between the gene expression profiles of DNMTs-Tg and WT mice. (b) Volcano plot of 1,070 differentially expressed genes (1,313 probe entities) between DNMTs-Tg and WT mice (Benjamini–Hochberg FDR, $p < 0.05$). Red and blue plots indicate upregulated and downregulated probe entities with statistic significance, respectively. DNMT: DNA methyltransferase; FDR: false discovery rate; Tg: transgenic; WT: wild type

Previous reports showed that cardiac hypertrophy positively correlates with collagen synthesis (Querejeta et al., 2004; Yang, Kandaswamy, Young, & Sen, 1997) and *Col1a1* expression is correlated with excess promoter methylation in rats (Pan, Chen, Huang, Yao, & Ma, 2013). Therefore, we focused on *Col1a1* encoding type I collagen as another candidate for DNA methylation analysis. Since differential expression in housekeeping genes, including *Pgk1*, was not observed in DNMTs-Tg mice, *Pgk1* was also analyzed in subsequent experiments as a control gene (fold change for DNMTs-Tg/WT = 1.09–1.22).

2.3 | DNA methylation in the promoters of candidate DNMT3A2 and DNMT3L-target genes

To assess whether changes in the expression levels of the candidate genes correlated with DNA hypermethylation, the DNA methylation statuses of their promoters were determined. Because the promoter CpG island of *Col1a1* is not annotated in the UCSC Genome Browser (http://genome.ucsc.edu), we investigated the methylation rate of CG-rich sequences around its TSS (Chr11: 94,936,216–94,936,384). Bisulfite sequencing analysis showed significant hypermethylation of *Apeh*, *Col1a1*, *Bdh1*, and *Rps4l* in the hearts of DNMTs-Tg mice, revealing correlations between gene expression and promoter hypermethylation (Figures 4 and S4). As *Bex1*, *Dcaf12l1*, and *Pgk1* were located on X chromosome, their DNA methylations are affected by the process of female X chromosome inactivation. However, when comparing the methylation levels among the same sex mice, *Bex1* and *Dcaf12l1* were significantly hypermethylated in hearts of DNMTs-Tg mice than that of WT mice. The methylation statuses of *Pgk1* did not differ between DNMTs-Tg and WT samples among the same sex mice, suggesting that *Pgk1* was not a target of ectopic DNMT3A2 and DNMT3L in the hearts of adult mice.

Next, to observe whether exogenous DNMT3A2 and DNMT3L induced aberrant DNA methylation specifically in the hearts, DNA methylation statuses of *Apeh*, *Bdh1*, *Bex1*, *Dcaf12l1*, and *Rps4l* genes were also analyzed in the brains. DNA methylation levels of *Apeh*, *Bdh1*, and *Rps4l* were significantly increased in the brains of DNMTs-Tg mice compared with those in WT mice (Figure S5a). However, there were no differences in the DNA methylation levels of *Bex1* and *Dcaf12l1* between DNMTs-Tg and WT mice. This indicates that alteration in DNA methylation by ectopic expression of DNMT3A2 and DNMT3L is common to the hearts and brains in some but not all genes. On the contrary, much higher expression of *Bex1* and *Dcaf12l1* genes was observed in the brains than hearts regardless of the mouse genotypes (Figure S5b). Thus, DNMTs did not bring de novo DNA methylation in the highly active TSS including housekeeping genes.

2.4 | Dynamics of promoter methylation during embryogenesis and postnatal growth

To determine when the *Apeh* and *Col1a1* promoters acquired abnormal de novo methylation in DNMTs-Tg mice, we performed DNA methylation analysis using whole embryos at embryonic day 9.5 (E9.5) and E12.5 and hearts of neonates and adults. All DNMTs-Tg embryos displayed normal methylation statuses at *Apeh* and *Col1a1* loci similar to those of WT samples throughout embryo development (Figure 5). In contrast, the *Apeh* promoter was partially methylated in

