INTRODUCTION

Nuclear transfer experiments demonstrate that the maternal and paternal sets of genome are nonequivalent and both are required for normal development of the mouse embryo (McGrath and Solter, 1984; Surani et al., 1984; Barton et al., 1984). The differences between the paternal and maternal genome are inherited from the gametes in which parental imprinting of the genome (or genomic imprinting) is established. Genomic imprinting results in differential expression of the paternal and maternal alleles of a small set of genes known as imprinted genes. Among the 40 or so known imprinted genes, some, such as \( H19 \), \( Igf2r \), and \( p57^{kip2} \) are expressed when inherited from the mother while others such as \( Snrpn \), \( Peg1 \), and \( Peg3 \) are expressed when inherited from the father. The imprinted genes appear to function in a wide range of developmental processes such as regulation of embryonic development, placenta function, fetal growth, and maternal behaviors, and various theories have been proposed to explain the existence and function of genomic imprinting (Moore and Haig, 1991; Barlow, 1995; Jaenisch, 1997; Tilghman, 1999).

DNA methylation is believed to be the epigenetic mechanism that controls genomic imprinting in mammals. The presence of differentially methylated regions (DMRs) between paternal and maternal alleles in almost all imprinted genes provides a molecular basis for regulation of allele-specific expression of imprinted genes (Neumann and Barlow, 1996; Ferguson-Smith and Surani, 2001). DNA methylation is a reversible epigenetic process and it can be reprogrammed during embryogenesis and gametogenesis (Reik et al., 2001). Parental methylation imprints are erased in the primordial germ cells during embryogenesis and are re-established during gametogenesis in male and female germ cells independently. Most imprinted genes, such as \( Igf2r \), \( Peg1 \) and \( Snrpn \), acquire their methylation imprints in the female germ cells (Stöger et al., 1993; Lefebvre et al., 1997; Shemer et al., 1997), whereas two imprinted genes, \( H19 \) and \( Rasgrfl1 \), acquire their methylation imprints in the male germ cells (Tremblay et al., 1995; Pearsall et al., 1999). Obata et al. demonstrated, by transferring nuclei from immature oocytes to full-grown oocytes, that maternal genomic imprinting is established during oocyte growth (Obata et al., 1998). Studies of the paternally imprinted \( H19 \) gene, in contrast, showed that paternal imprinting is established in male germ cells over a much longer period of germ cell development (Ueda et al., 2000; Davis et al., 2000). However, it remains unknown which DNA methyltransferases are required for de novo methylation of imprinted genes in the germ cells, and whether the methylation imprints acquired in the germ cells are essential for the establishment of genomic imprinting.

SUMMARY

Genomic imprinting is regulated by differential methylation of the paternal and maternal genome. However, it remains unknown how parental imprinting is established during gametogenesis. In this study, we demonstrate that Dnmt3L, a protein sharing homology with DNA methyltransferases, Dnmt3a and Dnmt3b, but lacking enzymatic activity, is essential for the establishment of maternal methylation imprints and appropriate expression of maternally imprinted genes. We also show that Dnmt3L interacts with Dnmt3a and Dnmt3b and co-localizes with these enzymes in the nuclei of transfected cells, suggesting that Dnmt3L may regulate genomic imprinting via the Dnmt3 family enzymes. Consistent with this model, we show that \([Dnmt3a^{+/–}, Dnmt3b^{+/–}]\) mice also fail to establish maternal methylation imprints. In addition, both Dnmt3a and Dnmt3L are required for spermatogenesis. Together, our findings suggest that Dnmt3L may cooperate with Dnmt3 family methyltransferases to carry out de novo methylation of maternally imprinted genes in oocytes.

Key words: Dnmt3L, Dnmt3a, De novo methylation, Genomic imprinting, Spermatogenesis, Mouse
Dnmt1 can carry out de novo methylation in vitro, there is little evidence that it alone can initiate de novo methylation in vivo. Inactivation of Dnmt1 in mice leads to global loss of methylation and bi-allelic expression or silence of imprinted genes (Li et al., 1992; Li et al., 1993; Caspary et al., 1998). In contrast, Dnmt3a and Dnmt3b, but not Dnmt1, are essential for de novo methylation in embryonic stem cells and early postimplantation embryos (Okano et al., 1999) and Dnmt3a can carry out de novo methylation in transgenic flies (Lyko et al., 1999). This unique property of Dnmt3a and Dnmt3b predicts that these enzymes are probably also responsible for carrying out de novo methylation of imprinted genes in the germ cells.

Dnmt1 and Dnmt3 family methyltransferases do not appear to have any sequence-specificity beyond CpG dinucleotides, the prime targets for both classes of enzymes (Okano et al., 1998; Dodge et al., 2002). How these enzymes find their target sequences in the genes to be imprinted and methylate them differently in the male and female gametes remains an enigma. Other proteins might be necessary to target DNA methyltransferases to specific sequences in vivo or local chromatin conformation of a gene may determine the accessibility of its DNA by various DNA methyltransferases. While a number of proteins capable of interacting with Dnmt1, Dnmt3a or Dnmt3b, including PCNA, DMAP1, HDAC1, HDAC2, pRB and RP58, have been identified (Robertson and Wolffe, 2000; Fuks et al., 2001; Bachman et al., 2001), their function in modulating DNA methyltransferase activity, targeting DNA methyltransferases to specific sequences, or repressing transcription in vivo has not been determined.

