



Epigenetic reprogramming in mouse primordial germ cells

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Abstract

Genome-wide epigenetic reprogramming in mammalian germ cells, zygote and early embryos, plays a crucial role in regulating genome functions at critical stages of development. We show here that mouse primordial germ cells (PGCs) exhibit dynamic changes in epigenetic modifications between days 10.5 and 12.5 post coitum (dpc). First, contrary to previous suggestions, we show that PGCs do indeed acquire genome-wide de novo methylation during early development and migration into the genital ridge. However, following their entry into the genital ridge, there is rapid erasure of DNA methylation of regions within imprinted and non-imprinted loci. For most genes, the erasure commences simultaneously in PGCs in both male and female embryos, which is completed within 1 day of development. Based on the kinetics of this process, we suggest that this is an active demethylation process initiated upon the entry of PGCs into the gonadal anlagen. The timing of reprogramming in PGCs is crucial since it ensures that germ cells of both sexes acquire an equivalent epigenetic state prior to the differentiation of the definitive male and female germ cells in which new parental imprints are established subsequently. Some repetitive elements, however, show incomplete erasure, which may be essential for chromosome stability and for preventing activation of transposons to reduce the risk of germline mutations. Aberrant epigenetic reprogramming in the germ line would cause the inheritance of epimutations that may have consequences for human diseases as suggested by studies on mouse models. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Epigenetic modifications of the genome such as DNA methylation and chromatin modifications are relatively stable in somatic cells, but are reprogrammed on a genome wide level in mammals in germ cells and in preimplantation embryos. The biological purposes of this methylation reprogramming likely include the erasure and reestablishment of parental genomic imprints in germ cells, the erasure of epimutations, and the generation of totipotent or multipotent cells (Reik et al., 2001; Surani, 2001; Rideout et al., 2001). In this reprogramming process the genome becomes demethylated by active (replication independent) or passive (replication dependent) mechanisms, and de novo methy-

lated during later stages of development. Little is known of the extent of reprogramming, and which sequences in the genome it occurs in.

Of particular significance is the reprogramming process in mammalian germ cells. Germ cells are first detected as a founder population of about 45 primordial germ cells (PGCs) in mice at 7.2 days post coitum (dpc) (Ginsburg et al., 1990). These PGCs then proliferate and migrate into the genital ridges between 10.5 and 11.5 dpc. PGCs continue to proliferate until about 13.5 dpc, when they enter into meiotic prophase in female gonads and mitotic arrest in male gonads. It is known that there is random X inactivation in XX germ cells during the migration phase of PGCs, which coincides with the time of X inactivation in somatic tissues (Tam et al., 1994). However, following the entry of PGCs into the genital ridge, the inactive X chromosome is reactivated in the majority of the PGCs by 13.5 dpc (Tam et al., 1994; Monk and McLaren, 1981).

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Despite the evident significance of reprogramming events in the germ line, little is known about the precise timing of epigenetic modifications in early PGCs. Previous work has mainly focused on the re-establishment of methylation in imprinted regions during oogenesis and spermatogenesis, or in cultured embryonic germ cells (EGCs) (Kafri et al., 1993; Tada et al., 1998; Ueda et al., 2000). The earliest time point at which the methylation of PGCs was studied by methylation sensitive PCR assays was 12.5 dpc at which the few CpGs analysed in imprinted and other single gene loci appeared to be grossly demethylated (Kafri et al., 1992). Most significantly, it remains unclear if PGCs carry normal methylation imprints and somatic methylation patterns prior to this developmental stage and if so, when and how these epigenetic patterns are reprogrammed. Indeed, there have been speculations previously that PGCs may be set aside during early development and escape from epigenetic modifications such as de novo methylation that occurs in somatic cells (Jaenisch, 1997; Monk et al., 1987).

