Published in final edited form as:
Dev Biol. 2008 December 1; 324(1): 139–150. doi:10.1016/j.ydbio.2008.09.015.

DNA methyltransferase 1o functions during preimplantation development to preclude a profound level of epigenetic variation

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Abstract

Most mouse embryos developing in the absence of the oocyte-derived DNA methyltransferase 1o (DNMT1o-deficient embryos) have significant delays in development and a wide range of anatomical abnormalities. To understand the timing and molecular basis of such variation, we studied pre- and post-implantation DNA methylation as a gauge of epigenetic variation among these embryos. DNMT1o-deficient embryos showed extensive differences in the levels of methylation in differentially methylated domains (DMDs) of imprinted genes at the 8-cell stage. Because of independent assortment of the methylated and unmethylated chromatids created by the loss of DNMT1o, the deficient embryos were found to be mosaics of cells with different, but stable epigenotypes (DNA methylation patterns). Our results suggest that loss of DNMT1o in just one cell cycle is responsible for the extensive variation in the epigenotypes in both embryos and their associated extraembryonic tissues. Thus, the maternal-effect DNMT1o protein is uniquely poised during development to normally ensure uniform parental methylation patterns at DMDs.

Keywords

imprinting; epigenetics; embryogenesis; DNA methylation; DNA methyltransferase

Introduction

Genomic imprinting is an epigenetic process that distinguishes parental alleles of a small number of genes that are essential for normal mammalian development (McGrath and Solter, 1984; Surani et al., 1984). In the post-implantation embryo and adult, parental alleles of imprinted genes are transcribed differently (one allele is usually silent and the other one expressed), and this difference in transcription is associated with different epigenetic modifications (Lucifero et al., 2002). These include different patterns of DNA cytosine

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methylation to generate differentially methylated domains (DMDs), as well as differences in the histone modifications of the alleles' resident nucleosomes (Carr et al., 2007; Delaval et al., 2007; Murrell et al., 2004; Verona et al., 2008; Yang et al., 2003). All of these allelic differences probably originate from processes that establish DNA cytosine methylation patterns during germ cell development and maintain some of the gametic methylation patterns during early embryogenesis. DNMT3A, and its accessory protein DNMT3L are important for establishing imprints in both germ lines, and disruptions in this mechanism adversely affect embryonic and fetal development (Bourc'his et al., 2001; Hata et al., 2002; Kaneda et al., 2004).

Less is known about the molecular mechanisms that ensure the inheritance of imprinted methylation patterns following fertilization. Inheritance of methylation imprints is necessary for normal development and may be a critical component of a two-step process of germline *establishment* and early embryonic *maintenance* of genomic imprints. Some insight into the functional importance of preimplantation inheritance of genomic imprints has come from examining the expression of DNMT1 proteins during this developmental window. The M_r 190,000 DNMT1s protein is expressed at low levels in all preimplantation cleavage stages, and at higher levels following implantation (Cirio et al., 2008). This protein maintains methylation patterns on DMD sequences in preimplantation staged embryos (Hirasawa et al., 2008). The M_r 175,000 DNMT1o protein is also expressed throughout preimplantation development; the protein is in the cytoplasm of all preimplantation stage embryos, but in the nuclei of only 8-cell embryos (Cardoso and Leonhardt, 1999; Carlson et al., 1992; Doherty et al., 2002; Howell et al., 2001; Ratnam et al., 2002). When the abundant DNMT1o protein is removed from the mouse oocyte by deletion of the promoter governing synthesis of the Dnmt1o transcript, DMD sequences still undergo *de novo* methylation during oogenesis. However, post-implantation embryos derived from DNMT1o-deficient oocytes lose methylation on a significant percentage of the normally methylated alleles of their DMDs (Howell et al., 2001). We concluded from these observations that DNMT1o is a maternal-effect protein that is synthesized in the oocyte, stored in high concentrations in the ooplasm, and then acts to maintain DMD methylation at the 4th S phase in 8-cell embryos.

A recent report suggests that DNMT1o is not present in the nuclei at any stage of preimplantation development (Hirasawa et al., 2008). The apparent absence of nuclear DNMT1o in the Hirasawa et al. (2008) study is puzzling because the same report confirms the previous finding of a genetic requirement for DNMT1o in maintaining DMD methylation during preimplantation development (Howell et al., 2001). Therefore, DNMT1o protein must come in contact with the nuclear genome during preimplantation development, and based on previous studies, we postulate this occurs during the 4th embryonic S phase (8-cell embryos). Based on assumptions that DNMT1o functions only in 8-cell blastomeres at the 4th S phase of embryogenesis, that DMDs on many autosomes are similarly affected by the loss of DNMT1o, and that chromosomes undergo independent assortment during preimplantation development, we hypothesized that loss of DNMT1o protein results in the generation of a multitude of epigenotypes (estimate of >4,000) (Howell et al., 2001; Toppings et al., 2008). If this model is correct, then each DNMT1o-deficient embryo is an epigenetic mosaic, composed of cells or epigenotypes with fundamentally different genomic methylation patterns. Furthermore, because each DNMT1o-deficient embryo would be comprised of a few epigenotypes out of the very large number of possible epigenotypes, each embryo is very likely to be different from other DNMT1o-deficient embryos. This variability in epigenotypes offers one explanation for the highly variable phenotypes observed among DNMT1o-deficient embryos of identical genetic backgrounds (Toppings et al., 2008).

There are considerable effects on both DMD methylation and on the phenotypes of embryos due to the loss of DNMT1o protein (Howell et al., 2001; Toppings et al., 2008). Because of this, it is imperative to understand the time at which DNMT1o functions to maintain DNA

methylation and to determine the molecular consequences of DNMT1o loss on post-implantation embryos and placentae.

Results

DNMT1o maintains genomic imprints between the 4-cell and morula stages

Because the DNMT1o protein is abundantly expressed during preimplantation development, this developmental window is likely to be the source of epigenetic defects in DNMT1o-deficient embryos. For this reason, we determined the preimplantation stage(s) at which the absence of DNMT1o protein affects the inheritance of genomic imprints. We first compared patterns of allele-specific DNA methylation in wild-type and DNMT1o-deficient morulae. F_1 hybrid embryos were obtained from crosses between wild-type and *Dnmt1*$^{\Delta 1o/\Delta 1o}$ 129/Sv strain females and wild-type consomic CAST7 male mice to distinguish maternal and paternal alleles (Mann et al., 2003). Imprinted methylation patterns were determined at the *Snurf/Snrpn* and *H19* DMDs using the bisulfite genomic sequencing method. All examined maternal alleles of the *Snurf/Snrpn* DMD were highly methylated and all paternal alleles were hypomethylated in wild-type morulae (Fig. 1). In contrast, there was a marked reduction of methylated maternal *Snurf/Snrpn* alleles in DNMT1o-deficient morulae. In a separate experiment, we showed a similar level of methylation loss from paternal alleles of the normally imprinted *H19* DMD in DNMT1o-deficient morulae compared to wild-type embryos (Fig. 1). In all cases, there were alleles with an intermediate level of methylation (30%–70% methylated CpGs) that cannot be easily classified as “fully” methylated or “unmethylated” alleles. These observations indicate that the DNMT1o protein functions before the morulae stage to maintain methylation on imprinted gene sequences. Because approximately one-half of the normally methylated *Snurf/Snrpn* and *H19* alleles had lost methylation, it is possible that in the absence of DNMT1o protein, there was a failure to maintain methylation of these DMDs at just one cell cycle.

To more accurately determine when DNMT1o protein is catalytically active, we attempted to measure *Snurf/Snrpn* methylation patterns in pools of 4-cell or 8-cell embryos obtained from crosses between homozygous *Dnmt1*$^{\Delta 1o/\Delta 1o}$ 129/Sv female mice and wild-type CAST7 males. More often than not, these attempts failed because of the absence of *Snurf/Snrpn* and/or *H19* PCR amplification products using the bisulfite-treated DNA samples as templates. Even when PCR products were obtained, such strong biases in the recovery of one parental allele over the other (data not shown) were observed that we were unable to reach any conclusions about the precise time of DNMT1o function. We attributed these experimental failures to the extremely small amounts of template genomic sequences obtained from pools of preimplantation embryos, and likely to other confounding factors specific to preimplantation embryos.