In this study, we report that Dnmt3L, a gene that shares homology with Dnmt3 family methyltransferase genes, is required for the establishment of methylation imprints in oocytes. Dnmt3L, which by itself has no detectable DNA methyltransferase activity, appears to regulate methylation of imprinted genes through its interaction with DNA methyltransferases, Dnmt3a and Dnmt3b. Consistent with this hypothesis, we show that Dnmt3L binds and colocalizes with Dnmt3a and Dnmt3b in the nuclei of mammalian cells. Like Dnmt3L–/– mutants, [Dnmt3a+/+, Dnmt3b+/+] female mice also fail to establish maternal methylation imprints. Our results thus provide genetic evidence that Dnmt3 family methyltransferases and a potential cofactor Dnmt3L are required for de novo methylation of imprinted genes in the female gametes.

MATERIALS AND METHODS

Targeted disruption of the Dnmt3L gene in ES cells and mice

To construct Dnmt3L targeting vectors, a 9 kb SacII-EcoRI fragment containing exon 1 and a 2.8 kb BamHI fragment containing exons 8, 9 and part of exon 10 were inserted into pBluescript II SK. The IRES-βgeo cassette with the splicing accept site (Mountford et al., 1994) or a PGK-hygromycin cassette was inserted into an Xho site between exons 1 and 2 in the same transcriptional orientation as Dnmt3L (Fig. 2A). The targeting vectors were transfected into ES cells and drug-resistant clones were selected as described (Li et al., 1992). Positively recombined clones were identified by Southern blot analysis. DNA from ES cell clones was digested with NsiI, blotted and hybridized to an external probe. The wild-type, IRES-βgeo and hygromycin alleles give fragments of 6.8 kb, 17.4 kb and 12.6 kb, respectively (Fig. 2A).

Construction of myc- or GFP-tagged Dnmt3a, Dnmt3b and Dnmt3L

Dnmt3a, Dnmt3b and Dnmt3L were fused with EGFP or tagged with a myc-tag at their N termini. Dnmt3a, Dnmt3b and Dnmt3L coding regions were amplified by PCR with primers for Dnmt3a (5ʹ-aag-ccaggaccgccggggag-3ʹ and 5ʹ-cagacatggtcctccgcaatg-3ʹ), Dnmt3b (5ʹ-aatgacatcaacagcagtca-3ʹ and 5ʹ-cagacatggtcctccgcaatg-3ʹ), and Dnmt3L (5ʹ-tcgacatggtcctccgcaatg-3ʹ and 5ʹ-tgaatccagaggagggc-3ʹ). The amplified DNA fragments were subcloned into EcoRI and SacII sites of pEGFP-C1 (Clontech) or EcoRI and XbaI sites of myc-pCDNA3.1 (Chen and Richard, 1998).

Co-transfection of COS cells and immunoprecipitations

Myc- or GFP-tagged proteins were expressed in COS cells by transfection of cDNA expression vectors using LIPOFECTAMINE PLUS (Invitrogen). The COS cells were lysed in the lysis buffer (1% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl (pH 8.0), 50 mM NaF, 100 mM sodium vanadate, 0.01% phenylmethylsulfonyl fluoride, 1 μg aprotinin per ml and 1 μg leupeptin per ml), and the cellular debris and nuclei were removed by centrifugation. The cell lysates were incubated with anti-GFP antibody or anti-myc antibody (Roche) at 4°C for 1 hour, then 40 μl of 50% slurry of Protein A Sepharose CL-4B (Pharmacia Biotech) were added and incubated at 4°C for 1 hour. The beads were washed twice with lysis buffer and once with phosphate-buffered saline. The samples were analyzed by SDS-PAGE and transferred to PVDF membrane. The designated primary antibody (anti-GFP or anti-myc antibody) was used followed by goat anti-mouse IgG antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch).

Co-transfection of NIH3T3 cells and immunofluorescence microscopy

NIH 3T3 cells were plated 12 hours before transfection, typically at a density of 10⁵ cells/48 mm² glass coverslip. Cells were transfected with DNA constructs, including GFP vector, GFP-Dnmt3a, GFP-Dnmt3b, myc vector and myc-Dnmt3L, using LIPOFECTAMINE PLUS (Invitrogen). 12 hours after transfection, the cells were fixed with 4% paraformaldehyde in PBS for 15 minutes and permeabilized with 1% Triton X-100 in PBS for 5 minutes. The fixed cells were incubated with anti-myc antibody (Roche; 1:1000) at room temperature for 1 hour and subsequently with a rhodamine-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch; 1:300) for 30 minutes. The nuclei were stained with 3 μg of 4,6-diamidino-2-phenylindole (DAPI)/ml. The staining patterns of the cells were examined by fluorescence microscopy.