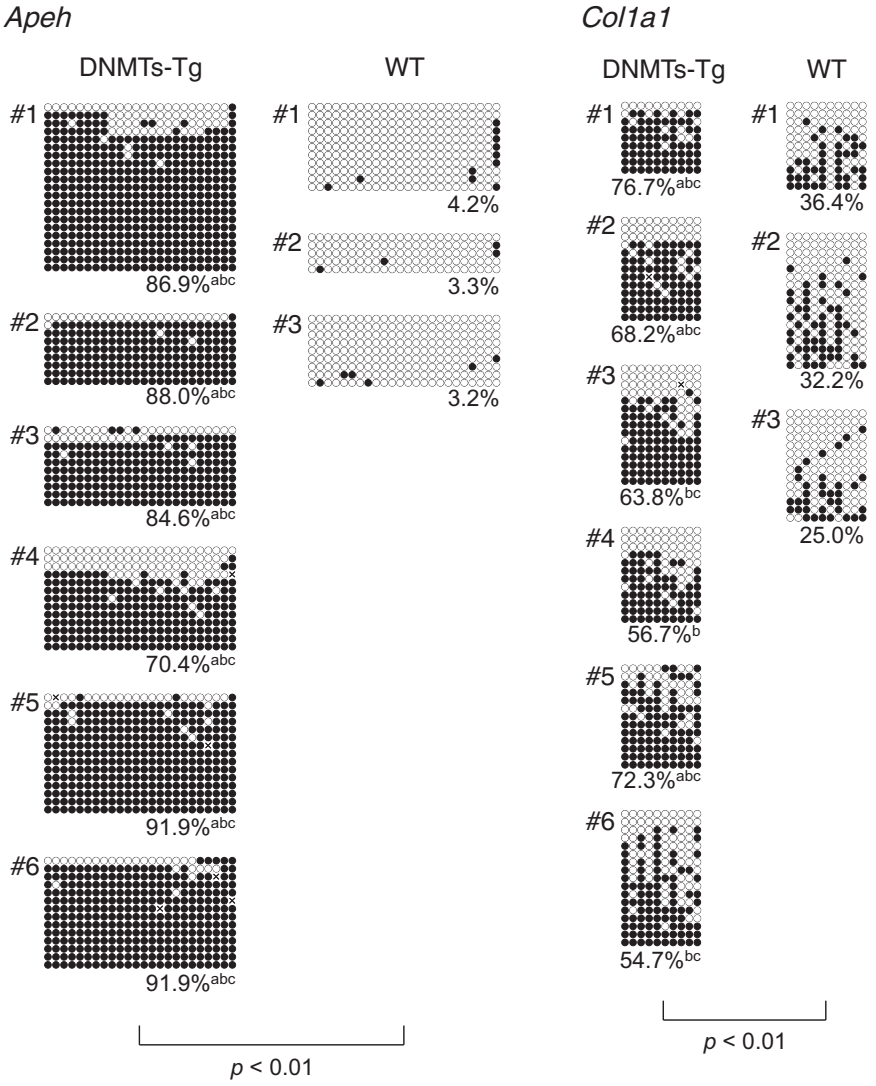

FIGURE 4 Abnormal hypermethylation of the *Apeh* and *Col1a1* promoters in hearts of DNMTs-Tg mice. DNA methylation rates of *Apeh* and *Col1a1* promoters were analyzed by bisulfite sequencing. Each line of circles indicates one DNA strand. *p* values were calculated by the Mann–Whitney *U* test between DNMTs-Tg and WT mice. Superscripted characters in DNMTs-Tg samples indicate significant differences against WT #1 (a), #2 (b), and #3 (c). DNMT: DNA methyltransferase; Tg: transgenic; WT: wild type

the hearts of neonatal mice in one of three mice. *Col1a1* was significantly hypermethylated in all of the neonatal samples ($N = 3$). Thus, abnormal DNA methylations were induced in the perinatal stage, and in turn, gene expression patterns were altered.