To circumvent the difficulties we encountered in measuring *Snurf/Snrpn* and *H19* DMD methylation on single-copy genomic sequences in early-stage preimplantation embryos, we instead examined the inheritance of DMD methylation of one of the many imprinted transgenes we have constructed (Reinhart et al., 2002). The advantages of using these imprinted transgenes for studies of preimplantation DMD methylation are their presence in multiple copies in the genome and their unambiguous inheritance as a hemizygous locus from a single parent. The transgene we chose to study in detail is designated *TR2+3Igmyc* (Fig. 2A). This imprinted transgene contains *Igf2r* intronic DMD2 sequences on which maternal-specific methylation is established during oogenesis, and then maintained during preimplantation development (Reinhart et al., 2006). *TR2+3Igmyc* models the acquisition and inheritance of a maternal methylation imprint that occurs on endogenous mouse *Igf2r* sequences (Brandeis et al., 1993). We observed that transgene *Igf2r* DMD sequences were methylated in MII oocytes (Fig. 2B, left panel). A similar level of methylation was present in 8-cell embryos and in blastocysts that had inherited only *TR2+3Igmyc* maternal alleles, consistent with the inheritance of oocyte

TR2+3Igmyc methylation. In contrast, when *TR2+3Igmyc* sequences were transmitted through DNMT1o-deficient oocytes, there was a loss of methylation during preimplantation development (Fig. 2B, right panel). Transgenic *Igf2r* sequences were highly methylated in DNMT1o-deficient MII oocytes and 4-cell embryos. However, in 8-cell, morula and blastocyst stage embryos, there was a significant reduction in CpG methylation to ~50% of the number of methylated CpGs in oocytes and 4-cell embryos (Figs 2B, 2C). Many of the transgenic maternal *Igf2r* alleles, much like many alleles of endogenous *Snurf/Snrpn* and *H19* genes in DNMT1o-deficient morulae (Fig. 1), were not easily classified as methylated or unmethylated. Nevertheless, the percentage reduction was similar to the 46% reduction in maternal *Snurf/Snrpn* methylation and 73% reduction in paternal *H19* methylation seen in DNMT1o-deficient morulae (Fig. 1). These findings are consistent with DNMT1o maintenance methyltransferase activity between the 4-cell and morulae stages. Based on previous observations that DNMT1o is present in nuclei of only 8-cell embryos (Cardoso and Leonhardt, 1999; Carlson et al., 1992; Chung et al., 2003; Doherty et al., 2002; Howell et al., 2001; Ratnam et al., 2002), this result supports the notion that the functional requirement of DNMT1o is associated with the single DNA replication cycle during the fourth S phase of embryonic development.

DNMT1o-deficient ES cell lines are mosaic for imprinted methylation

In a model in which each DNMT1o-deficient embryo loses one-half of DMD methylation during the fourth S phase, all embryos are epigenetic mosaics (Howell et al., 2001; Toppings et al., 2008). An alternative explanation however, for the loss of DMD methylation on normally methylated alleles in pooled samples of either 8-cell embryos, morulae or blastocysts (Fig 1, Fig 2) is that there is loss of methylation in ~1/2 of the embryos but retention of methylation in the other half. To distinguish between these two very different possibilities, we examined *Snurf/Snrpn* and *H19* DMD methylation in a series of ES cell lines from DNMT1o-deficient 129/Sv blastocysts. We chose to examine ES cells because they are the earliest developmental stage in which individual cells (clones) can be compared and are representative of the embryo's stem cells, which are established very soon after DNMT1o's maintenance function. Using Southern blot analysis, we determined that *Snurf/Snrpn* DMD methylation was lower in the representative DNMT1o-deficient ES cell line (Δ1o L2) than in the wild-type RI ES cell line, and significantly greater than in the *Dnmt1*-null ($Dnmt1^{c/c}$) ES cell line (Fig. 3A, compare lanes 7, 13 and 14). The reduction in the ratio of intensity of the methylated 1.9-kb and 0.9-kb bands to the unmethylated 0.5-kb band in the Dnmt1o-deficient ES cell line compared to the wild-type cell line indicates a significant reduction in methylation. To determine if this reduction in methylation represents an epigenetic mosaicism, individual cell clones of two DNMT1o-deficient and one wild-type ES cell lines were examined. We observed that ES clones distributed into two types: those with *Snurf/Snrpn* methylation similar to that seen in wild-type ES cell clones (Fig. 3A, compare lanes 4, 8, 10 and 12 to lane 15 and 16), and those with a hypomethylated pattern (Fig. 3A, compare lanes 1–3, 5, 6, 9 and 11 to lane 14). Clones of wild-type ES cell lines with a hypomethylated pattern were not seen. We conclude from this analysis of *Snurf/Snrpn* DMD methylation that each DNMT1o-deficient embryo is an epigenetic mosaic comprised of two distinct *Snurf/Snrpn* epigenotypes.

An analysis of *H19* DMD methylation showed a similar mosaic pattern to that seen for the *Snurf/Snrpn* DMD. The same wild-type and DNMT1o-deficient ES cell lines were used for *Snurf/Snrpn* and *H19* DMD methylation analysis. The methylation associated with the *H19* DMD is on paternal chromosome 7, whereas the methylation associated with the *Snurf/Snrpn* DMD is on maternal chromosome 7 (Lucifero et al., 2002; Tremblay et al., 1995). The DNMT1o-deficient ES cell line showed a similar reduction in *H19* DMD methylation to the reduction in *Snurf/Snrpn* DMD methylation (Fig. 3B, lane 7), and clones of the DNMT1o-deficient lines were either normally methylated or were hypomethylated (Fig. 3B, compare lanes 1, 2, 4–6, 8, 10–12 to lanes 3 and 9). Despite alterations in *Snurf/Snrpn* and *H19* DMD

methylation, all of the examined ES cell lines and clones showed normal or nearly normal levels of LINE-1 and IAP methylation (Fig. S1). Similar findings were seen in a carefully analyzed post-implantation embryo (Howell et al., 2001). Taken together, these observations suggest that, of the sequences studied here, the only lasting genomic effect of DNMT1o deficiency is the loss of methylation on imprinted DMD sequences.

Four epigenotypes are predicted from the analysis of *Snurf/Snrpn* and *H19* DMD methylation (Figure 3C). We recovered DNMT1o-deficient clones with epigenotype 1 (Fig. 3C) that showed normal *Snurf/Snrpn* and *H19* DMD methylation (lanes 4, 8,10 and 12), epigenotype 3 that were hypomethylated on the *Snurf/Snrpn* DMD but methylated on the *H19* DMD, (lanes 1, 2, 5, 6 and 11) and epigenotype 4 that were hypomethylated on both the *Snurf/Snrpn* and the *H19* DMD (lanes 3 and 9). The inability to recover one of the four epigenotypes (epigenotype 2 in Fig. 3C) could be due to the small number of clones screened or the inability of epigenotype 2 to proliferate in culture. We previously reported the isolation of this distinct epigenotype in mouse embryonic fibroblasts isolated from a DNMT1o-deficient post-implantation embryo, supporting the first explanation (Toppings et al., 2008).

Discordance between allele-specific methylation and expression in DNMT1o-deficient blastocysts

Because each DNMT1o-deficient ES cell line we examined was an epigenetic mosaic comprised of roughly equal numbers of cells with normally imprinted methylation and loss of methylation at a particular DMD, we assumed that each DNMT1o-deficient blastocyst was similarly composed. In principle, the cells in these mosaic blastocysts that were unmethylated on both parental alleles of certain DMDs should promote the biallelic expression of gene(s) regulated by those DMDs. We tested this hypothesis by comparing the levels of paternal- and maternal-allele expression of three genes (*Snurf/Snrpn*, *H19* and *Peg3*) known to be imprinted in blastocysts (Lucifero et al., 2002; Shemer et al., 1997; Tremblay et al., 1995). As shown in Fig. 4, *H19* was expressed from only the maternal allele in wild-type as well as DNMT1o-deficient blastocysts. The *Snurf/Snrpn* gene also showed an unexpected retention of imprinted gene expression in blastocysts (Fig. 4). Although DNMT1o-deficient blastocysts showed a slight increase in the ratio of expression from the normally silent maternal *Peg3* allele compared to its paternal allele, the imprinted expression of *Peg3* was largely unaffected by the removal of DNMT1o (Fig. 4). We conclude from these observations that, despite the significant reduction in DNA methylation, there was no corresponding change in expression from those parental alleles that have become unmethylated in the absence of DNMT1o.

Imprinting abnormalities in E7.5 DNMT1o-deficient embryos

The consistent loss of approximately one-half of DMD methylation in DNMT1o-deficient ES cell lines (Fig. 3) suggests that each DNMT1o-deficient blastocyst had undergone the same or nearly the same loss in DMD methylation. To determine if this overall mosaic composition is maintained during further embryonic development, we measured *H19* and *Snurf/Snrpn* methylation in a group of individual E7.5 embryos produced by crossing a homozygous *Dnmt1*$^{\Delta 1o/\Delta 1o}$ 129/Sv female mouse and a CAST7 male mouse. DNA was extracted from the entire E7.5 embryo for this analysis, and the findings were compared to the allele-specific patterns of DNA methylation seen in wild-type 129/Sv × CAST7 F_1 E7.5 embryos. We observed the expected pattern of *H19* (paternal allele methylated) and *Snurf/Snrpn* (maternal allele methylated) DMD methylation in a representative wild-type embryo (Fig. 5). This finding contrasts with the extent of *H19* and *Snurf/Snrpn* DMD methylation observed in six different E7.5 DNMT1o-deficient embryos (four embryos shown in Fig. 5). *H19* and *Snurf/Snrpn* methylation patterns of the DNMT1o-deficient E7.5 embryos ranged from normal or nearly normal to a complete absence of methylation. In DNMT1o-deficient embryos 1 and 2, *H19* paternal methylation was present on all examined alleles, whereas *Snurf/Snrpn* maternal

methylation was significantly reduced. In embryo 3, there was a partial loss of methylation on both DMDs, and in embryo 4, there was a complete loss of DMD methylation. These findings indicate that despite the consistent loss of approximately one-half of the methylation associated with the normally methylated parental alleles of *H19* and *Snurf/Snrpn* in pools of DNMT1o-deficient morulae/blastocysts (Fig 1, Fig 2) and in DNMT1o-deficient ES cell lines (Fig. 3), the extent of methylation loss was not maintained into postimplantation development. Rather, there was statistically significant variation in the extent of the measured epigenetic abnormalities among E7.5 embryos, suggesting that there were events following the initial establishment of mosaic preimplantation embryos that contributed to the cellular composition of the post-implantation embryo.