Ovary transplantation

Ovaries were removed from 2-week old Dnmt3a+/+ or Dnmt3a−/−; Dnmt3b+/− females and transplanted into 4-week old C57BL/6J × 129S1/SvlmJ F1 females (The Jackson Laboratory) as described previously (Hogan et al., 1994).

X-gal staining and histology

Testes and ovaries were removed and fixed with Bouin’s solution (Sigma) for Hematoxylin and Eosin staining or with 0.2% parafomaldehyde for staining with X-gal followed by counter-staining with Nuclear Fast Red as described previously (Hogan et al., 1994).

Northern blot analysis, whole-mount in situ hybridization

Total RNA prepared from ES cells, embryoid bodies, or E9.5 embryos was analyzed by northern hybridization. Full length Dnmt3L cDNA (AF220524) was used as a probe. cDNA probes for Pegl (a gift from A. Surani) and Snrnp (amplified by RT-PCR) were used for northern analysis. For whole-mount in situ hybridization, E7.5 and E8.5 embryos were dissected out and fixed in 4% paraformaldehyde in PBS with 0.1% Tween 20 at 4°C overnight. Antisense RNA probes were generated by transcription using digoxigenin UTP (Roche) with
full length Dnmt3L cDNA as a template. Whole-mount in situ hybridization was performed as described previously (Hogan et al., 1994).

**DNA methylation analysis**

E9.5 wild-type and mutant embryos were removed from pregnant mothers. Yolk sac DNA was used for genotype analysis. DNA isolated from embryos (or ES cell lines) was digested with methylation sensitive or methylation insensitive enzymes, and analyzed by Southern hybridization as previously described (Lei et al., 1996). The probes used for methylation studies were: pMO for endogenous C-type retroviruses (Lei et al., 1996); the Igf2r region 2 probe (Stöger et al., 1993); the H19 upstream region (Tremblay et al., 1995); Igf2 DMR2 (Feil et al., 1994); 5’ region of Xist CDNA (Sado et al., 2000); Rasgrf1 Sp-4 repeat region (Pearshall et al., 1999); Pegl (Lefebvre et al., 1997); and Snrpn DMR1 (Shemer et al., 1997). Bisulfite sequencing analysis of the Igf2r region 2 and the Peg3 DMR was performed as described previously (Clark et al., 1994). The primer sequences and PCR conditions were kindly provided by J. Walter and A. Paldi and are available upon requests.

**RESULTS**

**Expression of Dnmt3L in ES cells, placenta and gametes**

In the search for the Dnmt3 family DNA methyltransferase related genes in the dbEST database, we identified and cloned a mouse gene (GeneBank number: AF220524) that shared some degree of homology with Dnmt3a and Dnmt3b (Fig. 1A). The human homologue, termed DNMT3-like (Dnmt3L), was

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Fig. 1. Protein structure, sequence alignment and expression of Dnmt3L. (A) Schematic diagrams of mouse Dnmt3L (421 a.a.), Dnmt3a (908 a.a.), and Dnmt3b (859 a.a.) protein structures. The red block represents the PHD-like domain. The yellow bars represent the five highly conserved cysteine methyltransferase motifs in Dnmt3a and Dnmt3b. Dnmt3L contains motifs I, IV, VI, and part of IX, but not motif X. (B) Sequence alignment of the PHD-like domain. Conserved amino acid residues are highlighted in red while similar residues are highlighted in gray. The PHD-like domains are highly conserved among the Dnmt3 family members and the X-linked ATRX gene. (C) Sequence alignment of motifs I, IV, VI and IX. Note that Dnmt3L lacks the consensus amino acid residues PC in motif IV and ENV in motif VI, which form the catalytic center of cysteine methyltransferases. (D) A northern blot of total RNA (20 μg/lane) from undifferentiated ES cells (undiff) and differentiated embryoid bodies (diff) was hybridized with a Dnmt3L full-length cDNA probe. +, n, s and c represent wild-type and various Dnmt1 mutant alleles (Okano et al., 1998). The blot was rehybridized with a β-actin probe as a RNA loading control. (E) Whole-mount in situ hybridization of E7.5 and E8.5 embryos using a Dnmt3L cDNA probe reveals high expression predominantly in the chorion. (F) X-gal staining of Dnmt3L+/- adult mice carrying the IRES-βgeo marker driven by the endogenous Dnmt3L promoter shows Dnmt3L expression in the oocyte and in male germ cells in the seminiferous tubule. Scale bar: 40 μm.
previously reported (Aapola et al., 2000). The human and mouse Dnmt3L proteins share ~60% of amino acid identity. Dnmt3L contains a PHD-like cysteine-rich domain that is most closely related to that of Dnmt3a, Dnmt3b and a protein encoded by the X-linked ATRX gene (Fig. 1B). Its carboxy-terminal domain is related to the enzyme domain of Dnmt3a and Dnmt3b, but it lacks the conserved PC and ENV residues in motifs IV and VI, respectively (Fig. 1C), which are known to form the catalytic center of DNA cytosine methyltransferases (Cheng et al., 1993). Recombinant Dnmt3L protein produced using the baculovirus expression system failed to methylate DNA in standard methyltransferase assays (data not shown). Therefore, it seemed unlikely that Dnmt3L would encode a functional DNA cytosine methyltransferase.

