The cis-acting regulatory sequences of imprinted genes are subject to germline-specific epigenetic modifications, the imprints, so that this class of genes is exclusively expressed from either the paternal or maternal allele in offspring. How genes are differentially marked in the germlines remains largely to be elucidated. Although the exact nature of the mark is not fully known, DNA methylation [at differentially methylated regions (DMRs)] appears to be a major, functional component. Recent data in mice indicate that Dnmt3a, an enzyme with de novo DNA methyltransferase activity, and the related protein Dnmt3L are required for methylation of imprinted loci in germ cells. Maternal methylation imprints, in particular, are strictly dependent on the presence of Dnmt3L. Here, we show that, unexpectedly, methylation imprints can be present in some progeny of Dnmt3L^{-/-} females. This incomplete penetrance of the effect of Dnmt3L deficiency in oocytes is neither embryo nor locus specific, but stochastic. We establish that, when it occurs, methylation is present in both embryo and extra-embryonic tissues and results in a functional imprint. This suggests that this maternal methylation is inherited, directly or indirectly, from the gamete. Our results indicate that in the absence of Dnmt3L, factors such as Dnmt3a and possibly others can act alone to mark individual DMRs. However, establishment of appropriate maternal imprints at all loci does require a combination of all factors. This observation can provide a basis to understand mechanisms involved in some sporadic cases of imprinting-related diseases and polymorphic imprinting in human.

INTRODUCTION

Genomic imprinting is a form of non-Mendelian inheritance in mammals, where some genes are expressed depending on whether they are inherited from the mother or the father (1). To date, roughly 80 imprinted genes have been discovered in human and mouse. About half of these are exclusively expressed from the maternal allele, and the other half from the paternal allele only. Many imprinted genes are involved in the regulation of cellular proliferation and growth in the placenta and embryo. Other imprinted genes play key roles in neurological processes and in behaviour (2,3). Not surprisingly, therefore, deregulation of imprinted genes gives rise to abnormal development and is causally involved in a number of growth and behavioural syndromes in humans (4).

Several lines of evidence indicate that imprinted mono-allelic expression relies on the acquisition of germline-specific epigenetic modifications, of which DNA methylation is a major component (4 and references therein). Most, if not all, imprinted loci are characterized by the presence of a differently
methylated region (DMR) harbouring allelic DNA methylation inherited from the male or female gamete (the germine DMR). Importantly, the cis-acting elements that control imprinting (ICRs) have now been defined for several imprinted loci and these elements coincide with germine DMRs. There is an expectation that germine DMRs identified at other imprinted loci correspond to ICRs as well. Following acquisition in either the male or female germ line, DNA methylation marks are maintained in the zygote and are reliably transmitted throughout development to all somatic tissues. Ultimately, the epigenetic features at germine DMRs/ICRs are read in differing ways to ensure proper parental-allele-specific expression of the genes or imprinted clusters (5).

Imprints need to be reset between each generation. This crucial step occurs in the developing germ cell lineage, with the first erasure of the existing imprint followed by acquisition of a new imprint mark according to the sex of the new embryo. Erasure occurs in primordial germ cells (PGCs). Following the entry of PGCs into the genital ridge of both male and female embryos, there is a rapid and possibly active erasure of DNA methylation at germine DMRs (6). Acquisition of new imprints occurs at a late stage of mouse germ cell development. In males, imprint establishment starts before birth in prospermatogonia and is completed postnatally by the pachytene stage of meiosis (7,8). In females, methylation acquisition at germine DMRs occurs after birth during the growth phase of oocytes. These maternal methylation marks are acquired asynchronously at different loci but are, in all cases, completed by the metaphase II stage (9,10).

Elegant nuclear experiments have shown that, similar to DNA methylation, competence for imprinting is acquired during the postnatal growth of the oocyte (11,12). This observation emphasizes the role of DNA methylation in acquisition of functional imprints.