2.5 | Effects of ectopic DNMT3A2 and DNMT3L on DNA methylation at imprinted loci

We next investigated whether methylation imprints of DMRs were influenced by ectopic DNMT3A2 and DNMT3L. DNA methylation analysis showed hypomethylated and hypermethylated strands in equal proportions (methylation rates of nearly 50%) in *Igf2r*, *Kcnq1ot1*, *Mest*, *Slc38a4*, *Snrpn*, and *Zac1* DMRs in the hearts of DNMTs-Tg and WT mice (Figures 6a,b and S6a). However, the methylation of *H19* DMR was slightly but significantly increased in adult DNMTs-Tg mice compared with that in WT mice (DNMTs-Tg = 67.3% vs. WT = 55.4%, $p = 0.027$). In contrast, we found that most imprinted regions, including *H19*, maintained allele-specific methylation imprints both in DNMTs-Tg and WT embryos (Figures 6c and S6b). These results suggest that abnormal DNA methylation does not occur during embryo development.

3 | DISCUSSION

It is not fully understood how global DNA methylation patterns are created by DNA methyltransferases during ontogeny. In this study, to gain a better understanding of the mechanisms underlying the establishment and maintenance of normal DNA methylation patterns, including imprinted regions, during the life cycle of mammals, we analyzed how ectopic expression of DNMT3A2 and DNMT3L affects DNA methylation in somatic cell lineage in vivo.

Coexpression of DNMT3A2 and DNMT3L is observed only in germ cell lineage in vivo and is essential for germ cell-specific DNA methylation including genomic imprinting. A previous report showed that an episome containing a *Snrpn* DMR or *Igf2r* DMR, or nonimprinted sequences was methylated in HEK293 cells by coexpression of DNMT3A and DNMT3L but not by DNMT3A alone (Chedin, Lieber, & Hsieh, 2002). However, because an episome lacks a chromatin structure, it remained unclear whether DNMT3A and DNMT3L catalyzed de novo DNA methylation irrespective of target sites. On the contrary, our previous study demonstrated that overexpression of DNMT3A2 and DNMT3L did not induce de novo methylation in