Northern blot analysis showed that Dnmt3L was highly expressed in embryonic stem (ES) cells and was down regulated in differentiated embryoid bodies (Fig. 1D). Wholomount in situ hybridization using a Dnmt3L cDNA probe showed that Dnmt3L transcripts were primarily detected in the chorion in E7.5 and E8.5 embryos (Fig. 1E). Dnmt3L expression was also detected in the oocytes and differentiating spermatocytes of newborn and adult mice as shown by X-gal staining of the knockout mice containing an IRES-βgeo cassette (see below) (Fig. 1F). The positive X-gal staining indicates that Dnmt3L is transcribed in the oocytes, but the expression level and localization of Dnmt3L protein in oocytes remains to be determined. The expression pattern of Dnmt3L in the ES cells, chorion, and gonads is strikingly similar to that of Dnmt3a and Dnmt3b (Okano et al., 1998; Okano et al., 1999), suggesting that Dnmt3L may play an important role in regulation of Dnmt3a and Dnmt3b during mouse development.

Zygotic Dnmt3L is not essential for embryonic development

To investigate the function of Dnmt3L in mouse development, we disrupted the Dnmt3L gene in ES cells and mice by replacing the Dnmt3L genomic DNA (exons 2-7) with a promoterless IRES-βgeo cassette through gene targeting (Fig. 2A). Mice carrying Dnmt3L mutations were derived from two independently derived Dnmt3L+/– ES cell clones. Mice homozygous for the Dnmt3L mutation were viable and survived to adulthood without discernible morphological abnormalities. We also generated Dnmt3L–/– ES cell lines by knocking out the remaining wild-type allele in Dnmt3L+/– ES cells with a second targeting construct containing a hygromycin selection marker (Fig. 2A,B). Northern hybridization using a full-length cDNA probe did not detect transcripts from the IRES-βgeo allele, but it detected a weak
transcript of smaller size from the hygromycin allele in ES cells (data not shown). Since the entire PHD domain and part of motif I were deleted in both alleles and the mutant allele was expressed at very low levels, we believe that these mutations should be functionally null. Although Dnmt3L was highly expressed in ES cells, Dnmt3L−/− ES cell lines grew normally in culture and differentiated into various tissues in teratomas (data not shown).

To determine whether Dnmt3L played any role in regulation of DNA methylation, we carried out DNA methylation analysis of several repetitive elements and imprinted genes in mutant ES cells and mice. No significant changes in DNA methylation patterns of the endogenous retrovirus DNA, DMR2 of Igf2, and region 2 of Igf2r were detected in Dnmt3L−/− ES cell lines (Fig. 1F). Thus, we concluded that the zygotic function of Dnmt3L is not essential for global methylation in ES cells and mice. However, these experiments do not exclude the possibility that Dnmt3L may play a role in regulation of DNA methylation in a tissue-specific or developmental stage-specific manner.

**Dnmt3L mutation results in male sterility and a maternal effect on embryonic development**

Since Dnmt3L expression was detected in the germ cells (Fig. 1F), we investigated the function of Dnmt3L in reproduction by breeding Dnmt3L−/− mice with wild-type mice. We found that the male Dnmt3L−/− mice were completely infertile. Examination of testes in Dnmt3L−/− mice revealed that the size of the testis of mutant mice was normal at birth, but significantly reduced at 8 weeks of age compared to wild-type mice (Fig. 3A). In the testis of wild-type mice, the mitotically active spermatogonia reside near the periphery of the seminiferous tubule (Fig. 3B). They migrate towards the lumen of the tubule as they differentiate into spermatocytes and undergo meiosis (Fig. 3B), and then further differentiate into spermatids or spermatozoa (Fig. 3D). Testes from 1-week old Dnmt3L−/− appeared to be normal in size and contained a relatively normal number of spermatogonia in the seminiferous tubules (Fig. 3E). However, the spermatogonia either failed to differentiate into spermatocytes or in some cases differentiated into abnormal spermatocytes (Fig. 3F). In the testes of adult Dnmt3L−/−, very few differentiated spermatids or spermatozoa were found although a small number of spermatogonia were still present (Fig. 3F). These results suggest that Dnmt3L function is probably required for the differentiation of spermatogonia, consistent with the expression pattern of Dnmt3L in the testis. Whether disruption of Dnmt3L leads to meiotic defects is under investigation.

Unlike the male Dnmt3L−/− mice, the female homozygous mice were fertile. However, no live pups were born from female Dnmt3L−/− mice. To determine when embryos died in the uterus of Dnmt3L−/− mothers, we dissected embryos at different developmental stages. We found that embryos were grossly normal at E8.5, but were slightly smaller than normal embryos at E9.5 and died around E10.5. The most striking developmental defects were found in the head region with most embryos showing mild defects in neural tube closure and some displaying severe rostral neural tube defects (Fig. 4C). The cardiac development appeared to be normal. The chorion-placenta was smaller than that of a normal E9.5 embryo, suggesting that placental defects might be the cause of embryonic lethality (data not shown). These results demonstrate that the function of maternally expressed Dnmt3L is essential for embryonic development.

