Role of De Novo DNA Methyltransferases in Initiation of Genomic Imprinting and X-Chromosome Inactivation

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DNA methylation is an epigenetic mechanism that plays a key role in regulation of developmental gene expression, maintenance of genomic integrity, genomic imprinting, and X-chromosome inactivation (X-inactivation) in mammals. Methylation of mammalian genomic DNA occurs almost exclusively at the cytosine of CpG dinucleotides. The CpG methylation pattern of the mammalian genome is created and maintained by a combination of de novo DNA methyltransferases, Dnmt3a and Dnmt3b, and a maintenance DNA methyltransferase Dnmt1. Targeted disruption of these DNA methyltransferase genes in mice results in embryonic or early postnatal lethality, indicating that they are essential for normal mammalian development (Li et al. 1992; Okano et al. 1999).

Genomic imprinting and X-inactivation are the well-characterized, major epigenetic phenomena of mammals that regulate allelic expression of autosomal genes and X-linked genes, respectively (Lyon 1961; Reik and Walter 2001). Both phenomena are known to be crucial for normal mammalian development. Imprinting is initiated during male and female gametogenesis, marking a subset of autosomal genes (up to a few hundred) in a sex-specific way (paternal and maternal imprinting). The imprinted genes show either paternal-specific or maternal-specific monoallelic expression in the offspring (Reik and Walter 2001). Thus imprinting is dependent on the sex of the parent from which the gene is derived, but not on the sex of the individual that carries the gene. By contrast, X-inactivation is a dosage compensation mechanism found only in females, which equalizes the X-linked gene dosage between males (with one X and one Y chromosome) and females (with two X chromosomes) (Lyon 1961). In the embryo proper (the epiblast lineage), X-inactivation is initiated during early development, leading to random inactivation of either the paternal or the maternal X chromosome. However, in the extraembryonic lineages (trophoblast and primitive endoderm derivatives) of mice, preferential inactivation of the paternal X chromosome occurs (Takagi and Sasaki 1975). Thus X-inactivation can be subject to genomic imprinting (imprinted X-inactivation). Like the imprinting of autosomes, the imprinting of X chromosome is thought to occur in the parental germ line.

Previous studies with the mouse embryos and ES cells deficient for Dnmt1 showed that DNA methylation plays an essential role in the maintenance of genomic imprinting and X-inactivation in the embryo proper (Table 1) (Li et al. 1993; Beurd et al. 1995; Panning and Jaenisch, 1996, Sado et al. 2000). By contrast, in the trophoblast, the role of DNA methylation seems more relaxed (Table 1) (Carper et al. 1998; Tanaka et al. 1999, Sado et al. 2000). However, whether DNA methylation is involved in their initiation has not been addressed. If DNA methylation were to play a role in the initiation step, Dnmt3a or Dnmt3b (or both) should be the key players because these are the enzymes that establish new genomic methylation patterns (Okano et al. 1999). We therefore asked whether Dnmt3a or Dnmt3b is involved in the initiation of autosome imprinting and X-inactivation using the cells and embryos deficient for these genes. The Cre-loxP conditional gene knockout system was particularly useful because of the early lethality of conventional Dnmt1-/- knockout mice (Okano et al. 1999). In this article, we summarize the results obtained from these experiments and discuss the role of de novo DNA methylation in the initiation of the two epigenetic phenomena.

ROLE FOR DNA METHYLATION IN IMPRINTING OF AUTOSOMAL GENES IN THE PARENTAL GERM LINE

Several lines of evidence suggest a role for DNA methylation in the initiation of autosomal imprinting. Mice deficient for Dnmt3L, a protein sharing homology with Dnmt3a and Dnmt3b but lacking methyltransferase activity, showed a failure in establishment of X-chromosomal imprinting (Bourc’his et al. 2001; Hata et al. 2002). Offspring from such females showed loss of monoallelic expression of the maternally imprinted genes and died around embryonic day 10.5 (E10.5). Since Dnmt3L protein can interact with Dnmt3a and Dnmt3b enzymes in transfected cells (Hata et al. 2002), it has been
male mutants). For details, see Kaneda et al. (2004).

