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 lineages), 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; Beard 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) (Caspery 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* and/or *Dnmt3b* is involved in the initiation of autosomal 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 *Dnmt3a* or *Dnmt3b* 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 oocyte-specific methylation imprints (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

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Table 1. DNA Methylation in Autosomal Imprinting and X-Chromosome Inactivation

Phenomenon	Step	Tissue	DNA methylation required?
Autosomal imprinting	Initiation	Germ line	<u>Yes</u>
	Maintenance	Embryo proper	<u>Yes</u>
	Maintenance	Extraembryonic	<u>Yes/No^a</u>
Random X-inactivation	Initiation	Epiblast	<u>No</u>
	Maintenance	Embryo proper	<u>Yes</u>
Imprinted X-inactivation	Initiation	Germ line	<u>No?/?^b</u>
	Maintenance	Extraembryonic	<u>No</u>

The findings described in this paper are underlined.
^aDNA methylation is required for the maintenance of imprinting of some genes such as *p57^{kip2}* but not for the other genes such as *Mash2*.
^bDNA methylation is unlikely to be involved in the imprinting of Xm but its role in the imprinting of Xp has not been tested.

proposed that *Dnmt3L* may regulate maternal imprinting via the de novo methyltransferases. Consistent with this model, a [*Dnmt3a*^{-/-}, *Dnmt3b*^{+/-}] ovary transplanted into a wild-type female failed to establish the oocyte-specific (maternal) methylation imprints (Hata et al. 2002).

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 *Dnmt3* 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 ([*Dnmt3a*^{2lox/1lox}, TNAP-Cre] and [*Dnmt3b*^{2lox/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 [*Dnmt3a*^{2lox/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 *Snrpn*, *Igf2r*, and *Peg1*, were found to be unmethylated (Kaneda et al. 2004). By contrast, the methylation status of the paternally methylated *H19* and *Rasgrf1* DMRs was unaffected. We then examined the expression of the maternally imprinted genes in the same embryos and found that *p57^{kip2}* (*Cdkn1c*) and *Igf2r* are silenced, consistent with a loss of expression from the normally active maternal alleles (Kaneda et al. 2004). We also found that expression of *Peg1*, *Snrpn*, and *Peg3* 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.

The [*Dnmt3a*^{2lox/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 in the seminiferous tubules at postnatal day 11 (P11) (Kaneda et al. 2004). However, at 11 weeks of age, they contained only few spermatogonia and no spermatocytes, spermatids, or spermatozoa (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 by bisulfite sequencing. We found that the spermatogonia from the *Dnmt3a* conditional mutant males lacked methylation at the normally methylated *H19* DMR and *Dlk1-Gtl2* intergenic DMR (Kaneda et al. 2004). The normally methylated DMR of *Rasgrf1* was slightly less methylated than in wild-type spermatogonia (Kaneda et al. 2004). These results indicate that *Dnmt3a*

Figure 1. Conditional knockout of the de novo DNA methyltransferase genes *Dnmt3a* and *Dnmt3b* in mouse germ cells. The conditional mice with two loxP sites were produced by gene targeting and crossed with TNAP-Cre mice, which express the Cre recombinase in germ cells. The resulting conditional knockout mice survived to adulthood, and thus we could examine their germ cells (in both male and female mutants) and offspring (derived from the female mutants). For details, see Kaneda et al. (2004).

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

We also generated [*Dnmt3b*^{2lox/1lox}, TNAP-Cre] mice, which were found to be phenotypically normal. When the [*Dnmt3b*^{2lox/1lox}, TNAP-Cre] 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* (*Ascl2*) IN THE MATERNAL GERM LINE