Although this key role of DNA methylation is well recognized, the precise mechanism by which germine DMRs/ICRs are differentially marked in the germlines is still poorly understood. A key factor identified in recent years is the Dnmt3L protein. Dnmt3L belongs to the Dnmt3 methyltransferase family, although it lacks a functional methyltransferase domain (13,14). Interestingly, development of mouse embryos, produced from Dnmt3L−/− females, is arrested by embryonic day 10.5. These embryos completely lack maternal methylation at germine DMRs, resulting in deregulated expression of associated imprinted genes, whereas methylation of the remainder of the genome is apparently unaffected (15,16). Biochemical studies have shown that Dnmt3L can interact with Dnmt3a and Dnmt3b, thereby enhancing their de novo methyltransferase activities (17–19). That also Dnmt3a is essential for the acquisition of maternal imprints has been shown in vivo (16,20). Thus, Dnmt3L is postulated to be a key regulator of maternal imprint establishment, through the interaction with Dnmt3a. In the male germ line, conditional knock-out of Dnmt3a causes severely impaired spermatogenesis and lack of DNA methylation at two of the three described paternally methylated ICRs (20). The involvement of Dnmt3L in imprinted methylation seems to be less critical in the male germ line and could be ICR specific (20–22). Instead, Dnmt3L is responsible for de novo methylation and transcriptional silencing of dispersed repeated sequences in spermatogonial stem cells (21–23).

The precise mechanism involved in the germline specificity of Dnmt3L targeting is currently unknown. One possibility is that epigenetic modifications other than DNA methylation are already present and are used as a mark to indicate which sequences need to become methylated. This notion is supported by the observation that subsequent to methylation erasure in the male germ line, methylation at the H19 ICR is re-established differentially on the two alleles, with the paternal allele acquiring DNA methylation prior to the maternal allele (24). A similar observation has been made in the female germ line for the Surprn ICR. This ICR acquires first methylation on the maternally inherited allele in growing oocytes, with methylation on the paternally inherited allele occurring later (10). This difference in DNA methylation reacquisition timing could be due to an allelic chromatin structure that is not fully erased during gametogenesis.

In the present study, we aimed to investigate whether Dnmt3L is a strict obligatory factor for acquisition of maternal methylation imprints. In this purpose, we have analyzed DNA methylation at seven maternally methylated DMRs/ICRs in individual progeny of Dnmt3L−/− females. Contrary to our expectations, we observed that methylation imprints associated with appropriate imprinted expression can be present in such embryos. This incomplete penetrance of the effect of Dnmt3L deficiency in the female germline was neither embryo nor locus specific, but stochastic.

RESULTS

Stochastic ICRs methylation in Dnmt3L−/− embryos

DNA methylation was analyzed in eight different germline DMRs/ICRs present in seven different imprinted domains (with Gnas domain containing two germline DMRs) (Fig. 1).

![Figure 1. Schematic representation of the eight germline DMRs/ICRs analyzed.](http://hmg.oxfordjournals.org/)
Seven of these ICRs have DNA methylation that is acquired in the female germline. The H19 ICR, with sperm-derived methylation, served as a control. We performed analyses on 17 embryos at 9.5 days post-coitum (d.p.c.), derived from Dnmt3Lm−/− females. We refer to these as Dnmt3Lm−/− embryos. These embryos arose from four independent litters, consisting of, respectively, embryos e1–e2, e3–e9, e10–e11 and e12–e17. In the purpose to use DNA polymorphisms for allele discrimination, e3–e17 are intra-specific hybrids recovered after crossing Dnmt3Lm−/− females (on a 129, SvJae;C57BL/6 genetic background) with wild-type JF1 (Mus musculus molossinus) males. In addition, for embryos e10–e17, we recovered trophoblast tissue (t10–t17). For these conceptuses (10–17), DNA was prepared separately from the lower part of the embryo and half of the trophoblast, whereas the remaining halves of the embryos and trophoblasts were used for expression analyses (discussed subsequently).

For each individual conceptus, methylation at DMRs was analyzed in two different ways (Supplementary Material, Tables S1 and S2). One approach was by methylation-sensitive PCR. Where possible, the parental origin of amplicons was determined by electrophoretic separation of single-strand conformation polymorphisms (SSCPs) (Supplementary Material, Table S1). In a parallel approach, methylation was assessed by restriction digestion of PCR products obtained from bisulphite-treated DNA (Supplementary Material, Table S2) and confirmed by sequencing of randomly selected cloned products (Supplementary Material, Table S3). These two different approaches gave concordant results and the combined data are summarized in Table 1. In most Dnmt3Lm−/− embryos, the maternally methylated DMRs had the expected lack of methylation. Nevertheless, although limited to a few cases, germline DNA methylation was found to be present, apparently in a stochastic fashion as it was neither embryo nor germline DMR specific (Table 1).