Imprinting abnormalities in E9.5 DNMT1o-deficient embryos

To better define the epigenetic variability among DNMT1o-deficient embryos, we also examined E9.5 embryos, a developmental stage in where we could reliably measure effects of DNMT1o protein loss in both embryos and placentae. In a representative wild-type embryo, the *H19* gene is expressed solely from the maternal allele in both the embryo proper and in the placenta (Fig. 6), consistent with previous studies (Tremblay et al., 1997). In the embryo, the maternal-specific expression of *H19* was tightly associated with paternal-specific methylation, whereas in the placenta, the correlation was less precise. Although paternal *H19* alleles were highly methylated in the placenta, maternal *H19* alleles exhibited variable degrees of methylation (Davis et al., 1998).

In contrast to the strict paternal-specific *H19* DMD methylation seen in control E9.5 embryos, DNMT1o-deficient E9.5 embryos exhibited variability in the extent of paternal-allele methylation. DNMT1o-deficient embryos 1 and 2 had an approximately normal level of paternal-allele methylation, whereas approximately one-half of the paternal alleles of DNMT1o-deficient embryo 3 were unmethylated or hypomethylated. In all three embryos, there was biallelic expression of *H19*, with variable ratios of maternal-to-paternal expression. Abnormalities in DNA methylation and allele-specific expression of *H19* were also evident in the placentae of the three mutant embryos. There was significant loss of DNA methylation on paternal alleles in two of the three placentae (DNMT1o-deficient placentae 2 and 3), and loss of monoallelic *H19* expression in all three. Notably, in the case of DNMT1o-deficient embryo 2, there was a marked degree of discordance between the extent of paternal-allele methylation in the embryo and in the placenta.

Snurf/Snrpn methylation and expression were measured in the same wild-type and DNMT1o-deficient E9.5 embryos and placentae used to measure *H19* methylation and expression (Fig. S2). There was strict maternal-specific methylation and paternal-specific expression of *Snurf/Snrpn* in the wild-type embryo and its placenta, but a high degree of variability in both allele-specific expression and methylation among the three Dnmt1o-deficient embryos examined (Fig. S2). Whereas DNMT1o-deficient embryo 1 exhibited nearly normal patterns of allele-specific *H19* methylation in both embryo and placental tissue (Fig. 6), *Snurf/Snrpn* methylation was nearly absent in both the embryo and placenta. The other two DNMT1o-deficient embryos also showed discordance between the extent of allele-specific *H19* and *Snurf/Snrpn* methylation in embryo and placenta, although not to the extent seen in DNMT1o-deficient embryo 1 (Fig 6, Fig S2).

Discussion

Timing of DNMT1o function and random chromosome segregation as a primary source of epigenetic variation

The findings presented here indicate that there are molecular and cellular processes that work sequentially during embryogenesis to produce epigenetic variation and a wide range of anatomical phenotypes in DNMT1o-deficient mice. The primary effect of DNMT1o loss is the generation of preimplantation epigenetic mosaic embryos. These are produced by the combined effects of a transient loss of DNMT1o maintenance methyltransferase activity followed by the normal process of random chromosome segregation (Fig. 7). We define different epigenotypes by the different methylation states of DMDs. Thus, mosaic embryos are comprised of cells with different epigenotypes, with each unique epigenotype representing a collection of normally methylated and unmethylated DMDs.

The duration of one cell cycle and the time of action are intriguing aspects of DNMT1o deficiency. Because we observed that ~50% of methylation remains on normally methylated DMDs in DNMT1o-deficient morulae and blastocysts (Fig 1, Fig 2), DNMT1o probably functions at a single S phase. This is based on interpretation of multiple localization studies (Cardoso and Leonhardt, 1999;Carlson et al., 1992;Doherty et al., 2002;Howell et al., 2001;Ratnam et al., 2002) which concluded that DNMT1o protein localizes to the nuclei of 8-cell blastomeres. The only reported exception to these results is the negative staining for DNMT1o of 8-cell nuclei by Hirasawa et al. (2008).

We interpret the 8-cell embryo nuclear localization and the timing of DNMT1o's effect (Fig. 2) to mean that loss of methylation at the fourth S phase, followed by DNA replication, maintenance methylation and chromosome segregation at the subsequent cell cycle, would produce mosaics that are first evident at the 32-cell stage. If loss of maintenance methylation occurred for two or more cell cycles much higher number of cells with unmethylated DMDs, and a much lower number of distinct epigenotypes would result. Furthermore, loss of maintenance methylation for a single cell cycle at a later stage of preimplantation development, when many more cells are present, would produce a mosaic embryo with much greater cell-to-cell variation. Thus based on our data, we conclude that the timing of DNMT1o function is at the 8-cell stage, consistent with the fact that DNMT1o is only present in the nucleus at the 8-cell cleavage stage.

In light of this, questions arise as to why DNMT1o function at the fourth embryonic S phase is necessary. There are two likely possibilities. First, the 8-cell embryo is the latest embryonic stage in which all cells are morphologically, and probably functionally identical (Fleming et al., 2001; Pratt et al., 1982; Reeve and Ziomek, 1981), and maintenance methylation at a distinct S phase might only be possible if all the cells are identical. Second, although cell-to-cell epigenetic complexity is much less than maximal when maintenance methylation is lost at the 4^{th} S phase (as in a DNMT1o-deficient embryo), the embryo-to-embryo variation will be much greater because each DNMT1o-deficient embryo will have <1% of possible epigenotypes. That is, the chances of two given embryos having one or more identical epigenotypes is very low, and each DNMT1o-deficient embryo is likely to be very different from every other DNMT1o-deficient embryo.

In addition to transient loss of DNMT1o maintenance methyltransferase activity, random chromosome segregation likely plays a major role in the generation of epigenetically mosaic embryos. In this study, we examined the methylation status of two DMDs in DNMT1o-deficient preimplantation embryos. Four distinct *Snurf/Snrpn-H19* chromosome 7 epigenotypes were predicted to occur according to a model of random (independent) assortment of chromosome 7 sister chromatids. This randomization of chromosome 7 maternal and

paternal epigenotypes would occur during anaphase of the cell cycle immediately following the DNMT1o-dependent one (Fig. 3C). These cellular epigenotypes would therefore first appear in DNMT1o-deficient morula, a stage at or just preceding the appearance of cells destined to become the definitive stem cells of the embryo. If, as expected, all other chromosomes containing DMDs undergo random assortment of their chromatids, we would expect a very large number of epigenotypes to be produced due to the loss of DNMT1o protein (Toppings et al., 2008). More specifically, as there are 12 autosomes containing imprinted genes, we estimated that there are greater than 4000 possible epigenotypes that can be produced as a consequence of DNMT1o deficiency (Toppings et al., 2008).

The severe epigenetic consequences of DNMT1o deficiency indicate that preimplantation development is a potent source of variation and that an important role of DNMT1o protein is in precluding epigenetic variation. In light of this, we can speculate that DNMT1o may, in some circumstances, be regulated in order to increase epigenetic and phenotypic variation among embryos. For example, a partial decrease in the level of DNMT1o in nuclei of 8-cell embryos might increase epigenetic variation, but not lead to fetal death. Epigenetic mosaicism has been reported in humans, and in a pair of familial cases, pronounced phenotypic differences between mosaic siblings was observed (Boonen et al., 2008). In this regard, it is interesting to consider that a partial decrease in DNMT1o may underlie epigenetic defects in mouse embryos cultured in various synthetic media (Doherty et al., 2000; Khosla et al., 2001). Moreover, there is evidence that cases of Beckwith-Wiedemann Syndrome (BWS) associated with assisted reproductive technology (ART) are epigenetic mosaics (DeBaun et al., 2003), consistent with an ART-induced disruption in the normal process whereby inherited methylation is maintained in the early embryo. Taken together, observations on the highly variable phenotypes among DNMT1o-deficient mice and the emerging evidence of similar imprint-related epigenetic mosaic humans indicate a need to understand the etiology of these effects.