To further determine whether the maternal effect of Dnmt3L mutation resided in the egg or in the uterus, we performed embryo transfer experiments. We mixed 31 blastocysts from pregnant Dnmt3L−/− mothers and 26 blastocysts from wild-type mothers and transferred them into the uteri of seven wild-type females. We dissected embryos at E9.5 or E10.5 and recovered 11 Dnmt3L−/− (from Dnmt3L−/− mothers mated with wild-type males) and 18 wild-type embryos. While wild-type embryos...
were normal, all 11 mutant embryos displayed similar developmental defects as conceptuses from Dnmt3L\(^{+/–}\) mice. These results strongly suggest that the maternal function of Dnmt3L resides in the egg or preimplantation embryos rather than in the uterus.

Since Dnmt3L is expressed in early embryos, it is possible that the maternal Dnmt3L may compensate the loss of zygotic Dnmt3L during early development of Dnmt3L\(^{+/–}\) embryos. To test this possibility, we crossed male Dnmt3L\(^{+/–}\) mice with female Dnmt3L\(^{+/–}\) mice. If the maternal Dnmt3L transcripts present in preimplantation embryos are required for preimplantation or early postimplantation development, we would expect to recover no viable Dnmt3L\(^{+/–}\) embryos at E9.5 or to observe more severe defects in Dnmt3L\(^{+/–}\) embryos than in Dnmt3L\(^{+/–}\) embryos. We dissected a total of 29 embryos and found 13 Dnmt3L\(^{+/–}\) embryos and 16 Dnmt3L\(^{+/–}\) embryos with indistinguishable phenotypes. Since both maternal and zygotic Dnmt3L transcripts were abolished in the Dnmt3L\(^{+/–}\) embryos, this result strongly suggested that Dnmt3L was not essential for early development of the mouse embryo.

**Dnmt3L\(^{+/–}\) females fail to establish methylation imprints in the oocytes**

We then investigated whether Dnmt3L was required for the establishment of genomic imprinting in the oocytes and appropriate expression of maternally imprinted genes. We analyzed the methylation status of differentially methylated regions (DMRs) of several imprinted genes in embryos of Dnmt3L\(^{+/–}\) mothers (we designated such embryos as Dnmt3L\(^{+/–}\) to distinguish them from embryos from wild-type and Dnmt3L\(^{+/–}\) mothers). We showed that the maternally imprinted regions such as the region 2 CpG island of Igf2r, the DMR of Peg3, the DMR1 of Srrpn, and the DMR of Peg1 are almost completely demethylated in Dnmt3L\(^{+/–}\) embryos, as shown either by bisulfite sequencing or southern blot analysis, while these regions were partially methylated in the control embryos as expected since only the maternal alleles were normally methylated (Fig. 5A-D) (Stöger et al., 1993; Li et al., 2000; Shemer et al., 1997; Lefebvre et al., 1997). In contrast, the methylation patterns of paternally imprinted genes such as H19 and Rasgrf1 were unaffected in the Dnmt3L\(^{+/–}\) embryos as compared to wild-type embryos (Fig. 5E,F), consistent with the fact that methylation imprints of H19 and Rasgrf1 are inherited from the male germ cells (Tremblay et al., 1995; Pearsall et al., 1999). Methylation of the 5′ region of Xist and the endogenous retrovirus DNA was also unaffected in Dnmt3L\(^{+/–}\) embryos (data not shown). This is expected as de novo methylation of these sequences occurs in blastocysts around implantation, erasing the difference in methylation between the paternal and maternal genome. These results indicate that Dnmt3L is required for the establishment of maternal methylation imprints.

We then analyzed expression of several maternally imprinted genes, including Igf2r, p57Kip2, Peg1 and Srrpn in Dnmt3L\(^{+/–}\) embryos to see whether disruption of methylation imprinting in the oocytes lead to abnormal expression of these genes in the offspring. Previous studies showed that maternal methylation of Igf2r region 2 CpG island is associated with expression of the gene and abrogation of methylation leads to silencing of the maternal allele (Stöger et al., 1993; Li et al., 1993). Other experiments showed that blocking maternal epigenetic modification by transferring nuclei from the non-grown oocyte into enucleated mature oocytes represses expression of the maternal alleles of Igf2r and p57Kip2 in the parthenotes (Obata et al., 1998). We showed that expression of both Igf2r and p57Kip2 was diminished in the Dnmt3L\(^{+/–}\) embryos (Fig. 5G). Opposite to Igf2r and p57Kip2, the paternal alleles of Peg1 and Srrpn are expressed and the maternal alleles are repressed by methylation (Lefebvre et al., 1997; Leff et al., 1992). We showed, by northern analysis, that the expression level of both Peg1 and Srrpn in Dnmt3L\(^{+/–}\) embryos was increased to approximately twice the level in wild-type embryos, as determined by phosphoimaging (Molecular Dynamics) (Fig. 5H). Collectively, these results suggest that the failure of Dnmt3L\(^{+/–}\) female mice to establish methylation imprints leads to aberrant expression of maternally imprinted genes.