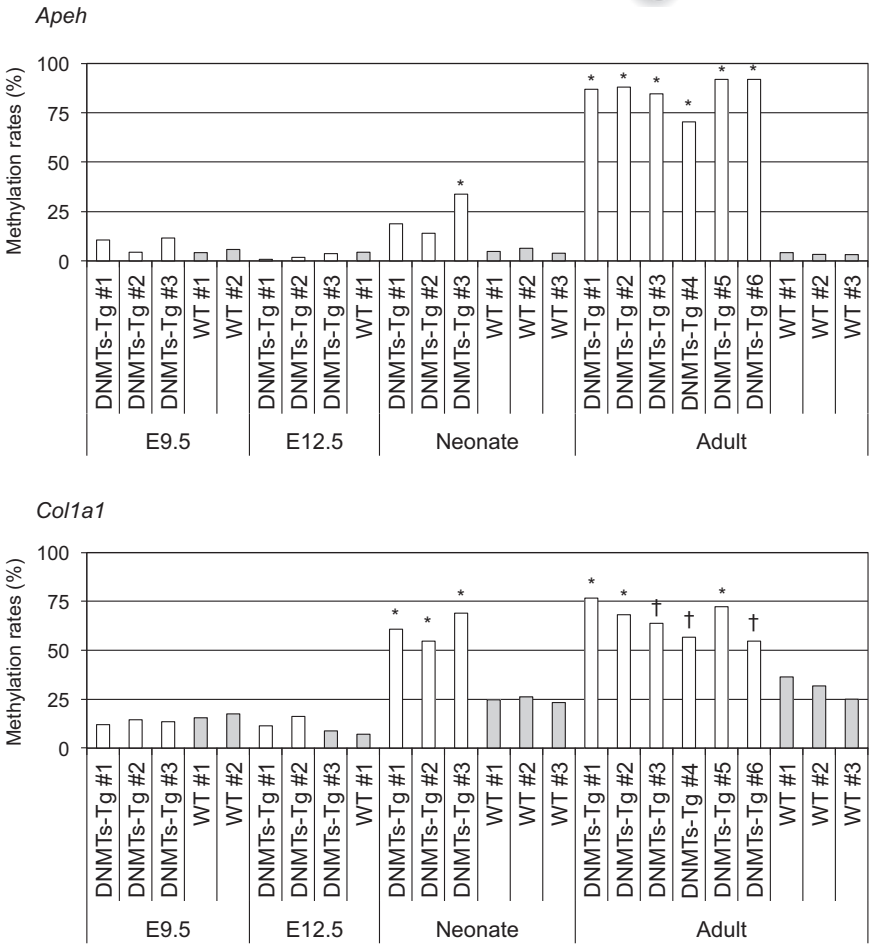

FIGURE 5 DNA methylation statuses of the *Apeh* and *Col1a1* promoters during the lifetime of DNMTs-Tg mice. DNA methylation levels of *Apeh* and *Col1a1* promoters were analyzed in embryos at E9.5 and E12.5, and in the hearts of neonatal pups and 8-week-old adult mice. Graphs represent the methylation rates of each sample. White and gray bars indicate DNMTs-Tg and WT samples, respectively. * and † show significant differences against all WT samples and at least one WT sample at the same developmental stage, respectively ($p < 0.05$). DNMT: DNA methyltransferase; Tg: transgenic; WT: wild type

nongrowing oocytes but promoted the earlier acquisition of DNA methylation imprints in growing oocytes compared with naïve growing oocytes (Hara, Takano, Fujikawa, et al., 2014). This indicated that de novo methylation occurs in oocytes after chromatin dynamics from nonpermissive into permissive states for DNMT3A2 and DNMT3L. In the current study, we showed that de novo methylation was induced in somatic cell lineage (e.g., heart and brain) in vivo by ectopic expression of DNMT3A2 and DNMT3L. The methylation levels of at least six nonimprinted regions were generally increased in the hearts derived from DNMTs-Tg mice compared with those from WT mice. Among them, three regions were also significantly methylated in the brains derived from DNMTs-Tg mice. The hypermethylated regions, identified by the ectopic expression of DNMT3A2 and DNMT3L in hearts, differed from the germline-specific DNA methylated regions that were identified through our previous study (Kobayashi et al., 2012). Thus, DNMT3A2 and DNMT3L-targeting and/or -permissive regions differ between germ cell and each somatic cell lineage, for example, heart and brain. In the brains, much higher expression of *Bex1* and *Dcaf12l1* was observed than in the hearts of DNMTs-Tg mice. Significant de novo methylation did not occur in the TSSs of *Bex1* and *Dcaf12l1*. Thus, transcriptionally active chromatin states, such as those characterized by tri-methylation of histone H3 at lysine 4 (H3K4) and acetylated histone, would not permit to binding of DNMT3A2 and DNMT3L. It is known that DNMT3L fails to bind to a chromatin that shows methylation of H3K4 in cell-free conditions (Hu et al., 2009). Oocyte-specific deletion of KDM1B that encodes H3K4 demethylase also leads to a lack of DNA methylation imprints at four of seven imprinted loci (Ciccone et al., 2009). However, the specific features of ectopically methylated regions remain unclear in this study.

Here, we identified 549 downregulated genes (666 probes) and 521 upregulated genes (647 probes) in the hearts of DNMTs-Tg mice. Some of them are known to be indispensable for postnatal life, and some of them are involved in normal cardiac function after birth (Tables S2 and S3). Aberrant gene expression of these genes would detrimentally affect the viability of DNMTs-Tg mice, although the cause of death of DNMTs-Tg mice remains unknown in the present study. On the contrary, constitutive expression of DNMT3A2 and DNMT3L did not have a critical effect on embryonic development. This result is consistent with the fact that native DNA methylation patterns are altered particularly after birth in DNMTs-Tg mice. TET1 −3, members of ten-eleven translocation (Tet) methylcytosine dioxygenases, function to change 5-methylcytosine into 5-hydroxymethylcytosine, 5-formylcytosine, 5-carboxylcytosine, and finally unmodified cytosine (Ito et al., 2010, 2011; Iyer, Tahiliani, Rao, & Aravind, 2009; Rougier et al., 1998). Expression levels of *Tet1* and *Tet2* are remarkably decreased until the early gestational stage