Both male and female mutants) and offspring (derived from the female males lacking methylation at the normally methylated DMRs by bisulfite sequencing. We found that the spermatogonia from the Dnmt3a conditional mutant males contained only few spermatogonia and no spermatocytes, p57kip2 and p57kip2 were silenced, consistent with a loss of expression of p57kip2 (Calles) and p57kip2 are silenced, consistent with a loss of expression of the normally active maternal alleles (Kaneda et al. 2004). Thus, the Dnmt3a conditional mutant females fail to establish the oocyte-specific imprints at the maternally imprinted loci.

The [Dnmt3a2lox/1lox, TNAP-Cre] males showed impaired spermatogenesis (Kaneda et al. 2004). Histological examinations showed that the testes from the mutant males contain a slightly reduced number of spermatogonia and no spermatocytes, spermatids, or spermatocytes (Kaneda et al. 2004). Because of the azoospermia, we could not obtain offspring to examine. We therefore used the laser-microdissection technology to collect spermatogonia from histological sections of the P11 testes and analyzed three paternally methylated DMRs of the imprinted genes in the E10.5 embryos. The DMRs normally methylated on the maternal allele, such as those of Stum, Igf2r, and Peg1, were found to be unmethylated (Kaneda et al. 2004). By contrast, the methylation status of the paternally methylated H19 and Rasgev1 DMRs was unaffected. We then examined the expression of the maternally imprinted genes in the same embryos and found that p57kip2 (Calles) and p57kip2 are silenced, consistent with a loss of expression of the normally active maternal alleles (Kaneda et al. 2004). We also found that expression of Peg1, Stum, and Peg1 is increased, with a derepression of the normally silent maternal alleles (Kaneda et al. 2004). Thus, the Dnmt3a conditional mutant females fail to establish the oocyte-specific imprints at the maternally imprinted loci.

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We wanted to investigate the role of Dnmt3a and/or Dnmt3b in the initiation step of imprinting in more detail by disrupting the genes in male and female germ cells. A big problem was that the conventional Dnmt3b knockout mice are embryonic lethal and the conventional Dnmt3a knockout mice die around 3–4 weeks of age, before reaching the reproductive stage (Okano et al. 1999). We therefore could not examine the gametes of these mice or the offspring derived from them. To overcome this problem, we took advantage of the Cre-loxP conditional knockout technology and disrupted the Dnmt3a genes in a germ-cell-specific way, leaving the genes intact in most somatic cells (Fig. 1). We used the tissue nonspecific alkaline phosphatase (TNAP)-Cre knockin mice, which express the Cre recombinase in germ cells from E9.5 to late gestation (Lomeli et al. 2000). Although expression of TNAP-Cre was not strictly germ-cell-specific, we could derive conditional knockout mice ([Dnmt3a2lox/1lox, TNAP-Cre] and [Dnmt3b2lox/1lox, TNAP-Cre], where 2lox represents the functional allele and 1lox represents the nonfunctional allele) that can survive to adulthood (Fig. 1) (Kaneda et al. 2004).

When the [Dnmt3a2lox/1lox, TNAP-Cre] females were crossed with wild-type males, no live pups were obtained. Subsequent studies revealed that all embryos died around E10.5 with various developmental defects, such as open neural tube, lack of branchial arches, and impediment of blood circulation (Kaneda et al. 2004). We examined the methylation status of the differentially methylated regions (DMRs) of the imprinted genes in the E10.5 embryos. The DMRs normally methylated on the maternal allele, such as those of Stum, Igf2r, and Peg1, were found to be unmethylated (Kaneda et al. 2004). By contrast, the methylation status of the paternally methylated H19 and Rasgev1 DMRs was unaffected. We then examined the expression of the maternally imprinted genes in the same embryos and found that p57kip2 (Calles) and p57kip2 are silenced, consistent with a loss of expression of the normally active maternal alleles (Kaneda et al. 2004). We also found that expression of Peg1, Stum, and Peg1 is increased, with a derepression of the normally silent maternal alleles (Kaneda et al. 2004). Thus, the Dnmt3a conditional mutant females fail to establish the oocyte-specific imprints at the maternally imprinted loci.