**Dnmt3L may regulate maternal imprinting by interacting with Dnmt3a and Dnmt3b**

Since Dnmt3L protein possessed no detectable methyltransferase activity, it would have to effect methylation of imprinted genes through one of the known DNA methyltransferases. Dnmt3a and Dnmt3b were favored candidates as both had been shown to carry out de novo methylation in vivo (Okano et al., 1999; Lyko et al., 1999). We first investigated whether Dnmt3L could interact with Dnmt3a and Dnmt3b by transfecting GFP-tagged Dnmt3L and myc-tagged Dnmt3a or Dnmt3b cDNA into COS cells followed by immunoprecipitation. We found that GFP-Dnmt3L was co-precipitated with myc-Dnmt3a and myc-Dnmt3b by the antimyc antibody (Fig. 6A). The GFP-Dnmt3L signal was weak in myc-Dnmt3b transfected cells because of poor expression of the...
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myc-Dnmt3b protein (Fig. 6A, lanes 3 and 9). Similar results were obtained when myc-Dnmt3L and GFP-Dnmt3a (or GFP-Dnmt3b) were cotransfected (data not shown).

To further test whether Dnmt3L could interact with Dnmt3a or Dnmt3b in live cells, we analyzed subcellular localization of Dnmt3L in the presence or absence of Dnmt3a or Dnmt3b by immunostaining of 3T3 cells expressing both myc-tagged Dnmt3L and GFP-tagged Dnmt3a or GFP-tagged Dnmt3b. We showed that Dnmt3L localized predominantly in the cytoplasm in the absence of Dnmt3a or Dnmt3b, whereas GFP-Dnmt3a or GFP-Dnmt3b alone localized predominantly to the heterochromatin foci in the nucleus (data not shown). Interestingly, cotransfection of myc-Dnmt3L and GFP-Dnmt3a resulted in relocation of Dnmt3L to the heterochromatin foci in >80% of the cells that expressed both proteins (Fig. 6B). Similar results were obtained with myc-Dnmt3L and GFP-Dnmt3b cotransfected cells (Fig. 6B, last line). These results suggest that Dnmt3L may be capable of forming a complex with Dnmt3a or Dnmt3b in vivo. Further studies will be necessary to determine whether endogenous Dnmt3L and Dnmt3a (or dntmt3b) colocalize in the oocytes.

Since disruption of Dnmt3b was embryonic lethal and Dnmt3a+/- mice were growth retarded and failed to breed before death at 4-8 weeks after birth, we could not directly test the function of Dnmt3a and Dnmt3b in establishing genomic imprinting in the germ cells. To overcome this problem, we performed ovary transplantation by implanting ovaries from Dnmt3a-/- or [Dnmt3a-/-, Dnmt3b+/+] female mice to wild-type recipients. We then crossed the recipient females with wild-type male mice. From one recipient female, we obtained two embryos at E9.5 of gestation, which were morphologically normal. Genotype analysis indicated that one embryo was Dnmt3a+/+ and the other was [Dnmt3a+/-, Dnmt3b+/-]. We confirmed by

Fig. 5. Disruption of methylation and expression of maternally imprinted genes in Dnmt3Lmat-/- embryos. (A-D) Methylation of four maternally imprinted genes (Igf2r, Peg3, Snrpn and Peg1) were analyzed by either bisulfite sequencing or southern blot hybridization with genomic DNA isolated from E9.5 wild-type (+/+), Dnmt3L+/- (+/-), Dnmt3L-/- (-/-) and Dnmt3Lmat-/- (mat-/-) embryos. (A,B) Bisulfite sequencing analysis (methylation shown as black circles) shows that the Igf2r region 2 and Peg3 DMR are almost completely unmethylated in Dnmt3Lmat-/- embryos, whereas they are partially methylated in wild-type and Dnmt3L+/- embryos. (C,D) Southern blot analysis of the Snrpn DMR1 and the Peg1 DMR shows that the maternal alleles (m) of both genes are methylated in wild-type and Dnmt3L+/- embryos, but unmethylated in Dnmt3Lmat-/- embryos. (E,F) No change in methylation of paternally imprinted genes, Rasgrf1 and H19, was observed in Dnmt3Lmat-/- embryos. (G,H) Expression of four maternally imprinted genes (Igf2r, p57kip2, Peg1 and Snrpn) in E9.5 embryos was analyzed by either RT-PCR (G) or northern blot hybridization (H). RT-PCR analysis of expression of Igf2r and p57kip2 in two wild-type, two Dnmt3L+/-, and four Dnmt3Lmat-/- embryos showed that expression of Igf2r and p57kip2 was drastically reduced in all Dnmt3Lmat-/- embryos. Northern analysis of Peg1 and Snrpn expression with total RNA isolated from a pool of 5-6 E9.5 embryos for each genotype showed an increased expression (approximately twice the wild-type level) of Peg1 and Snrpn in Dnmt3Lmat-/- embryos as compared to the normal embryos. m, maternal allele; p, paternal allele.
Southern blot analysis that the donor ovary originated from a [Dnmt3a+/–, Dnmt3b+/-] female mouse. This result suggests that oocyte maturation and meiosis in [Dnmt3a+/-, Dnmt3b+/-] female mice are probably normal as in Dnmt3L–/– females.