For example, this was observed at the Gnas imprinted domain. Gnas is a complex, compact cluster where multiple imprinted promoters control biallelic, maternally and paternally expressed coding and non-coding transcripts (25 and references therein). Interestingly, this complex cluster contains two candidate ICRs, both of them acquiring their DNA methylation in the female germline: the promoter/exon 1A region (26–28) and the promoters for Gnasxl (X1) and exon 1A DMR (1A).

Table 1. Summary of DNA methylation in Dnmt3Lm−/− conceptuses

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DNA methylation analysis performed on Dnmt3Lm−/− embryos (e1 to e17) and respective trophoblast (t10 to t17). Imprinted loci analysed are indicated on the left: all contain maternally methylated germline DMRs, except H19. For Gnas the results refers to the Gnasxl (X1) and exon 1A DMR. The symbol – indicates absence of methylation; + indicates presence of methylation. Where it was ascertained, the methylated parental allele is indicated (M: maternal; P: paternal). N/D: not determined.

For embryos e2–e17, we observed a complete absence of methylation at both putative maternally methylated ICRs at the Gnas domain (Table 1 and Fig. 2) (data not shown). In contrast, we observed in the same conceptuses that the Nesp DMR was fully methylated, presumably on both alleles (Fig. 2) (data not shown). Therefore, in embryos from Dnmt3Lm−/− mothers, both alleles of the Gnas locus have a paternal epigenotype. These observations indicate that the two oocyte-specific germline methylation marks in this locus are dependent on the presence of Dnmt3L. In addition, the gametic maternal methylation of one or both of these two regions is likely to be required for zygotic methylation at the Nesp DMR. These findings confirm those of Liu et al. (28).

Unlike the observation made in conceptuses e2–e17, the methylation patterns observed in e1 at the Gnas locus were similar to that of a wild-type 9.5 d.p.c. embryo, with equal proportions of methylated and unmethylated sequences at all three Gnas DMRs, suggesting that methylation had been established normally in the absence of Dnmt3L (Table 1 and Fig. 2). Among the five maternally imprinted loci analyzed in embryo e1, only the Gnas cluster displayed such a wild-type like pattern (Table 1).
hybrid embryos e3–e17. Representative results are shown in Figure 2. DNA methylation patterns at the Gnas locus in Dnmt3Lm−/− embryos. Examples of bisulphite sequences of the Gnas locus obtained from two 9.5 d.p.c. Dnmt3Lm−/− embryos (e1 and e2) and from one wild-type 9.5 d.p.c. embryo (wt). For each region and each embryo, the sequences shown derive from a single PCR product. Similar patterns were observed in sequences obtained from at least one additional independent PCR product (data not shown) (Supplementary Material, Table S3). The Gnas locus contains two maternally methylated germline DMRs (X1 and 1A: grey boxes) and one paternally methylated somatic DMR (Nesp: hatched box). Each row of dots represents the series of CpGs in an individual sequence molecule, in which methylated CpGs are shown as solid circles and unmethylated CpGs as open circles.

Stochastic methylation in Dnmt3Lm−/− embryos is faithfully imprinted

We next tested whether such methylation had the expected parental allele specificity for normal imprinting. In this purpose, we took advantage of sequence polymorphisms to distinguish maternal and paternal alleles in the intra-specific hybrid embryos e3–e17. Representative results are shown in Figs 3 and 4.