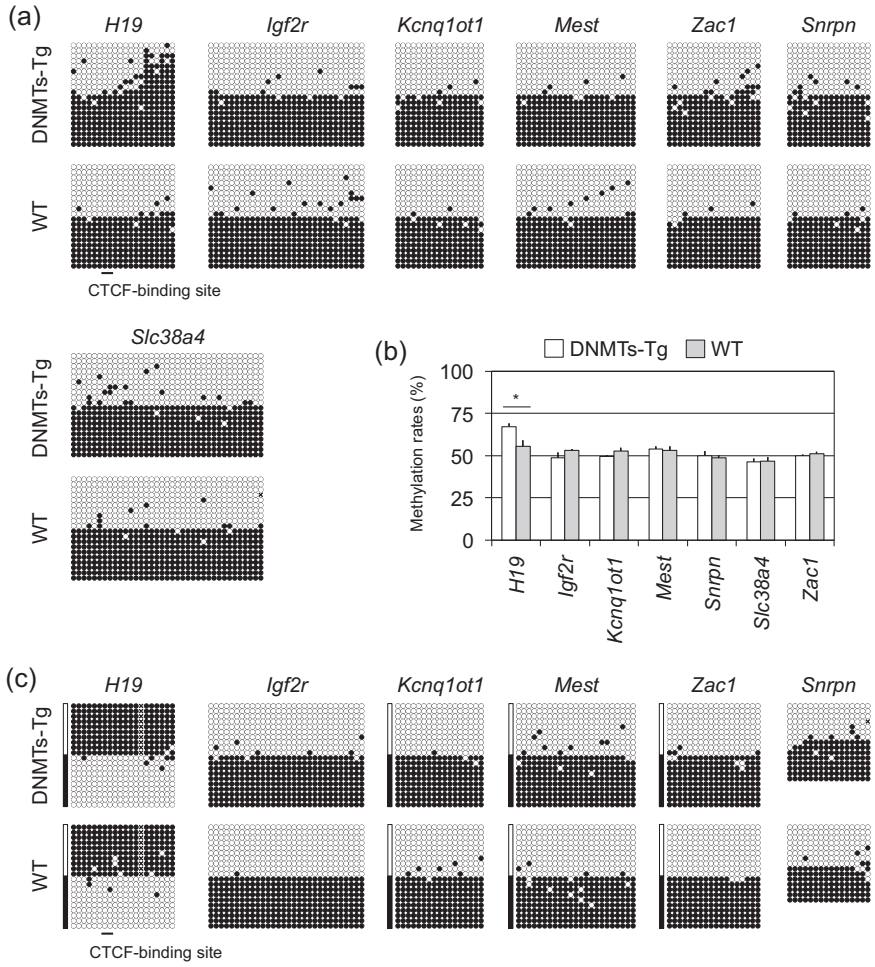

FIGURE 6 Methylation imprints of differentially methylated regions during embryogenesis and postnatal growth. Bisulfite sequencing analyses revealed invariable methylation statuses of imprinted DMRs in DNMTs-Tg and WT in hearts of 8-week-old adults (a) and embryos at E12.5 (c). White and black lines indicate JF1-inherited paternal alleles and maternal 1lox alleles, respectively. Circles represent the CpG sites in imprinted loci (open, unmethylated; closed, methylated). (b) DNA methylation rates of imprinted regions of adult DNMTs-Tg mice. White and gray bars indicate DNMTs-Tg and WT samples, respectively. Only *H19* DMR of DNMTs-Tg was slightly more hypermethylated than that of WT. DNMT: DNA methyltransferase; Tg: transgenic; WT: wild type

(Sohni et al., 2015). Therefore, the abnormal DNA methylation after birth might not be attributed to the ablation of TETs.