We then examined the methylation status of several imprinted genes. Similar to the Dnmt3L–/– embryos, we found that the DMRs of four maternally imprinted genes, Igf2r, Peg1, Peg3 and Surp were unmethylated in the two embryos derived from the transplanted ovary (Fig. 7A-D). As controls, we showed that methylation of the paternally imprinted H19 gene and repetitive DNA sequences such as the endogenous provirus DNA was unaffected (Fig. 7E,F). These results suggest that Dnmt3a and/or Dnmt3b are required for establishing maternal methylation imprints. Since female [Dnmt3a+/–, Dnmt3b+/-] mice were normal and fertile, we believed that Dnmt3a mutation was primarily responsible for the loss of methylation imprints in [Dnmt3a+/-, Dnmt3b+/-] oocytes. Interestingly, Dnmt3a+/- male mice display similar but less severe defects in spermatogenesis as Dnmt3L–/–. Together, our results suggest that Dnmt3L probably cooperates with the Dnmt3 family de novo methyltransferases to regulate genomic imprinting during oocyte development.

**DISCUSSION**

Genomic imprinting is a developmentally regulated process that controls expression of a small set of genes, estimated to be around a few hundreds, in the mammalian genome in a parent-of-origin-specific manner. Differential methylation of the paternal and maternal alleles has been detected in most known imprinted genes, and it is essential for the maintenance of mono-allelic expression of imprinted genes in development (Neumann and Barlow, 1996; Jaenisch, 1997; Tilghman, 1999; Ferguson-Smith and Surani, 2001). While DNA methylation is believed to be at least part of the imprinting mechanism, it is largely unknown as to how imprinted methylation is established during gametogenesis. In this study, we have provided genetic evidence that Dnmt3L, a Dnmt3a/Dnmt3b methyltransferase related protein, is required for the establishment of maternal genomic imprinting in the oocyte. Similar results were reported recently by Bourc’his et al., but it was not determined how Dnmt3L might regulate DNA methylation (Bourc’his et al., 2001). We show further that Dnmt3L can interact with Dnmt3a and Dnmt3b in mammalian cells and [Dnmt3a+/-, Dnmt3b+/-] female mice also fail to establish imprinted methylation patterns in their oocytes (Figs 6 and 7). Thus, our study has identified the Dnmt3 family DNA methyltransferases as the major enzymatic machinery responsible for establishing maternal genomic imprinting. DNA methylation is believed to be the epigenetic marker in the gametes for genomic
imprinting. However, no evidence is available to show that such methylation markers are necessary for transmission of the imprinting signal from gametes to offspring. Nuclear transfer experiments provided compelling evidence that epigenetic modification in the growing oocytes is necessary for establishing genomic imprinting and appropriate expression of some imprinted genes in the offspring (Obata et al., 1998). However, it was not clear whether DNA methylation or other heritable epigenetic mechanisms such as histone modification and chromatin conformation was responsible for the establishment of the imprinting signals. Bourc’his et al. (Bourc’his et al., 2001) reported that several imprinted genes on chromosome 7 were expressed from both paternal and maternal alleles and two other genes, Lp1 and Cdkn1, were silent in embryos from Dnmt3L−/− mothers. We showed here that disruption of imprinted methylation patterns in Dnmt3L−/− mice led to bi-allelic expression of Peg1 and Snrpn and bi-allelic silence of Igf2r and p57Kip2 in the offspring. Together, these results provide compelling evidence that the methylation markers of imprinted genes established in the oocytes are essential epigenetic signals for the establishment and transmission of genomic imprinting.

Among the 40 or so imprinted genes identified in the mouse, half are maternally expressed and the other half are paternally expressed. However, the majority of these imprinted genes inherit methylation imprints from the mother, whereas only two genes, H19 and rasgrf1, are so far known to inherit the methylation imprints from the father (Reik and Walter, 2001). Although the functional significance of such asymmetric inheritance of methylation imprints is unclear, this intriguing phenomenon indicates that methylation imprints are primarily established in the oocytes. Dnmt1 was previously predicted to be the enzyme responsible for de novo methylation of imprinted genes in oocytes because a maternal form of Dnmt1 (Dnmt1o) was found to be highly expressed in the oocytes (Mertineit et al., 1998). However, recent experiments failed to support such a notion, as inactivation of the maternal Dnmt1o does not perturb the establishment of genomic imprinting in the oocytes; instead, the maintenance of imprinted methylation patterns in preimplantation embryos was partially impaired in embryos lacking the maternal Dnmt1o (Howell et al., 2001). These results are consistent with Dnmt1 being primarily responsible for the maintenance of DNA methylation patterns during mammalian development.