The Peg3 DMR was found to display methylation in two hybrid embryos (e8 and e9) and the Snrpn ICR in three (e8, e13 and e16) (Figs 3 and 4; Table 1). Parental origin of methylation was first determined by methylation-sensitive PCR coupled with SSCP analysis. Following the digestion of native DNA from embryos e3–e9 with HpaII (5′-CCGGG-3′), only the maternal allele was amplified at Peg3 in e8 and e9 and at Snrpn in e8, consistent with maternal-allele-specific methylation in these embryos (Figs 3A and 4A). Similar results were obtained on FnuDII (5′-CGCCG-3′) digested DNAs from embryos e3–e9 (data not shown). Bisulphite sequencing of the same regions confirmed the presence of methylation on the maternal allele and the absence on the paternal one (Figs 3B and 4B). Importantly, the patterns observed following bisulphite sequencing indicate strict allelic methylation, as all maternally derived sequences were methylated rather than just a subpopulation. Similarly, maternal methylation was observed at the Snrpn ICR in e13 and e16 (Fig. 4B). In all embryos, the paternally methylated H19 ICR displayed a wild-type pattern, with methylation detected only on the paternally inherited allele (Table 1) (data not shown).

To confirm these results with a non-PCR-based approach, we performed Southern analysis on the Peg3 locus. Genomic DNAs from Dnmt3Lm−/− embryos e3 + e4 and embryos e8 + e9 were pooled, and the two pools digested with AvrII and AvrII + Eco52I, blotted and hybridized with a probe for the Peg3 DMR. As Eco52I (5′-CCGGCCG-3′) digestion is blocked by CpG methylation, this assays methylation at two CpG sites in the 3′ part of Peg3 DMR. In the e3 + e4 DNA pool, complete digestion by Eco52I is consistent with the lack of methylation on both alleles. In contrast, the digestion pattern observed in e8 and e9 lanes was similar to that for wild-type adult liver DNA, suggesting that in these two embryos the Eco52I site is methylated on half of the DNA strands (Fig. 3C).

Together, these results indicate that, although limited, faithfully imprinted DNA methylation is present in some progeny of Dnmt3Lm−/− females. In addition, they reinforce our observation that such methylation is neither embryo nor locus specific, but stochastic (Table 1).

A similar pattern of DNA methylation in embryo and trophoblast of Dnmt3Lm−/− conceptuses

Acquisition of methylation imprints is tightly regulated and occurs only during germline development (29). The stochastic imprinted methylation we observed in Dnmt3Lm−/− embryos could have resulted from incomplete penetrance of Dnmt3L deficiency in the female germline and could thus have been acquired during oocyte development in the Dnmt3L−/− females. Alternatively, the DMRs could have become methylated in these conceptuses during the major wave of de novo methylation occurring from the implantation stage. To test this latter possibility, we compared methylation in some Dnmt3Lm−/− embryos with that of their trophoblast. We based this approach on the fact that the initiation of de novo methylation occurs after the fifth cell cycle, coinciding with the first differentiative event, and leads to an asymmetrical pattern in the first two cell-lineages. The inner cell mass, which gives rise to all the tissues of the embryo proper, is hypermethylated, whereas the trophoderm, which forms most of the extra-embryonic tissues, is under-methylated (30). Thus, the DMRs/ICRs methylation patterns observed in trophoblast are assumed to reflect their methylation state prior to implantation.

DNA methylation was analyzed in trophoblast tissue collected from conceptuses e10–e17, using the same approaches as for the embryos. In all cases, the methylation status of the DMRs/ICRs in trophoblast matched that in the corresponding embryo (Table 1), with the lack of methylation for most regions analyzed. For the Snrpn ICR, which was maternally methylated in e13 and e16, the same allelic methylation pattern was observed in the corresponding trophoblasts (Fig. 4B). Although based on a limited number of samples, these results suggest that ICR methylation in Dnmt3Lm−/− embryos is present before implantation.
**Figure 3.** DMR methylation features at the Peg3 locus in Dnmt3L−/− embryos. (A and B) Allelic methylation patterns of Peg3 DMR in Dnmt3L−/− embryos. Embryos are from matings of Dnmt3L−/− females (129.SvJae;C57BL/6 background) with wild-type JF1 males. (A) Methylation-sensitive PCR coupled with SSCP electrophoresis performed on undigested (−) or HpaII digested (+) DNAs from 9.5 d.p.c. Dnmt3L−/− embryos e3−e9 and a 9.5 d.p.c. wild-type embryo (wt) as comparison. The SSCP profiles expected from maternal allele (Mat.) and paternal allele (Pat.), obtained by amplification, respectively, from undigested C57BL/6 and JF1 DNAs, are shown on the left. (B) Bisulphite sequence analysis of DNAs from e3, e8, e9 and wt 9.5 d.p.c. embryos. Parental allele, as shown, was based on the presence of sequence polymorphisms (Supplementary Material, Table S2). Mat., maternal allele; Pat., paternal allele. For e8 and e9, the sequences obtained from two independent bisulphite treatments are shown in the upper and lower panels. The Peg3 DMR displays maternal-allele-specific methylation in e8 and e9. (C) Southern-blot analysis of the Peg3 DMR in e3+e4 and e8+e9 DNA pools. DNAs were digested with AvrII alone (A) or with AvrII and Eco52I (A+E) and hybridized with a probe for the Peg3 DMR (indicated as black bar in the scheme above). DNA from wild-type (C57BL/6 × JF1) (B6 × J) adult liver served as a control. Digestion at the Eco52I site (5′-CGGCCG-3′) is inhibited by CpG methylation. At the top, a representation of the region analyzed: the fragment produced by AvrII (A) digestion and location of the Eco52I site are shown (numbers refer to positions in sequence AF105262). The region analyzed by bisulphite sequencing in (B) is shown in grey.