Discordance between loss of methylation and loss of expression in DNMT1o-deficient embryos

Another prominent message that emerges from our analysis of the observed sources of phenotypic variation in DNMT1o-deficient mice is that there is a delay between the developmental time of methylation loss and a change in allele-specific expression (Fig 1, Fig 2 and Fig 4). Methylation defects are present in both DNMT1o-deficient blastocysts and E9.5 embryos and yet we only observed biallelic expression of imprinted genes in E9.5 embryos. Although we do not know when in the intervening six days biallelic expression of chromosome 7 imprinted genes first occurred, it likely takes place after implantation.

The mechanism of such discordance between imprinted methylation and expression might be the presence of other chromatin modifications regulating expression of these genes. In a strict sense, the parental alleles in a DNMT1o-deficient blastocyst must be distinguished by an epigenetic mark (other than DNA methylation) that has an absolute controlling effect on transcription. We speculate that there are differences between the maternal and paternal alleles in the chromatin composition of the *Snurf/Snrpn, H19* and *Peg3* genes in DNMT1o-deficient blastocysts. Perhaps the relevant differences are histone modifications that are known to distinguish the maternal and paternal alleles in preimplantation-stage embryos (Liu et al., 2004; Santos et al., 2005). Precedents for this type of effect have been described in extraembryonic tissues of the mouse; imprinted genes expressed only in the placentae and located in a cluster of imprinted genes associated with the *Kcnq1* gene remain imprinted even in placentae of embryos homozygous for a null-allele of *Dnmt1* (*Dnmt1*^{C} allele) (Lewis et al., 2004). Interestingly, in the absence of DNA methylation, parental allele-specific differences in chromatin were observed, suggesting that chromatin differences maintain an imprinted state of imprinted gene expression in the absence of DNMT1-dependent maintenance methylation.

An alternative explanation for the discordance between allele-specific DMD methylation and expression is that the absence of DNMT1o has not affected methylation patterns at locations in the *H19* and *Snurf/Snrpn* genes that control expression in blastocysts. However, because a concordance between *H19* and *Snurf/Snrpn* DMD methylation and expression is realized with further embryonic development, in this case the absence of DNMT1o must eventually lead to more extensive methylation changes in these genes.

Subsequent developmental events and the generation of variable phenotypes

Epigenetic mosaicism may be related to the widely ranging anatomical phenotypes seen in DNMT1o-deficient embryos. Different epigenotypes have different developmental potential, and cells with abnormal epigenotypes have restricted developmental potentials compared to cells with a normal epigenotype. Data from a variety of experiments in the mouse support this notion. Uniparental disomies can be produced experimentally in mice, and a given uniparental disomy will reproducibly give rise to a consistent abnormal phenotype. For example, paternal uniparental disomy of chromosome 11 is associated with a predictable effect on *in utero* growth. Fetal and placental overgrowth was associated with paternal disomy and maternal nullisomy for chromosome 11, whereas fetal and placental growth retardation was associated with chromosome 11 maternal disomy and paternal nullisomy (Cattanach and Kirk, 1985). Thus, the chromosome 11 epigenotype predicts the outcome of fetal development. Similar arguments can be made for the abnormal development seen in gynogenotes and androgenotes (McGrath and Solter, 1984; Surani et al., 1984). This includes the restricted distribution and development of cells of uniparental origin in both gynogenic-biparental and androgenic-biparental chimeras (Norris et al., 1990).

Our data also suggest that other processes, besides the altered developmental potential of epigenotypes produced by the absence of DNMT1o, may account for the wide range in phenotypes apparent in DNMT1o-deficient embryos (Fig. 7) (Toppings et al., 2008). We observed a remarkable degree of statistically significant variation in the extent of DMD methylation among E7.5 and E9.5 DNMT1o-deficient embryos and placentae (Fig 5, Fig 6 and Fig S2). For example, whereas some DNMT1o-deficient E7.5 and E9.5 embryos had normal levels of *H19* methylation, other E7.5 and E9.5 embryos showed highly significant losses of *H19* methylation. These differences in the degree of variation in methylation between late-stage preimplantation and post-implantation DNMT1o-deficient embryos suggest that primary mosaicism established in early development evolves through one or more post-implantation processes to expand the range of molecular defects associated with imprinted genes.

One explanation is the nonrandom distribution of cells of different epigenotypes in the developing DNMT1o-deficient embryo. Non-random distribution of cells of different epigenotypes likely occurs because these cell types are present at a very early stage of development (32-cell stage). At such a time, cells of these early mosaics will most likely distribute to trophectoderm and inner cell mass in a random fashion that could account for the observed discordance in methylation between embryo and placenta (Fig 6, Fig S2). Non-random distribution of cells within a DNMT1o-deficient embryo may also account for the discordance between *H19* and *Snurf/Snrpn* expression and methylation (Fig 6, Fig S2) because expression and methylation were measured in different sagital halfs. In conclusion, although we cannot reach a firm conclusion about the source(s) of embryo-to-embryo variation in methylation, there are some probable explanations for this that extend beyond the events associated with the initial generation of the epigenetic mosaics.

In summary, the primary effect of DNMT1o loss is the generation of epigenetically mosaic embryos. While it is not known how DNMT1o functions specifically during the 8-cell stage, it is clear that DNMT1o function during this critical time cannot be compensated by other methyltransferases (Chung et al., 2003; Cirio et al., 2008). Further investigation is needed to

determine whether disruptions in DNMT1o-dependent maintenance methylation underlie cases of DMD mosaicism associated with assisted reproduction technology (ART) in humans and hybrid dysgenesis in mice (DeBaun et al., 2003; Shi et al., 2005).

Materials and methods

Mouse genotyping and embryo collection

The mutant *Dnmt1*$^{\Delta 1o}$ allele (Howell et al., 2001) was maintained in the inbred 129/Sv background, and the *TR2+3Igmyc* transgene was maintained in the inbred FVB/N background (Reinhart et al., 2006). *Dnmt1*$^{\Delta 1o}$ mice were genotyped using a PCR assay as previously described (Howell et al., 2001). *TR2+3Igmyc* transgenic mice were genotyped using a PCR assay in which the PCR primers spanned the unique *IgA-myc* junction of the transgene construct: 5'-ctattccagcctagtctgct-3' (IgA) and 5'-agtcagaagctacggagcct-3' (c-myc).

Copulation was determined by the presence of a vaginal plug, and embryonic day zero (E0) was assumed to be midnight, the midpoint of the dark portion of the dark-light cycle. Embryos were collected from the oviducts or uteri of the female mice at various times (embryonic days) after mating. For the determination of methylation of *TR2+3Igmyc* maternal transgene alleles, metaphase II (MII) oocytes from carrier females were collected following superovulation or carrier females were crossed with wild-type FVB/N males and embryos from various preimplantation stages collected as previously described (Clarke et al., 1992). For allele-specific methylation and expression studies, morulae, blastocysts and post-implantation embryos were obtained from crosses with *Dnmt1*$^{\Delta 1o/\Delta 1o}$ 129/Sv females and CAST7 males, and from crosses with 129/Sv females and CAST7 males. CAST7 is a mouse strain in which two *Mus musculus castaneus* (CAST) chromosomes 7 reside on a C57BL/6 background (Mann et al., 2003). All experiments were performed in compliance with guidelines established by the Canadian Council of Animal Care and the Institutional Animal Care and Use Committee of the University of Pittsburgh.

Determination of allele-specific methylation from morulae, post-implantation embryos and placentae

DNA was isolated from pools of 8–13 morulae using the QIAamp DNA Micro Kit (Qiagen) and from whole E7.5 embryos or one sagital half of E9.5 embryos and placentae using the DNeasy Kit (Qiagen). The whole DNA from pools of morulae or 100 ng from the post-implantation embryos was used for bisulfite treatment using the EpiTect Bisulfite Kit (Qiagen) according to the manufacturer's protocol. Following bisulfite treatment nested PCR amplification of the *H19* and *Snurf/Snrpn* DMDs was performed as previously described (Lucifero et al., 2002). The presence of both alleles in the PCR product was confirmed using restriction fragment length polymorphisms generated by SNPs between 129/Sv and CAST7 strains. Five μl of the PCR products purified with MinElute Gel Extraction Kit (Qiagen) were digested with either *Dpn*II for *H19* or *Swa*I for *Snurf/Snrpn* and run on 1.5% agarose gels. For each sample, at least two different PCR amplification products were used to generate the results. The DNA was subcloned using the TOPO TA Cloning Kit (Invitrogen) and plasmids containing the appropiate insert were sequenced using the Big Dye Terminator v3.1 Kit (Applied Biosystems) and run on the ABI 3700 capillary sequencer. Statistically significant differences among samples were determined using the Mann-Whitney U-test comparing wild-type vs. DNMT1o-deficient samples. In Fig. 5, Fig 6 and Fig S2, * denotes significantly different levels of CpGs methylation ($p < 0.05$), ** denotes highly significant differences ($p < 0.01$) and ns = not statistically significant differences ($p > 0.05$).