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is required for the initiation of imprinting in both paternal and maternal germ lines.

We also generated [Dnmt3a\textsuperscript{lox/1lox}, TNAP-Cre\textsuperscript{\textminus}] mice, which were found to be phenotypically normal. When the [Dnmt3a\textsuperscript{lox/1lox}, TNAP-Cre\textsuperscript{\textminus}] males and females were crossed with wild-type partners, healthy pups were obtained (Kaneda et al. 2004). We analyzed the DMRs for the allelic methylation difference in these pups but all was found to be normal (Kaneda et al. 2004). Thus, so far there is no evidence that Dnmt3b is involved in the initiation of imprinting in the parental germ line.

**ROLE FOR DNA METHYLATION IN IMPRINTING OF Mash2 (Ass2) IN THE MATERNAL GERM LINE**

The mouse Mash2 (Ass2) gene encodes a transcription factor of the basic helix-loop-helix class that is essential for extraembryonic development (Guillemot et al. 1994). Mash2 is located within a large imprinted cluster on mouse distal chromosome 7 and is exclusively expressed from the maternal allele (Guillemot et al. 1995). However, the imprinting of this gene is unique in that its maintenance is highly resistant to hypomethylation, as shown by the analysis of the Dnmt1-deficient embryos (Caspery et al. 1998; Tanaka et al. 1999). It is therefore interesting to ask whether the imprinting of this gene is initiated normally in the absence of Dnmt1.

- **Figure 2.** A maternally imprinted gene Mash2 is silenced in the trophoblast of embryos derived from [Dnmt3a\textsuperscript{lox/1lox}, TNAP-Cre\textsuperscript{\textminus}] mothers. Expression of Mash2, as well as p57\textsuperscript{kip2}, was examined by RT-PCR in the embryo proper (em) and trophoblast (tb) of two conceptuses (1, 2). The data suggest that the initiation of Mash2 imprinting in the maternal germ line requires DNA methylation. Gapd is a nonimprinted housekeeping control.

- **Table 2.** Summary of results showing the role of Dnmt3a and Dnmt3b in the initiation of imprinting of Mash2.

- **Role for DNA Methylation in Initiation of Random X-Chromosome Inactivation**

  We next asked whether the initiation of random X-inactivation in the embryo proper (epiblast lineages) and ES cells requires de novo DNA methyltransferases. In the initiation step of X-inactivation, a noncoding RNA, X-inactive specific transcript (Xist), is upregulated on the future inactive X chromosome. The Xist RNAs then coat the entire chromosome and presumably recruit factors required for heterochromatin formation (Brockdorff 2002). By contrast, Xist is stably silenced on the active X chromosome. Although the maintenance of the silenced state of Xist requires Dnmt3a, monoallelic Xist expression and subsequent X-inactivation can occur normally in ES cells deficient for Dnmt3b (Beard et al. 1995; Panning and Jaenisch 1996). We therefore examined whether X-inactivation can occur normally in the epiblast lineages of [Dnmt3a\textsuperscript{\textminus}, Dnmt3b\textsuperscript{\textminus}] embryos and in differentiating [Dnmt3a\textsuperscript{\textminus}, Dnmt3b\textsuperscript{\textminus}] ES cells (Sado et al. 2004).

  We found by RNA fluorescence in situ hybridization (FISH) that monoallelic Xist expression is appropriately initiated in most cells of the [Dnmt3a\textsuperscript{\textminus}, Dnmt3b\textsuperscript{\textminus}] female embryos at E9.5 (Sado et al. 2004). Furthermore, a cytological analysis showed that one of the two X chromosomes unexpressively replicates late in S phase. In addition, one X chromosome was hypomethylated at histone H4 in the mutant female embryos as in wild-type female embryos, as revealed by immunostaining. These results indicate that random X-inactivation occurred appropriately in the absence of the de novo methyltransferases (Sado et al. 2004). The Xist promoter was extensively hypomethylated in these embryos.