**Figure 4.** DMR methylation feature at the Snrpn locus in Dnmt3L−/− embryos and trophoblast. Allelic methylation patterns of Snrpn DMR in Dnmt3L−/− embryos were investigated, respectively, by (A) methylation-sensitive PCR coupled with SSCP electrophoresis on undigested (−) or HpaII digested (+) DNAs from 9.5 d.p.c. Dnmt3L−/− embryos e3−e9 and a 9.5 d.p.c. wild-type embryo (wt) as comparison. (B) Example of bisulphite sequence analysis DNAs from e3, e8, e9, e13 and e16 and wt 9.5 d.p.c. embryos. For each embryo and trophoblast, the sequences shown derive from a single PCR product. Similar patterns were observed in sequences obtained from additional independent PCR products (data not shown) (Supplementary Material, Table S3). Legends are as for Figure 3. The Snrpn ICR displays maternal allele methylation in e8, e13 and e16. e, embryo; t, trophoblast.
Imprinted DNA methylation in \textit{Dnmt3L}$^{m/-}$ conceptuses leads to imprinted expression

DNA methylation at ICRs regions is a major and functional component of the mechanism leading to mono-allelic expression. However, it probably does not constitute the only epigenetic modification of imprints. Indeed, germline DMRs are also differentially marked by chromatin structure and histone tail modifications, at least in somatic cells (31,32), and loss of factors involved in acquisition of such modifications has been shown to perturb imprinted expression (33,34). Therefore, we explored whether the DNA methylation imprints acquired in the absence of Dnmt3L contain all the information required for mono-allelic expression. This was done using the intra-specific \textit{Dnmt3L}$^{m/-}$ conceptuses 10–17, expression being assayed in individual embryos and their corresponding trophoblasts (e10–e17 and t10–t17, respectively).

For all loci and all embryos at which the germline DMR was unmethylated on both alleles, we observed the absence of imprinted expression. \textit{U2af1rs1}, \textit{Peg3} and \textit{Snrpn} were expressed biallelically, whereas \textit{Grb10} was biallelically repressed (Fig. 5 and Table 2). This pattern is in agreement with the proposed role of DNA methylation in silencing or activating the maternal allele at each of these loci. A similar expression pattern was observed in embryo and the corresponding trophoblast. This suggests that the germline methylation imprint is a prerequisite for imprinted expression in both embryonic and extra-embryonic lineages. The paternally imprinted \textit{H19} ICR was correctly methylated in all conceptuses (Table 1). The \textit{Igf2} gene it controls exhibited correct imprinted expression in all samples analyzed (Fig. 5). Importantly, the two conceptuses with maternally derived methylation at the \textit{Snrpn} ICR (e/t13 and e/t16) both exhibited correct imprinted expression: the paternal allele was specifically expressed, both in embryo and trophoblast (Fig. 5). Thus, imprinted DNA methylation acquired independently of Dnmt3L leads to functional imprinted gene expression.
DISCUSSION