Determination of TR2+3Igmyc transgene methylation

DNA was isolated from pools of 25 MII oocytes or 10–15 preimplantation embryos and used for sodium bisulfite mutagenesis using the EpiTect Bisulfite Kit (Qiagen). For *TR2+3Igmyc*, a semi-nested strategy was employed using outside primers 1A: 5'-gtattgaaattgagtttgaagtgg-3' and 2D: 5'-atactctaaataacctaaaaaatcc-3', and inside primers 1A and 2C: 5'-tatcttcacctaaaaaccctccac-3'. The PCR conditions used were 2 cycles of 94°C for 4 minutes, 55°C for 2 minutes, and 72°C for 2 minutes, followed by 35 cycles of 94°C for 1 minute, 55°C for 2 minutes, and 72°C for 2 minutes, with a final extension of 72°C for 10 minutes. For the nested round of PCR the cycling conditions were as follows: 5 minutes at 94°C followed by 35 cycles of 94°C for 1 minute, 55°C for 2 minutes, and 72°C for 2 minutes, with a final extension of 72°C for 10 minutes. The PCR products were cloned and analyzed with the same methods described before for endogenous genes. The Kruskal-Wallis test followed by Dunn's multiple comparison test were performed to test the differences between all DNMT1o-deficient samples. * denotes significantly different levels of CpGs methylation ($p < 0.05$). ** denotes highly significant differences ($p < 0.01$). ns = not statistically significant differences ($p > 0.05$).

Determination of allele-specific expression in single blastocysts

Individual blastocyst stage embryos were placed in 100 µl Dynal Lysis Buffer, and a solid-phase cDNA library was generated as described (Mann et al., 2003). The *H19* and *Snurf/Snrpn* expression assays were conducted on second-strand product using the LightCycler Real Time PCR System. The *H19* and *Snurf/Snrpn* assays was performed essentially as described (Mann et al., 2003) except for the *Snurf/Snrpn* assay where a final concentration of 10% DMSO was used, amplification was performed for 48 cycles and the melting curve analysis was performed as follows. A final denaturation step was conducted at 95°C for 1 minute, followed by a single annealing step at 40°C for 1 minute and a melting curve analysis with fluorescence acquisition occurring continuously as the temperature was increased from 40°C to 85°C in 0.2° C increments. Parental allele-specific expression patterns for all genes were calculated as the percent expression of the 129/Sv or CAST allele relative to the total expression of both alleles.

For PCR analysis of *Peg3*, 2× reverse primer and [^{32}P]dCTP (1 mCi) were added to a Ready-To-Go PCR Bead. Ten microliters of 2× reverse primer-PCR reaction mix was added to the second-strand product that contained 2× forward primer, resulting in a final concentration of 0.3 mM for each primer. *Peg3* primers, Pg11: 5'-aaggctctggttgacagtcgtg-3' and Pg12: 5'-ttctccttggtctcacgggc-3', were used to amplify a 224 bp fragment (94°C for 2 minutes followed by 34 cycles at 94°C for 15 seconds, 52°C for 10 seconds and 72°C for 20 seconds). This amplicon contains a polymorphism between 129/Sv (A) and CAST (G) at position 3451 (AF038939). Restriction digestion with *Taq*I resulted in 148 bp and 76 bp fragments in CAST while the 129/Sv amplicon was uncleaved. Products were resolved on a 7% polyacrylamide gel. The relative band intensities were quantitated after a 16 hour exposure using ImageQuant (Molecular Dynamics). Differences in the *Peg3* expression levels between DNMT1o-deficient and control blastocysts were evaluated by the student *t*-test.

Determination of allele-specific expression in post-implantation embryos and placentae

RNA was isolated from the other sagital half of the same E9.5 embryos and placentae and from whole E7.5 embryos using the HighPure RNA Tissue Kit (Roche Applied Science). cDNA was synthetized from the isolated RNA by using Superscript II Reverse Transcriptase and oligo-dT (Invitrogen). Allele-specific *H19* and *Snurf/Snrpn* expression were assessed on cDNA by fluorescent hybridization probe analysis using the LightCycler Real Time PCR system (Roche Applied Science) as described above without the addition of DMSO for the *Snurf/Snrpn* assay.

Derivation of DNMT1o-deficient ES Cell Lines

ES cell lines were generated from crosses between either wild-type or homozygous *Dnmt1*$^{\Delta 1o/\Delta 1o}$ 129/Sv female mice and 129/Sv wild-type male mice. Wild-type or DNMT1o-deficient E3.5 blastocysts from these crosses were collected from the uteri, and ES lines established using previously published methods (Nagy, 2003). Each line is representative of one blastocyst. Individual ES cell clones of both wild-type and DNMT1o-deficient lines were generated by plating ES cells at a very low density and allowing sufficient time for new colonies to form. Each ES cell clone is a single colony isolated from the original ES cell lines and expanded for isolation of DNA.

Southern blot analysis

For extraction of DNA from ES cells lines and clones, cells were plated in gelatinized plates without feeders. For *Snurf/Snrpn* methylation analysis, 5 μg DNA were digested with *Pst*I and the methylation sensitive enzyme *Hha*I, electrophoresed on 1% agarose gel and transferred to Genescreen nylon membrane (NEN, Boston MA). The membranes were hybridized with a [^{32}P]dCTP labeled probe to the 5'-end of the *Snurf/Snrpn* gene (GenBank accesion no. AF081460). For *H19* methylation analysis, 5 μg DNA were digested with *Msp*I and *Hha*I, electrophoresed on 1.5% agarose gels, transferred and hybridized with a [^{32}P]dCTP labeled probe to the 5' non-transcribed region of the *H19* gene between nucleotides 1,216 and 2,387 (GenBank accession no. U19619). For the analysis of repetitive sequences (IAP and LINE-1), 1 μg of DNA was used for *Msp*I or *Hpa*II digests, electrophoresed on 1% agarose gel and following transfer, hybridized with a [^{32}P]dCTP labeled probe to the LTR sequence of the agouti *A*^{iapy} allele for IAP (Michaud EJ, 1994), or a probe to LINE-1 repeats between nucleotides 6,477 and 7,092 (GenBank accession no. M29324).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from the NIH to J.R.C., J.T. and to M.B. and from CIHR to J.T. M.T. was a recipient of a studentship award from the Montreal Children's Hospital Research Institute of the McGill University Health Centre. J.T. is a James McGill Professor and a Scholar of the Fonds de la recherche en santé du Québec.

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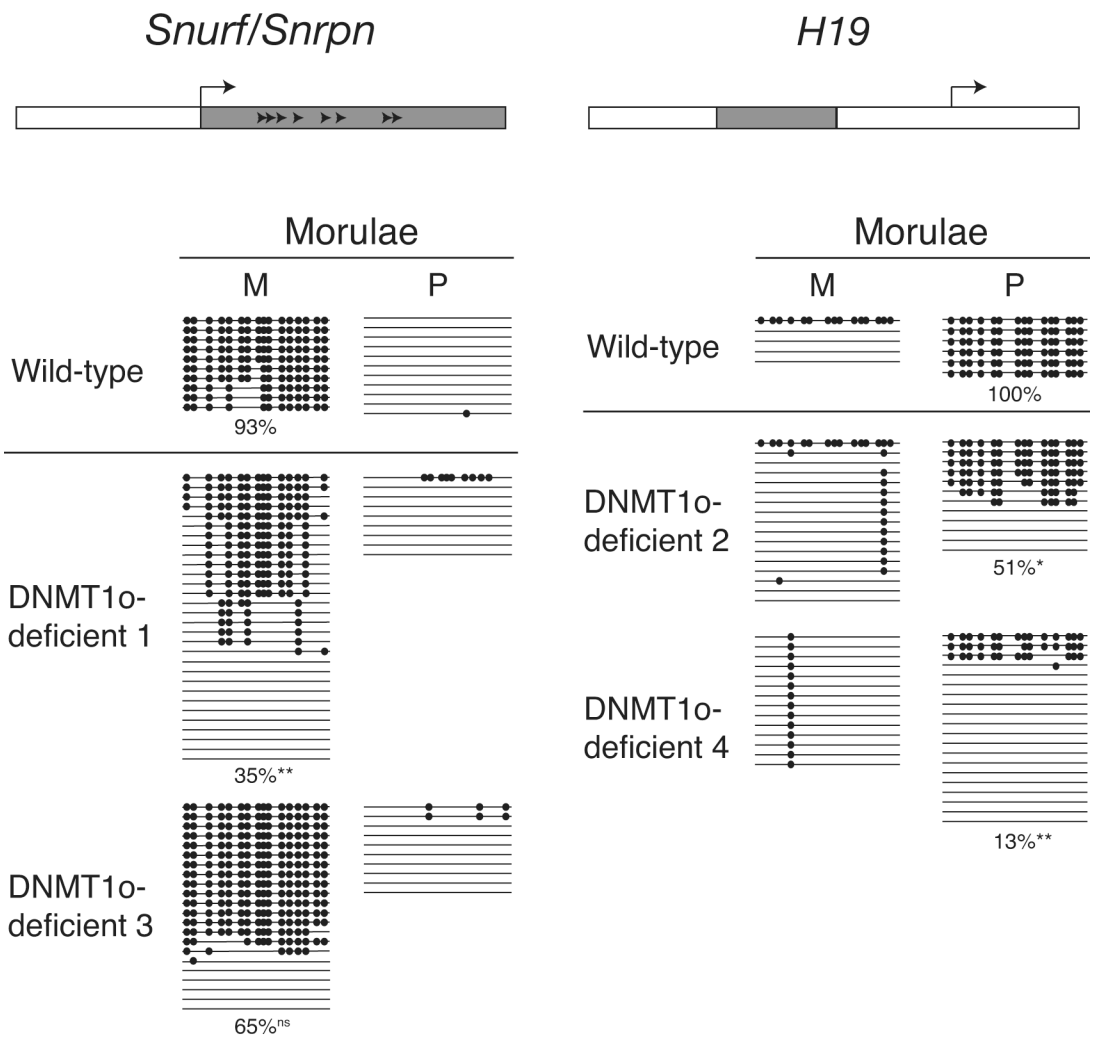