Surprisingly, de novo DNA methylation did not occur at imprinted loci in DNMTs-Tg embryos and mice. One exception was *H19*, which exhibited a slight increase in methylation level only after birth in DNMTs-Tg mice. It is known that CCCTC-binding factor (CTCF) boundary prevents maternal *H19* alleles from de novo DNA methylation and deletion of some nucleotides in the CTCF boundary sites leads to de novo methylation at maternal *H19* allele (Bell & Felsenfeld, 2000; Hark et al., 2000; Szabó, Tang, Rentsendorj, Pfeifer, & Mann, 2000). Overexpression of the small interfering RNA against *Ctcf* during oocyte growth also causes ectopic DNA methylation at *H19* in the resultant oocytes (Fedoriw, Stein, Svoboda, Schultz, & Bartolomei, 2004). However, our DNA methylation analysis showed that gain of methylation did not occur at the CTCF-binding sites (positions 7 and 8) on the maternal alleles. Genomic imprinting during oogenesis is regulated by both mechanisms, acquisition of DNA methylation at oocyte-specific DMRs and blocking of DNA methylation at sperm-specific DMRs, whereas genomic imprinting during spermatogenesis is regulated by the opposite mechanisms. Our results demonstrated that these blocking mechanisms at imprinted loci are perpetuated in somatic cell lineage and prevent de novo DNA methylation at imprinted loci rather than nonimprinted loci.

In humans, trisomy of chromosome 21 causes Down's syndrome. *DNMT3L* is encoded at the locus associated with the Down's syndrome and is ectopically expressed in the brains of neonatal patients with Down's syndrome, resulting in global hypermethylation at many genomic loci (Aapola et al., 2000; Lu et al., 2016). In our present study, DNMTs-Tg mice also exhibited hypermethylation of several genomic loci in the brain. Furthermore, it has been hypothesized that transgenerational inheritance of epimutation can lead to disorders or fetal miscarriages. Siklenka et al. (2015) reported that overexpression of H3K4 demethylase KDM1A caused epimutation, induced abnormal embryonic development, and resulted in small litter size in mice. These effects were transmitted from non-Tg (F2) mice derived from a heterozygous Tg (F1) father to the next generation (F3). Thus, ectopic expression of DNMT3A2 and DNMT3L may also be related to some epigenetic disorders and may serve as a model for understanding epigenetic disorders.

In conclusion, we showed that constitutive expression of DNMT3A2 and DNMT3L caused lethality within 20 weeks after birth accompanied by aberrant DNA methylation in several loci excluding almost all imprinted DMRs. These findings suggest that epigenetic programs are

protected from ectopic DNMTs during the embryonic stage but these machineries are weak after birth in nonimprinted regions.

4 | MATERIALS AND METHODS

4.1 | Ethical approval

All animal experiments were conducted under approval of the Tokyo University of Agriculture Institutional Animal Care and Use Committee, according to the Guidelines for Proper Conduct of Animal Experiments by the Science Council of Japan.

4.2 | Generation of DNMTs-Tg embryo and mice

The previously generated 2lox mice, harboring floxed alleles were used in this study (Hara, Takano, Fujikawa, et al., 2014; Hara, Takano, Ogata, et al., 2014). For the production of DNMTs-Tg mice (1lox mice), *Vasa*-Cre female mice, having FVB genetic background (purchase from the Jackson Laboratory, Bar Harbor, ME), were crossed with 2lox male mice. Two lines (ak and p) of 2lox mice were used for the phenotype analysis and line ak mice were used for all other experiments. The mCherry-positive DNMTs-Tg embryos were collected from the uteruses of pregnant *Vasa*-Cre females, at E9.5 and E12.5.

4.3 | Western blot analysis

Mouse whole embryos at E12.5 and adult tissues were fractionated by hypotonic and lysis treatment using IGEPAL CA-630 (Sigma-Aldrich, St Louis, MO) to obtain the nucleic protein. Approximately 20 μg of protein diluted in sample buffer containing 0.25 M Tris-HCl (pH 6.8), 40% glycerol, 0.8% sodium dodecyl sulfate (SDS), and 1% β-mercaptoethanol was subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE) in 4% stacking and 10% running acrylamide gel and transferred to polyvinylidene fluoride membranes. After blocking with 5% skim milk in TBS-T, the membrane was reacted with anti-DNMT3A mouse monoclonal IgG (ab13888, 1:500; Abcam, Cambridge, UK), anti-DNMT3L rabbit polyclonal IgG (immunizing rabbit with a synthetic peptide corresponding to 228 amino acids from position 194–421 of DNMT3L, 1:2,000; Sakai et al., 2004), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) rabbit polyclonal IgG (2275-PC; Trevigen, Gaithersburg, MD, 1:2,000). Secondary antibody treatments were performed using anti-mouse horseradish peroxidase (HRP)-linked whole Ab sheep (NA931; GE Healthcare, Little Chalfont, UK) for DNMT3A and anti-rabbit IgG, HRP-linked whole Ab donkey (NA934; GE Healthcare) for DNMT3L and GAPDH. Signals were visualized with the ECL Prime Western blot analysis Detection Reagent (RPN2232; GE Healthcare).