Unlike Dnmt1, Dnmt3a and Dnmt3b have been shown to be primarily responsible for de novo methylation of the mouse genome during early embryonic development (Okano et al., 1999). Here we provide genetic evidence that the Dnmt3 family methyltransferases are also required for de novo methylation of imprinted genes during oocyte development. The finding that [Dnmt3a+/−, Dnmt3b+/-] female mice are fertile, but [Dnmt3a−/−, Dnmt3b−/−] oocytes are defective in establishing proper methylation imprints suggests that Dnmt3a probably contributes

![Fig. 7. Methylation of imprinted genes in embryos derived from a transplanted [Dnmt3a+/-, Dnmt3b+/-] ovary. (A-D) Methylation of four imprinted genes (Igf2r, Peg1, Peg3, and Snrpn) was analyzed either by Southern blot hybridization (A,B) or bisulfite sequencing (C,D) with DNA isolated from E9.5 wild-type (+/+), Dnmt3a+/- (+/-), and Dnmt3a−/− (−/−) embryos, and two embryos derived from the [Dnmt3a−/−, Dnmt3b+/-] oocytes (labeled as 1, 2). In A and B, DNA was digested with designated restriction enzymes and Southern blots were hybridized with probes of Igf2r region 2 (A) or Peg1 DMR (B). Bisulfite sequencing analysis of methylation (black circles) of Peg3 DMR and Snrpn DMR1 in wild type and embryos 1 and 2 derived from ovary transplantation (C,D). The maternal alleles of Igf2r, Peg1, Peg3 and Snrpn were almost completely unmethylated in the two embryos derived from the [Dnmt3a+/-, Dnmt3b+/-] mother, but they were methylated in Dnmt3a−/− and Dnmt3a+/- embryos from Dnmt3a−/− mothers. (E,F) Methylation of H19 (E) and MMLV (F) remained unchanged. Methylation of the paternally imprinted H19 gene and non-imprinted endogenous MMLV DNA was unaffected in all embryos tested.](image-url)
more, if not entirely, to the de novo methylation of imprinted genes. Analysis of expression patterns of Dnmt3a and Dnmt3b during oocyte development and inactivation of individual enzymes specifically in primordial germ cells will determine the function of each enzyme in establishing genomic imprinting.

In spite of the fact that Dnmt3a and Dnmt3b can initiate de novo methylation in both early embryos and germ cells, they appear to have different target specificity at these two stages. In the early embryos, Dnmt3a and Dnmt3b actively methylate repetitive elements, endogenous retrovirus and retrotransposons and some unique genes such as Xist, but they omit the sequences of DMRs in imprinted genes such that the imprinted methylation patterns are preserved. In contrast, Dnmt3a and Dnmt3b appear to selectively methylate the DMRs of imprinted genes in oocytes. Such cell lineage-specific de novo methylation is probably regulated by proteins that are either involved in targeting Dnmt3 methyltransferases to different sequences or in chromatin remodeling that changes chromatin accessibility at imprinted loci. Dnmt3L is the first protein that appears to confer sequence specificity of DNA methylation. While the precise mechanism by which Dnmt3L regulates de novo methylation of imprinted genes remains to be elucidated, we would like to consider the following possible scenarios. First, Dnmt3L may facilitate genome-wide de novo methylation by Dnmt3a and Dnmt3b specifically in the oocytes. In other words, Dnmt3L mediates de novo methylation of both the DMRs of imprinted genes and the bulk genomic DNA. Gamete-specific methylation patterns of imprinted genes are maintained after fertilization simply because these sequences can escape genome-wide demethylation and de novo methylation during preimplantation development (Reik et al., 2001). To test this hypothesis, it will be important to determine whether de novo methylation of repetitive elements and non-imprinted genes is also disrupted in Dnmt3L−/− oocytes. Alternatively, Dnmt3L may target Dnmt3a and Dnmt3b specifically to the DMRs of imprinted genes in the oocytes. The ability of Dnmt3L to interact with Dnmt3a and Dnmt3b in mammalian cell provides the molecular basis for such targeting events. Dnmt3L may form a complex with the Dnmt3 family methyltransferases, which may selectively bind to DMRs of imprinted genes. Interestingly, many DMRs consist of direct repeats that may selectively bind to DMRs of imprinted genes. The H19 methylation imprint is erased and re-established differentially on the parental alleles during male germ cell development (Kojima et al., 2000).

In this study, we have also shown that Dnmt3L is required for spermatogenesis. The lack of Dnmt3L appears to prevent spermatogonia from undergoing differentiation or meiosis (Fig. 3). Interestingly, Dnmt3a is also required for spermatogenesis (data not shown) though the testicular phenotype of Dnmt3a−/− mice is less severe, likely due to functional compensation by Dnmt3b. These results suggest that the Dnmt3 family DNA methyltransferases and Dnmt3L may also cooperate to regulate spermatogenesis. Whether defects in spermatogenesis is caused by disruption of de novo methylation in the male germ cells is a question of great interest and will be addressed rigorously in future studies. It also remains to be determined whether the Dnmt3 family methyltransferases and Dnmt3L are required for establishing methylation imprinting of H19 and rasgrf1 in the male germ cells.

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