  In the above experiments, however, we observed ectopic accumulation of Xist RNA (on the single X chromosome in males and on both X chromosomes in females) in a subset of cells (typically 4–5%) (Sado et al. 2004). Do these ectopic signals arise from inappropriate activation of Xist at the onset of X-inactivation or from derepression of a once silenced Xist locus? To address this question, we examined Xist RNA accumulation in the course of differentiation of [Dnmt3a\textsuperscript{\textminus}, Dnmt3b\textsuperscript{\textminus}] male ES cells (Sado et al. 2004). Although the single X chromosome was never coated with Xist RNA in undifferentiated state, ectopic Xist accumulation was detected in 3.2% and 16.8% of cells at day 2 and day 5 of differentiation, respectively. At day 12 of differentiation, a surprisingly high percentage (68%) of cells from [Dnmt3a\textsuperscript{\textminus}, Dnmt3b\textsuperscript{\textminus}] embryoid bodies showed ectopic accumulation, suggesting a progressive derepression of the unmethylated Xist locus. These observations establish that de novo DNA methylation is not required for the initial silencing of Xist but is necessary for stabilizing the silenced state of Xist (Sado et al. 2004).
traembryonic lineages (Takagi and Sasaki 1975). This seems to be due to an imprint on the maternal X chromosome (Xm) to remain active, as well as an imprint on the paternal X chromosome (Xp) to inactivate. The paternal imprint on the Xp can be reversed. Xp0 mice are developmentally retarded but viable and fertile (which indicates that the Xp is active) (Thornhill and Burgoyne 1993) and androgenetic embryos with two Xp chromosomes can undergo random X-inactivation in the extraembryonic tissues (Okamoto et al. 2000). The maternal imprint on the Xm may be more rigid. Genetic experiments using Robertsonian translocations showed that, in embryos carrying two Xm chromosomes, both remain active in the extraembryonic tissues and that such embryos die early because of poor development of the extraembryonic tissues (Goto and Takagi 1998). Furthermore, nuclear transplantation experiments showed that the maternal imprint is set on the Xm during oocyte growth (Tada et al. 2000), just as the maternal imprints on autosomes. Recently, it was found that imprinted X-inactivation is observed from the two-cell or four-cell stage (Huynh and Lee 2003; Okamoto et al. 2004) and it is proposed that the preinactivated state of the Xp is carried over from the parental germ line (Huynh and Lee 2003).

We wanted to ask whether the de novo DNA methyltransferases play a role in imprinting X chromosome in the male and female germ lines. Since disruption of Dnmt1 in the male germ line results in azoospermia (Kaneda et al. 2004), we could ask only whether its disruption in the female germ line has an effect on imprinted X-inactivation. As described above, embryos conceived by the [Dnmt3a2lox/1lox, TNAP-Cre] females survived until E10.5 (Kaneda et al. 2004), indicating that the X-linked gene dosage was appropriately controlled, or nearly so, during early development. We recently obtained several embryos from [Dnmt3a2lox/1lox, Dnmt3b1lox/1lox, TNAP-Cre] females and found that their phenotype is almost identical with that of the above embryos. Two X-inactivation patterns are envisaged in these embryos. One is the normal imprinted X-inactivation, which suggests that the loss of the de novo DNA methyltransferases in the female germ line had no effect on the Xm. The other is a random X-inactivation, which suggests that the Xm had lost the imprint and then the counting and choice mechanisms were switched on. The previous findings that the Xm derived from nongrowing oocytes resembled the normal Xp (Tada et al. 2000) and the presence of two Xp chromosomes resulted in random X-inactivation (Okamoto et al. 2000) appear to support the latter idea.