4.4 | Microarray analysis

Total RNA from hearts of DNMTs-Tg mice and WT from line ak ($N = 3$) was extracted with RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany). The first-strand complementary DNA (cDNA) was synthesized using the Eukaryotic Poly-A RNA Control Kit (Affymetrix, Santa Clara, CA) and one-cycle cDNA synthesis kit (Affymetrix), purified using Sample Clean Up Module (Affymetrix), and subsequently converted to biotin-labeled cRNA using Gene Chip IVT labeling Kit (Affymetrix). The GeneChip Mouse Genome 430 2.0 Array (Affymetrix), which hybridized the cRNA, was scanned by the Affymetrix GeneChip Scanner 3000 (Affymetrix). The array data were analyzed in GeneSpring v12.4 (Agilent Technology, Santa Clara, CA) to filter out probes with raw value <200 in all the samples. Differentially expressed genes between DNMTs-Tg and WT samples were determined by moderated *t* test (Benjamini–Hochberg false discovery rate, $p < 0.05$). The microarray data was deposited on Gene Expression Omnibus (Accession number: GSE126566).

4.5 | DNA methylation analysis

Genomic DNA was extracted from embryos and tissues of neonates and adults to analyze DNA methylation status. Tissues and whole embryos were incubated overnight in 1% SDS solution containing 0.2 mg/ml of Proteinase K (Thermo Fisher Scientific, Waltham, MA) at 55°C. Eluted genomic DNA was purified by phenol–chloroform extraction and then treated using a EpiTect Bisulfite Kit (Qiagen) for bisulfite conversion. Subsequently, PCR was performed using TaKaRa EpiTaq HS (Takara Bio Inc., Shiga, Japan) with each primer pair specific for promoter CpG islands and DMRs. PCR amplicons were purified using Wizard SV Gel and PCR Clean-Up System (Promega, Fitchburg, WI), cloned into pGEM-T Easy vector (Promega), and sequenced on ABI PRISM 3100 (Applied Biosystems, Waltham, MA). DNA methylation rates were assessed using QUMA (Kumaki, Oda, & Okano, 2008). Primer information is given in Table S4.

4.6 | Polymorphism analysis

To conduct polymorphic DNA methylation analysis, 2lox female mice were crossed with *Vasa*-Cre male mice and the progeny double Tg female mice whose oocytes harbored 1lox allele were mated with WT males of JF1/Msf strain (*Mus musculus molossinus*; Koide et al., 1998). JF1/Msf mouse strain (RBRC00639) was provided by RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. DNA methylation statuses of the embryos at E12.5 were determined as described above. Maternal and paternal alleles were identified via the single nucleotide polymorphisms between B6FVBF1 and JF1 genomes (Figure S7).

ACKNOWLEDGEMENTS

We thank for Prof. Toshihiko Shiroishi (National Institute of Genetics) and RIKEN BioResource Research Center for providing JF1/Msf strain mice. We are also grateful to the members of the Animal Life Science Research Center at Tokyo University of Agriculture for their contributions to the animal care. This work was supported in part by the Grants-in-Aid for Scientific Research (Nos. 18H05547 and 18H02355 to Y. O. and 18J01481 to K. S.).

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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How to cite this article: Sasaki K, Hara S, Yamakami R, et al. Ectopic expression of DNA methyltransferases DNMT3A2 and DNMT3L leads to aberrant hypermethylation and postnatal lethality in mice. *Mol Reprod Dev*. 2019;86:614–623. https://doi.org/10.1002/mrd.23137