Fig. 1. DNMT1o is active in preimplantation development prior to the morula stage

Top: Schematics of the mouse *Snurf/Snrpn* and *H19* DMDs (grey boxes). The arrows represent the start of transcription and arrowheads indicate the tandem repeats within the *Snurf/Snrpn* DMD. Bottom: Patterns of DNA methylation on DMD sequences of the endogenous *Snurf/Snrpn* and *H19* genes were analyzed with the bisulfite genomic sequencing technique in pools of wild-type and DNMT1o-deficient morulae. One pool of wild-type and two pools of DNMT1o-deficient morulae were analyzed for each DMD. M = maternal allele; P = paternal allele. Each line represents one sequenced allele. Filled circles indicate the position of methylated CpG dinucleotides. The percentages of methylated CpGs are indicated. * denotes significantly different levels of CpGs methylation ($p < 0.05$). ** denotes highly significant differences ($p < 0.01$). ns = not statistically significant differences ($p > 0.05$).

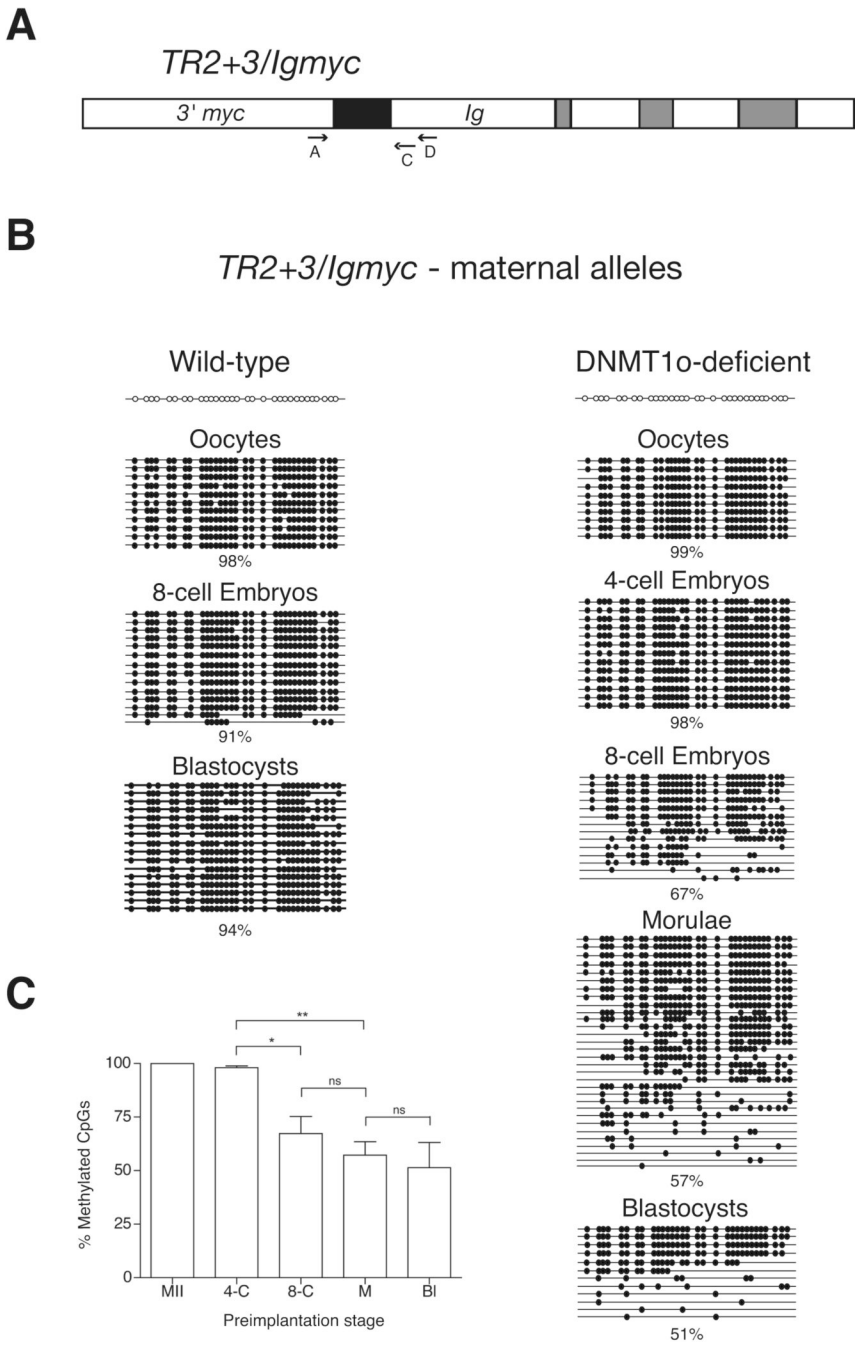

Fig. 2. Time of DNMT1o activity during preimplantation development

(A) Schematic of the *TR2+3Igmyc* transgene. The black box indicates *Igf2r* DMD sequences. The Cα and Sα regions of the mouse *IgA* locus (*Ig*) and the 3' UTR and the *c-myc* exons (grey boxes) are shown. The arrows indicate the three primers used for semi-nested PCR. **(B)** Analysis of DNA methylation of the maternal *TR2+3Igmyc* transgene in wild-type and DNMT1o-deficient oocytes and preimplantation embryos. The top line shows the position of the CpG dinucleotides analyzed (open circles). The filled circles represent the position of methylated CpG dinucleotides. **(C)** Summary of CpG methylation levels of the *TR2+3Igmyc* transgene on DNMT1o-deficient MII oocytes (MII) and preimplantation embryos. 4 = 4-cell embryos; 8 = 8-cell embryos; M = morulae; Bl = blastocysts. The percentages of methylated

CpGs are indicated. Error bars represent standard error of the mean (SEM). * denotes significantly different levels of CpGs methylation ($p < 0.05$). ** denotes highly significant differences ($p < 0.01$). ns = not statistically significant differences ($p > 0.05$).