Gene targeting experiments have revealed that Dnmt3L is required for the establishment of imprinting in the oocyte (15,16). Consequently, because of loss of imprinting and associated embryonic defects, heterozygous progeny of Dnmt3L homozygous null females fail to develop past 10.5 d.p.c. Our results are in agreement with these studies. By analyzing seven maternally methylated germ line DMRS in 17 individual progeny of Dnmt3L−/− females, we observed a general absence of DNA methylation at these DMRS correlated with loss of imprinting. Unexpectedly, however, in a limited number of conceptuses, we found that a functional imprint was present, suggesting that the penetrance in oocytes of Dnmt3L deficiency is incomplete. Nevertheless, this phenomenon, which occurs at a low frequency and is neither locus nor embryo specific, does not rescue the phenotype of these Dnmt3L−/− progeny. It might, however, account for some of the phenotypic variability among these embryos.

Acquisition of maternal germ line imprints independent of Dnmt3L

The methylation we observed in Dnmt3Lm−/− embryos for some germ line DMRS/ICRs appears to represent authentic imprints: the maternally derived allele is fully methylated, whereas the paternal allele is unmethylated, both in embryonic and extra-embryonic lineages. However, our analysis does not allow us to conclude firmly whether this methylation is acquired in oocytes or after fertilization.

In addition to growing oocytes and perinatal prospermatogonia, mouse Dnmt3L is expressed in the chorion and ES cells (15,16,35). This pattern is similar to that of Dnmt3a (36). As Dnmt3L is postulated to function in establishing maternal imprints through interaction with Dnmt3a, it is possible that the methylation we observed was acquired during the genome-wide de novo methylation occurring from implantation, because of the Dnmt3L expression in embryos from the paternal allele. We do not favour this possibility for several reasons. First, it has been shown that maternally methylated DMRS, such as the Surprn ICR, cannot be remethylated by expression of several isoforms of murine Dnmt3a or Dnmt3b in Dnmt3a−/−/Dnmt3b−/− ES cells, whereas other regions of the genome can be remethylated (37). This observation supports the notion that ICR methylation might require factors that are expressed in germ cells only (29). Second, if the stochastic methylation we observe was acquired during the implantation de novo methylation wave, we might expect to see a mosaic pattern of methylation not necessarily present in all lineages and without parental allele specificity. This was not the case. Thus, we suspect that the methylation we observed was determined in the oocyte. It is still possible that this is an indirect effect: a specific chromatin structure developing stochastically at ICRs in the oocyte could be maintained during pre-implantation stages and recognized and methylated by Dnmt3a and paternally derived Dnmt3L at the implantation stage. This seems rather speculative.

Instead, we favour a model in which the stochastic methylation is acquired in oocytes. This is supported by biochemical data on Dnmt3L function. It is known that Dnmt3L can interact with Dnmt3a and Dnmt3b (16). Co-transfection experiments in a human cell line have demonstrated that Dnmt3L stimulates DNA methylation by murine Dnmt3a and by several isoforms of human DNMT3A and DNMT3B (17,19). In vitro, stimulation by human Dnmt3L is 1.5–3-fold (18); for mouse Dnmt3L, stimulation of up to 15-fold has been reported (38). However, this effect is independent of the sequence of the substrate DNA and is observed on DMRS and non-imprinted regions, albeit to variable extents. It is proposed that Dnmt3L induces a more open conformation in the active site of Dnmt3a and accelerates binding of the methyl donor S-adenosyl-L-methionine (AdoMet) with the target sequences (38). Thus, Dnmt3L may function as an activator protein for the methylation of germline DMRS/ICRs by Dnmt3a, with specific targeting to these regions requiring other factors, probably germ cell specific. The weak activity of Dnmt3a towards ICRs in oocytes could, on its own, account for the stochastic methylation events observed. This could be facilitated by incomplete erasure of all components of the imprint mark in PGCs, as has been implied from the differential establishment of imprints on the maternal and paternal alleles in germ cells (10,24). In this view, we should expect that the maternal chromosome carrying maternal imprints in Dnmt3L−/− embryos is always derived from the maternal grandmother. Such assumption cannot be tested in our system.