To distinguish between the two possibilities, we made use of mice carrying an X-linked green fluorescent protein (GFP) transgene (XGFP) (Takagi et al. 2002). [Dnmt3a1lox/+TNAP-Cre] and [Dnmt1a1lox/+TNAP-Cre] females crossed with XGFPY males. Expression of GFP from the Xp is observed in the embryo proper (as a result of random X-inactivation) but not in the trophoblast (because of preferential Xp-inactivation) of both wild-type (top) and mutant (bottom) embryos ([Dnmt3a2lox/1lox, Dnmt3b1lox/1lox, XGFPY]). This suggests, although indirectly, that the Xm derived from the conditional mutant females has the imprint to be active in the trophoblast. em, embryo proper; ys, yolk sac; tb, trophoblast.

Based on the studies described here, we now begin to obtain a comprehensive view of the role of de novo DNA methyltransferases in the initiation of autosomal imprinting in both the paternal and maternal germ lines. Notably, we showed that a trophoblast-specific gene Mash2, which does not require Dnmt1 for the maintenance of its imprinted state, does require Dnmt3a for the initiation of imprinting in the maternal germ line. Second, we found that the initiation of random X-inactivation can occur normally in the absence of Dnmt1a and Dnmt3b. Third, we showed that a disruption of both Dnmt1a and Dnmt3b in the maternal germ line does not affect the imprinted X-inactivation.

CONCLUSIONS

Based on the studies described here, we now begin to obtain a comprehensive view of the role of de novo DNA methyltransferases in the initiation of autosomal imprinting in the trophoblast. First, we found that Dnmt1a is required for the initiation of autosomal imprinting in both the paternal and maternal germ lines. Notably, we showed that a trophoblast-specific gene Mash2, which does not require Dnmt1 for the maintenance of its imprinted state, does require Dnmt3a for the initiation of imprinting in the maternal germ line. Second, we found that the initiation of random X-inactivation can occur normally in the absence of Dnmt1a and Dnmt3b. Third, we showed that a disruption of both Dnmt1a and Dnmt3b in the maternal germ line does not affect the imprinted X-inactivation in the trophoblast. This seems to indicate that the Xm derived from the mutant females has the imprint to remain active. The role of Dnmt3b is more malleable. This suggests that the Xm derived from the mutant females has the imprint to remain active. The role of Dnmt1a and Dnmt3b in the imprinting of Xp, however, remains an open question. These findings are summarized in Table 1, in terms of necessity for DNA methylation, together with the previous findings on the maintenance of these phenomena. Autosomal imprinting and X-inactivation share a number of molecular features. These include cis-acting con-

Figure 3. Lack of expression of a GFP transgene in the trophoblast of E8.5 embryos derived from [Dnmt3a2lox/1lox, Dnmt3b1lox/1lox, TNAP-Cre] females crossed with XGFPY males. Expression of GFP from the Xp is observed in the embryo proper (as a result of random X-inactivation) but not in the trophoblast (because of preferential Xp-inactivation) of both wild-type (top) and mutant (bottom) embryos ([Dnmt3a2lox/1lox, Dnmt3b1lox/1lox, XGFPY]). This suggests, although indirectly, that the Xm derived from the conditional mutant females has the imprint to be active in the trophoblast. em, embryo proper; ys, yolk sac; tb, trophoblast.
trol centers, long-distance regulation, association with noncoding and antisense RNAs, involvement of histone modifications and chromatin-associated factors, and differential DNA methylation (Lee 2003). These features lead to the proposal that X-inactivation and autosomal imprinting may have a common origin (Lee 2003). Indeed, marsupials such as kangaroos show paternal-specific imprinting (Sharman 1971), just as the trophoblast of mice, and X-inactivation of this type is thought to be the early form of dosage compensation. Since de novo DNA methylation is required for the initiation of autosomal imprinting, it will be important to establish whether the same mechanism is involved in the initiation of imprinted X-inactivation. By contrast, we clearly showed that the initiation of random X-inactivation, which may be the more recent form of dosage compensation, does not require de novo DNA methylation. This indicates that, even in X-inactivation and autosomal imprinting have a common origin, a very different molecular mechanism evolved afterward to achieve random X-inactivation in the eplhab lineages.

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