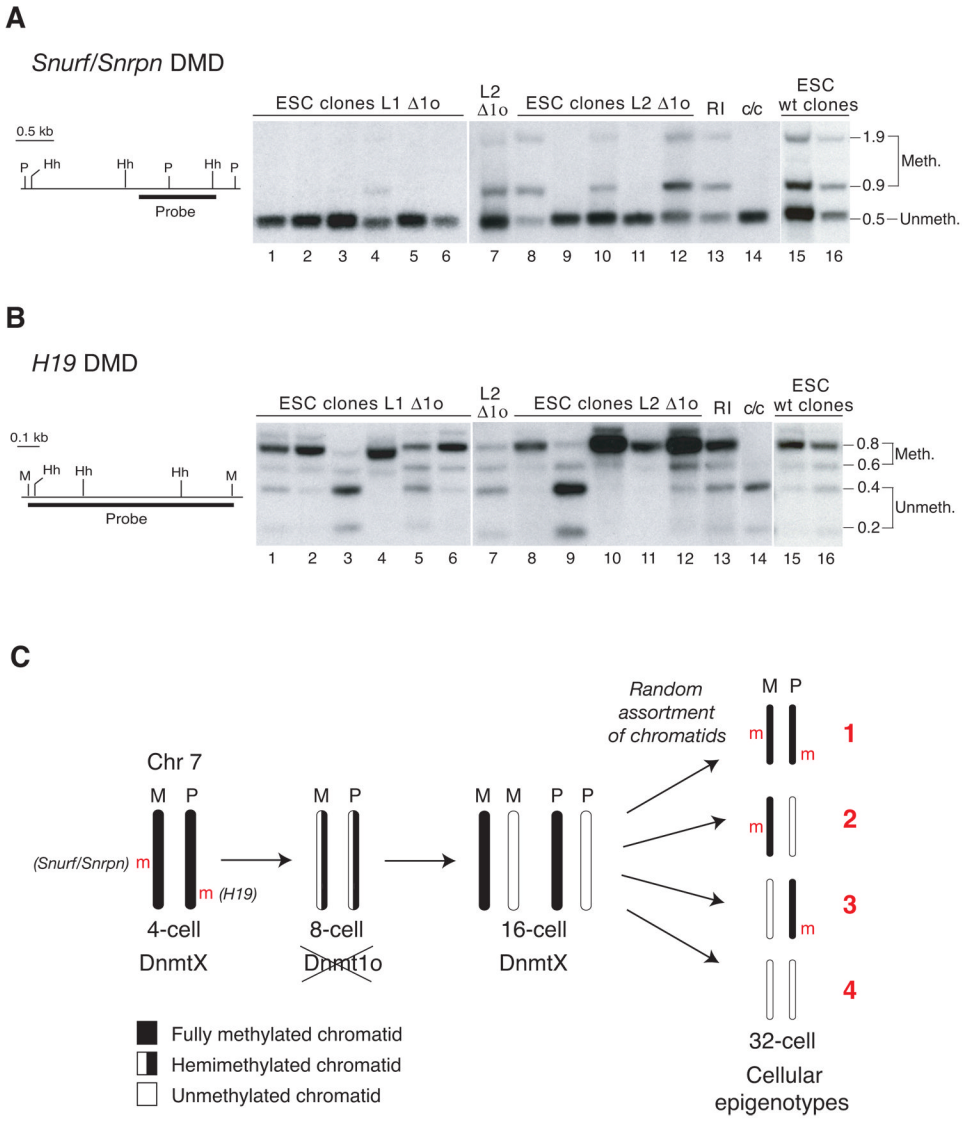

Fig. 3. DMD methylation in DNMT1o-deficient ES Cells

(A) Methylation analysis of the *Snurf/Snrpn* DMD on ES cells (ESC) derived from DNMT1o-deficient (Δ1o) blastocysts. Left: A restriction map of the 5'-end of *Snrpn* gene is shown. Hybridization probe is indicated by the dark bar. Right: DNAs were digested with *Pst*I (P) and the methylation sensitive *Hha*I (Hh) restriction enzymes. Lanes 1 to 6 are ESC clones from DNMT1o-deficient line 1 (L1). Lane 7 is DNA from DNMT1o-deficient ESC line 2 (L2 Δ1o) and lanes 8 to 12 are ESC clones from DNMT1o-deficient line 2. RI = RI ES cells, c/c = *Dnmt1*$^{c/c}$ ES cells. Lanes 15 and 16 are ESC clones from a wild-type line. **(B)** Methylation analyses of the *H19* DMD on ES cells derivated from DNMT1o-deficient (Δ1o) blastocysts. Left: A restriction map of the 5' non-transcribed region of *H19* gene is shown. Hybridization probe is indicated by the dark bar. Right: DNAs were digested with *Msp*I (M) and *Hha*I (Hh) restriction enzymes. Lanes 1 to 16 same as in A. Molecular weights are expressed in kb and the expected methylated and unmethylated bands are shown. **(C)** Prediction of four chromosome 7 epigenotypes following loss of DNMT1o maintenance methylation at the fourth embryonic S phase; these first appear in cells of 32-cell embryos after random assortment of chromatids. Each pair of vertical lines (M = maternal, P = paternal) represents the two

homologues of chromosome 7. m indicates a parent-specific imprinted methylation mark on the normally methylated chromosome.

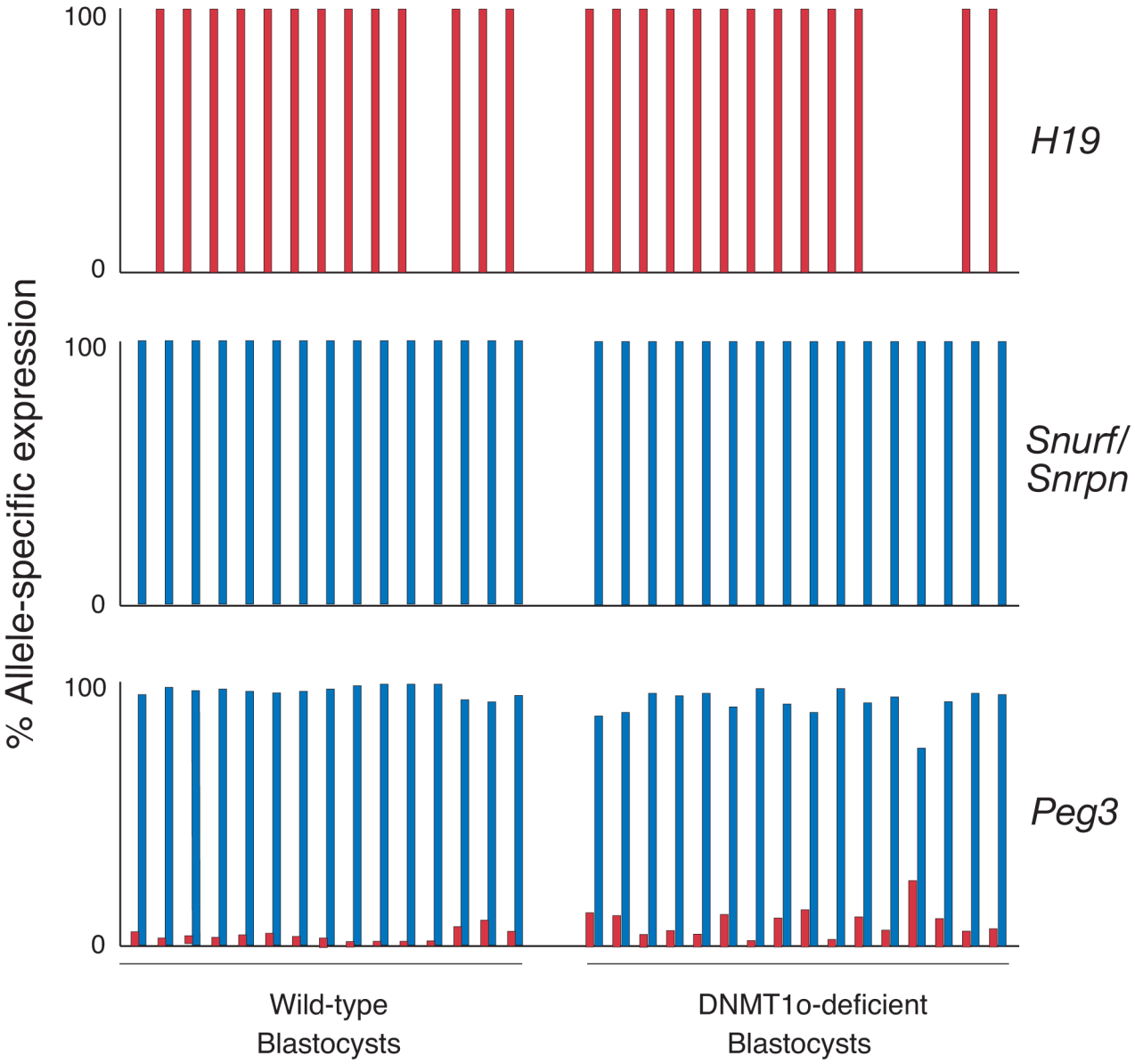

Fig. 4. Retention of imprinted gene expression in DNMT1o-deficient blastocysts

Allele-specific expression of *H19*, *Snurf/Snrpn* and *Peg3* imprinted genes were analyzed in individual wild-type and DNMT1o-deficient blastocysts using real time RT-PCR. Expression of maternal allele is shown in red and expression of paternal allele is shown in blue. Percentages of expression were determined as described in materials and methods.