Stochastic acquisition of imprints: a link with imprinting diseases and polymorphic imprinting

Our data establish that a maternal imprint can be acquired in the absence of Dnmt3L in female germ cells, possibly through the action of DNMT3A and other accessory factors. This is reminiscent of recent observations made in the gestational trophoblastic disease, the biparental hydatidiform mole. Most hydatidiform moles are conception events with two paternal genomes and no maternal nuclear genome (39,40). Molar pregnancies exhibit trophoblastic hyperplasia and grossly abnormal embryonic development (41). Exceptionally, some phenotypically indistinguishable moles have both a maternal and paternal genome contribution, and such biparental moles can be familial in origin. They are proposed to arise from a genetic defect preventing the establishment of maternal germline imprints (42). Molecular studies have excluded DNMT3L and other DNMT genes as candidate genes in one kindred (43). However, the presumed hydatidiform mole mutations are not always fully penetrant. In other families, linkage analyses have assigned a candidate locus to chromosome 19q13.4 (44,45). Mutations affecting the 19q13.4 locus are not always fully penetrant, as the loss of DNA methylation at DMRs is not complete in all conceptuses (46), and some presumed carriers of the mutation have had normal pregnancies (47). Together, these observations indicate a hierarchy of factors involved in the establishment and maintenance of maternal germline imprints, the loss of one can be rescued in a stochastic fashion by the activity of the others.

Stochastic acquisition of imprints occurring in the absence of one or more key factors may also be a mechanism in ectopic methylation of ICRs sporadically observed in...
imprinting-related diseases, where the normally unmethylated copy has acquired methylation. This might be most likely for those few DMRs normally acquiring their methylation in the male germline, as the majority of imprints are established in the female germline. Such an ectopic methylation change has been observed at the H19 ICR in the cases of the imprinted growth disorder Beckwith-Wiedemann syndrome (49). Aberrant ectopic methylation of DMRs occurring in and transmitted from the male germline may be a rare event, because of the profound and active demethylation of sperm- or oocyte-derived chromosomes occurring at the pronuclear stage in many (although not all) mammalian species, including mouse and human (50).

In addition, the recent observation that human DNMT3L is expressed in early embryo, but not in oocytes (51), could similarly counteract stochastic acquisition in germline by validating ICR methylation patterns after fertilization. Despite these apparent barriers, aberrant methylation of paternal copies of DMRs has been found. The SNRPN ICR is biallelically methylated in some cases of the neurodevelopmental disorder Prader-Willi syndrome, in the absence of detectable sequence mutation. In a detailed molecular analysis of such cases, it was shown that the paternal chromosome carrying an incorrect imprint was always derived from the paternal grandmother (52). It is possible that incomplete erasure of maternal imprint was always derived from the paternal grandmother (52). It is possible, therefore, that stochastic acquisition of imprints in germ cells could provide an additional source of phenotypic variation in the population and be a source of selection in the evolution of imprinting.

**MATERIALS AND METHODS**

**Material collection**

To use DNA polymorphisms for allele discrimination, we crossed homozygous Dnmt3L<sup>m<sup>-/-</sup></sup> female mice ([29SVJae × C57BL/6 hybrid genetic background]) (16) to wild-type JF1 male mice (Mus musculus molossinus). E9.5 Dnmt3L<sup>m<sup>-/-</sup></sup>/embryos (we designated such embryos as Dnmt3L<sup>m<sup>-/-</sup></sup>) to distinguish them from embryos from wild-type and Dnmt3L<sup>m</sup>/ mothers) were removed from pregnant mothers. In addition, we recovered trophoblast tissue from embryos e10–e17 (t10–t17): trophoblast tissue was dissected carefully from the embryo and maternal decidua, and then the trophoblast tissue was washed in PBS twice to remove contaminating maternal blood. Yolk sac DNAs were used for genotype analysis by PCR as described previously (16).

**DNA extraction**

Whole or half 9.5 d.p.c. embryos and trophoblasts were suspended in 200 µl of a solution containing 10 µg glycogen, 1 mM SDS and 280 mg/ml proteinase K, incubated for 120 min at 37°C and then for 15 min at 95°C in a thermocycler. DNA was recovered by phenol–chloroform extraction, ethanol precipitated and stored in 10 mM Tris:Cl pH 7.8.