The mouse *Mash2* (*Ascl2*) 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 germ line of [*Dnmt3a*^{2lox/1lox}, TNAP-Cre] females. (It is known that *Mash2* is a maternally imprinted gene.) Expression of *Mash2* and *p57^{kip2}* (*Cdkn1c*), as well as *Gapd* (a nonimprinted control), was examined in the trophoblast of E9.5 embryos obtained from the [*Dnmt3a*^{2lox/1lox}, TNAP-Cre] females crossed with wild-type males. The results showed that both *Mash2* and *p57^{kip2}* are silenced in the trophoblast whereas *Gapd* expression is unaffected (Fig. 2), indicating that the initiation of *Mash2* imprinting in the maternal germ line does require *Dnmt3a*. This finding is consistent with the fact that the imprinting of *Mash2* is controlled by an imprinting center located in an intron of *Kvlqt1* (*Kcnq1*) (Fitzpatrick et al. 2002), which is methylated in oocytes but not in sperm.

Figure 2. A maternally imprinted gene *Mash2* is silenced in the trophoblast of embryos derived from [*Dnmt3a*^{2lox/1lox}, TNAP-Cre] mothers. Expression of *Mash2*, as well as *p57^{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. wt, wild-type.

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 the 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 *Dnmt1*, monoallelic *Xist* expression and subsequent X-inactivation can occur normally in ES cells deficient for *Dnmt1* (Beard et al. 1995; Panning and Jaenisch 1996). We therefore examined whether X-inactivation can occur normally in the epiblast lineages of [*Dnmt3a*^{-/-}, *Dnmt3b*^{-/-}] embryos and in differentiating [*Dnmt3a*^{-/-}, *Dnmt3b*^{-/-}] 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*^{-/-}, *Dnmt3b*^{-/-}] female embryos at E9.5 (Sado et al. 2004). Furthermore, a cytological analysis showed that one of the two X chromosomes unanimously replicates late in S phase. In addition, one X chromosome was hypoacetylated 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*^{-/-}, *Dnmt3b*^{-/-}] 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*^{-/-}, *Dnmt3b*^{-/-}] 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).

ROLE FOR DNA METHYLATION IN INITIATION OF IMPRINTED X-CHROMOSOME INACTIVATION

In contrast to the random X-inactivation in the embryo proper, an imprinted X-inactivation occurs in the ex-

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: XpO 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 paternal germ line (Huynh and Lee 2003).

We wanted to ask whether the de novo DNA methyltransferases plays a role in imprinting X chromosome in the male and female germ lines. Since disruption of *Dnmt3a* 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 [*Dnmt3a*^{2lox/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 [*Dnmt3a*^{2lox/1lox}, *Dnmt3b*^{2lox/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 (X^{GFP}) (Takagi et al. 2002). [*Dnmt3a*^{2lox/1lox}, TNAP-Cre] and [*Dnmt3a*^{2lox/1lox}, *Dnmt3b*^{2lox/1lox}, TNAP-Cre] females were crossed with [X^{GFP}, Y] males, and embryos were recovered from the pregnant females at E8.5. In the embryo proper of female embryos derived from the mutant mothers, expression of the GFP transgene on the Xp was observed, just as in the embryo proper of control females, due to random X-inactivation (Fig. 3). By contrast, no GFP expression was ob-

Figure 3. Lack of expression of a GFP transgene in the trophoblast of E8.5 embryos derived from [*Dnmt3a*^{2lox/1lox}, *Dnmt3b*^{2lox/1lox}, TNAP-Cre] females crossed with X^{GFP}Y 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 ([*Dnmt3a*^{1lox/+}, *Dnmt3b*^{1lox/+}, X^{GFP}Y]). 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.

served from the Xp in the trophoblast of female embryos derived from the mutant mothers as well as those from the control mothers (Fig. 3). These results exclude the possibility of random X-inactivation in the trophoblast and suggest, although indirectly, that a loss of the de novo DNA methyltransferases in the female 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 and X-inactivation. First, we found that *Dnmt3a* 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 *Dnmt3a* and *Dnmt3b*. Third, we showed that a disruption of both *Dnmt3a* 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 *Dnmt3a* 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-

tol 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 imprinted X-inactivation (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 if X-inactivation and autosomal imprinting have a common origin, a very different molecular mechanism evolved afterward to achieve random X-inactivation in the epiblast lineages.

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