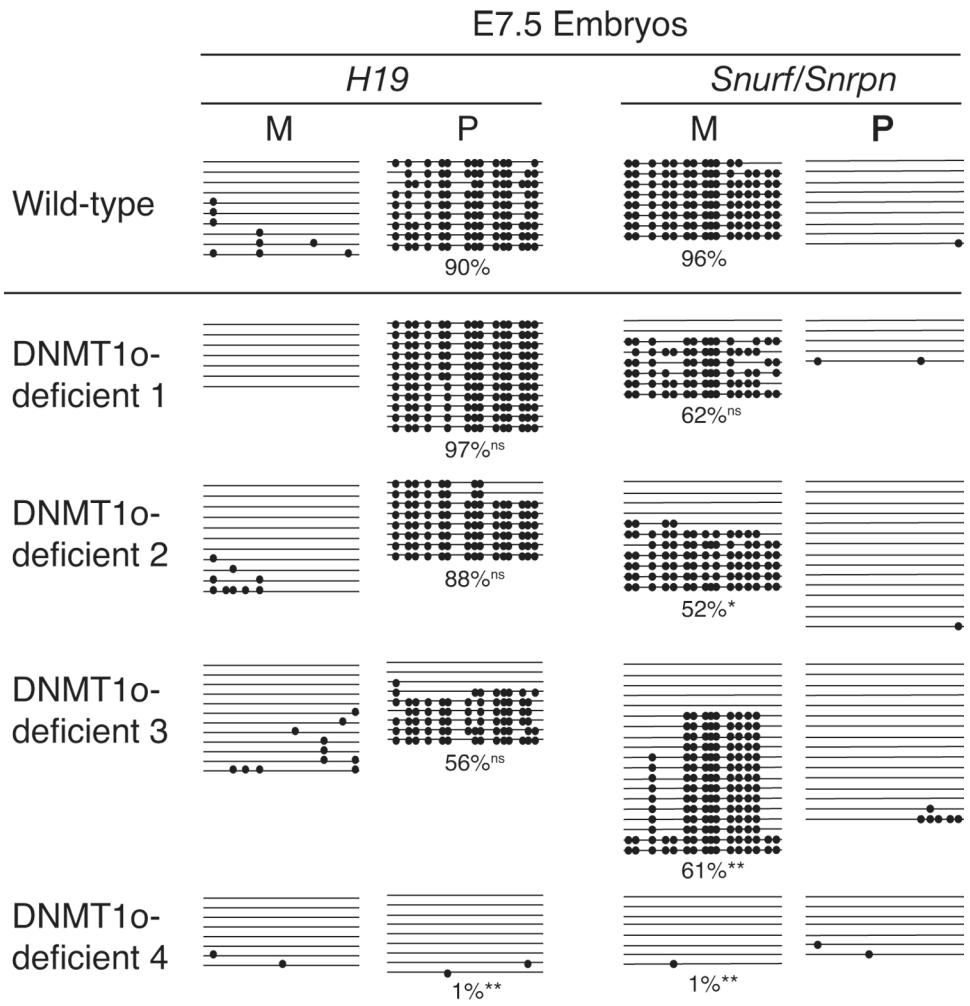

Fig. 5. Variable loss of DMD methylation of E7.5 DNMT1o-deficient embryos

Bisulfite genomic sequencing was used to establish allele-specific methylation of *H19* and *Snurf/Snrpn* DMDs in E7.5 embryos. A representative wild-type and 4 different DNMT1o-deficient embryos are shown. M=maternal allele; P=paternal allele. Position of methylated CpG dinucleotides are indicated by the filled circles. The percentages of methylated CpGs are indicated. * denotes significantly different levels of CpGs methylation ($p < 0.05$). ** denotes highly significant differences ($p < 0.01$). ns = not statistically significant differences ($p > 0.05$).

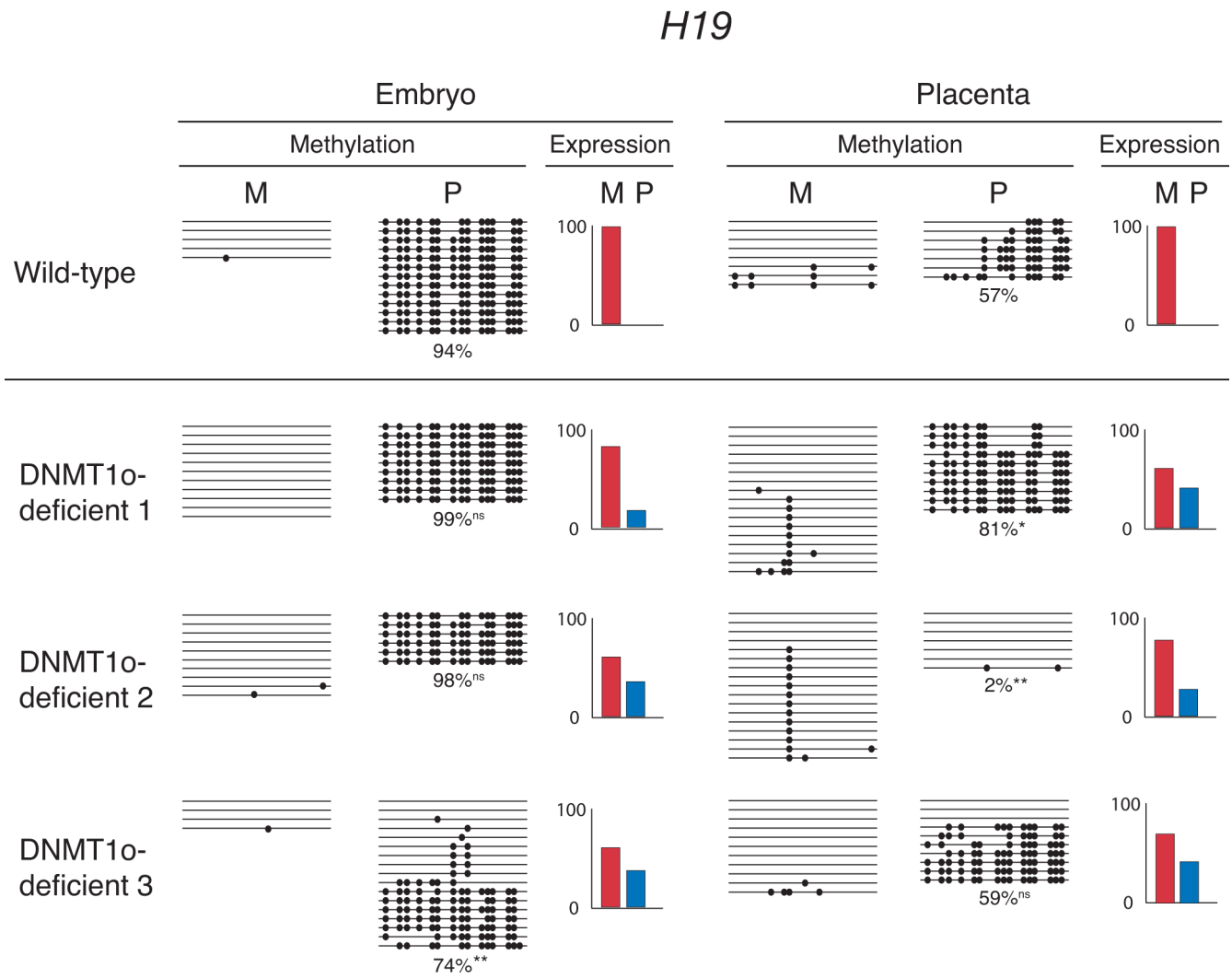

Fig. 6. Allele-specific *H19* methylation and expression in E9.5 DNMT1o-deficient embryos and placentae

Methylation patterns of a representative wild-type and three DNMT1o-deficient embryos were determined by the method of bisulfite genomic sequencing. Allele-specific expression for *H19* gene was analyzed using RT-PCR followed by restriction endonuclease digestion to distinguish parental alleles. M = maternal allele; P = paternal allele. Position of methylated CpG dinucleotides are indicated as filled circles. The percentages of methylated CpGs are indicated. * denotes significantly different levels of CpGs methylation ($p < 0.05$). ** denotes highly significant differences ($p < 0.01$). ns = not statistically significant differences ($p > 0.05$).

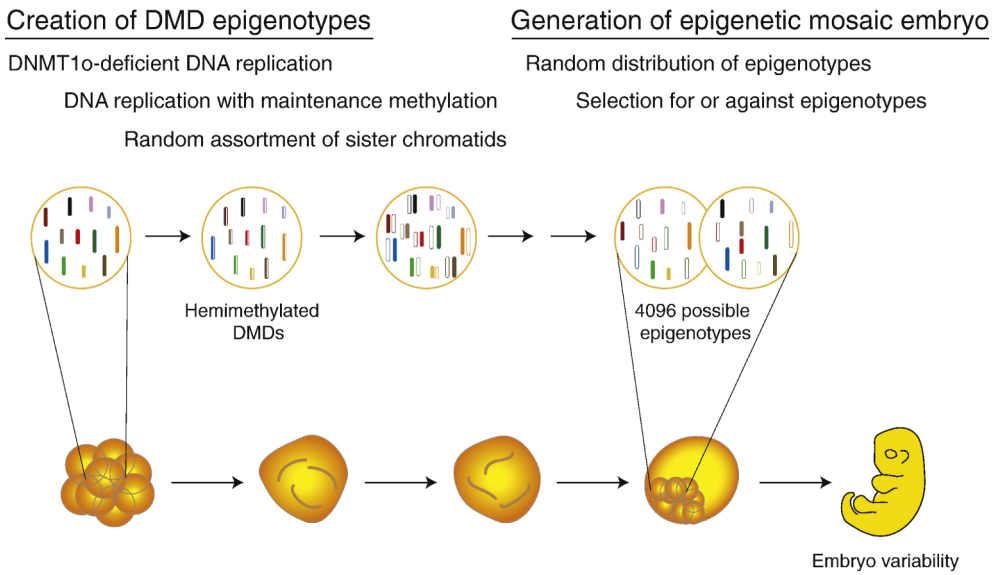

Fig. 7. Summary of molecular and cellular events leading to epigenetic mosaicism

The 12 autosomes known to contain imprinted genes are depicted as colored bars in 8-cell stage blastomeres (left). Methylated DNA single strands are chromatids indicated by filled areas and unmethylated DNA single strands and chromatids indicated by outlined areas. The fates of chromatids following replication, cell division and cell distribution within embryo are shown.