**Methylation-sensitive PCR and SSCP**

A 200 ng of DNA was digested with the appropriate restriction enzyme in a final volume of 25 µl. As control, duplicate sets of samples were incubated with restriction enzyme omitted. Aliquots of 2.5 µl were used per PCR. PCR amplifications were performed for 30–35 cycles, depending on the region analyzed, using HotStar Taq DNA polymerase (Qiagen) according to manufacturer’s recommendations. When allelic SNPs were present (Supplementary Material, Table S1), PCR amplifications were performed in the presence of [α-<sup>32</sup>P]dCTP at a concentration of 1/100 of the total dCTP, and products were analyzed by SSCP electrophoresis. For each conceptus, two independent experiments were performed and, when possible, two different restriction enzymes were used (Supplementary Material, Table S1).

**Bisulphite analysis**

Bisulphite treatments were performed as previously described (57). PCR amplifications were performed for 40 cycles in 25 µl containing 50 pmol of each primer (sequences available in Supplementary Material, Table S2), 0.2 mM each dNTP, 1.25 U HotStar Taq DNA polymerase (Qiagen) in the recommended buffer and 1.5–3 mM MgCl<sub>2</sub>, depending on the product. PCR products were cloned into pGemT-easy (Promega) and sequenced on an ABI310 (Perkin–Elmer) using vector primers and DYEnamic ET Terminator cycle sequencing kit (Amersham).

For each conceptus, at least two bisulphite treatments were performed (and between three and five treatments for Dnmt3L<sup>m<sup>-/-</sup></sup> embryos where methylation was detected at DMRs: i.e. e1, e2, e8, e9, e13 and e16). Each DMR was analyzed with between one and three primer sets (Supplementary Material, Table S2), and in each bisulphite treatment, amplification for the regions analyzed was repeated at least three times. The methylation pattern of the native DNA was first assessed by restriction digestion of PCR products obtained. Briefly, following bisulphite treatment, new restriction sites are created, whereby some sites appear only if they contain a methylated cytosine in the native DNA (therefore, unconverted in the PCR product). For example, ACGC in the native sequence will become ATGT if the first cytosine is methylated. The latter results in the creation of a Tail site. Thus, digestion indicates the ‘methylation pattern’ of the population of molecules in PCR products. Undigested PCR product suggests the absence of methylation in the native sequence, whereas complete digestion indicates a high degree of methylation in the native sequence. In addition, as such sites can be found frequently in the PCR products, a partially digested pattern indicates a heterogeneous pattern of methylation among the independent strands of the native sequence. When possible, restriction digestion was performed independently using two different enzymes (Supplementary Material, Table S2). This information can be used to ensure that the population of sequences
subsequently obtained through cloning is not substantially biased towards methylated or unmethylated molecules.

For each region analyzed, which gave ‘non-methylated’ amplicons based on restriction digestion analysis, PCR products were cloned and sequenced from amplicons randomly selected from at least three independent conceptuses, giving a minimum of 24 sequences for each region (Supplementary Material, Table S3). The same logic was applied to the control H19 locus. For all regions where methylation was detected by restriction digestion, PCR products were cloned and sequenced from two independent bisulphite treatments from each trophoblast sample and at least three from each embryo. Overall, for a region where methylation was detected, a minimum of 20 sequences was obtained for a given embryo and 16 for a given trophoblast (Supplementary Material, Table S3). In all instances, these sequence patterns were in agreement with the restriction enzyme analysis, thus validating this latter approach.

Conversion efficiency was determined, where possible prior to cloning by digestion with Drai (TTTAAA) or Msel (TTAA), in PCR products and was routinely found to be very high.

Furthermore, following sequencing, we estimated the overall conversion frequency as 98.2% (ranging from 93 to 100% according to the PCR product) on the basis of the number of unconverted non-CpG sites. A random distribution of residual unconverted non-CpG cytosines allowed us to conclude that most sequences with similar CpG methylation patterns arose from independent DNA strands. In addition, the presence of SNPs in most regions analyzed (Supplementary Material, Table S2) allowed us to exclude allelic bias in amplification.

**Supplementary Material**

Supplementary Material is available at HMG Online.

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**Conflict of Interest statement**

None declared.

**References**