To establish the precise timing of epigenetic reprogramming of the PGC genome, we analysed the fate of methylation in the developing PGCs in vivo at early developmental stages between 10.5 and 13.5 dpc in both male and female embryos. The evidence we present suggests that early PGCs initially possess a high level of methylation similar to somatic cells, which is rapidly erased at all single copy sequences shortly after entry into the gonadal anlagen. The observed demethylation takes place despite the presence of Dnmt1 (but not Dnmt3a and 3b) in the nuclei of PGCs.

2. Results

2.1. Reprogramming in PGCs detected by DNA methylation analysis

To follow the epigenetic changes in primordial germ cells, we first focused on methylation patterns of the differentially methylated regions (DMRs) of well-characterized maternally or paternally methylated imprinted genes. The genital ridges of transgenic embryos expressing an *Oct4-GFP* fusion protein (Yeom et al., 1996) were isolated and the PGCs purified by fluorescence-activated cell sorting. The highly purified population of PGCs were assayed for the extent of DNA methylation of a set of imprinted genes using the bisulphite genomic sequencing method (Oswald et al., 2000). Up to 10.5/11.5 dpc, we observed a substantial amount of methylated clones in most regions examined. The sequence patterns of individual clones showed an almost equal distribution of methylated and non-methylated chromosomes (see Fig. 2 as an example). This strongly suggests that in PGCs at 11.5 dpc, the DMRs of the maternally and paternally methylated genes, *Peg3*, *Lit1*, *Snrpn* (DMR1) and *H19* carry the appropriate parental epigenetic marks (Fig. 1a). Only 1 day later at 12.5 dpc, there was a striking reduction in the overall methylation state of most DMRs in the

PGCs, whereas stage matched somatic cells of the genital ridges retained normal methylation levels at this stage (Fig. 2). The demethylated state of those DMRs persisted in PGCs at 13.5 dpc as reported previously (Kafri et al., 1992; Brandeis et al., 1993). Similarly, the promoter regions of two non-imprinted genes, *mylC* and *a-actin*, were methylated to an extent comparable to somatic cells on 11.5 dpc, and also showed rapid demethylation between 11.5 and 12.5 dpc, concomitant with the loss of methylation in imprinted genes (Fig. 1a). Hence, the demethylation of single copy non-imprinted genes apparently follows the same kinetics as that observed for most of the imprinted genes. Exceptions to this rapid and synchronous demethylation kinetics of single copy sequences were found in the DMR2 of the *Igf2* gene, the 5' part of the H19 DMR and the DMR2 of *Snrpn* (see below). Whereas the *Igf2* DMR2 was clearly methylated on 10.5 dpc and became completely demethylated at 11.5 dpc, the 5' part of the H19 DMR was never found to be methylated. It is noteworthy in this context that both *Igf2* and H19 5' DMRs apparently do not represent the primary methylation imprints during early embryonic development (Oswald et al., 2000; Olek and Walter, 1997).

In order to examine if the extent and timing of the changes in DNA methylation occurred to the same extent in male and female embryos, PGCs of female and male embryos at stage 11.5 and 12.5 dpc were examined separately (see Fig. 2 for an example at 12.5 dpc). Both the timing and degree of demethylation was identical in PGCs from female and male embryos, suggesting that the initiation of this process is unaffected by the sex of the embryo. The undifferentiated male and female gonads should therefore provide an equivalent environment for epigenetic reprogramming of germ cells.

We went on to examine the fate of DNA methylation in the *Xist* promoter of PGCs isolated from male genital ridges as this gene plays a crucial role in X inactivation. We found that the kinetics of DNA methylation erasure within the *Xist* promoter was similar to that observed in all the imprinted genes. This loss of methylation seems surprising since the inactive X-chromosome becomes re-activated when PGCs enter the genital ridge, and remains so thereafter (Nesterova et al., 2002). However, demethylation of *Xist* may be necessary since the X-chromosome is also subject to imprinting in developing oocytes to ensure subsequent preferential inactivation of the paternal X chromosome in trophectoderm cells during early development (Tada et al., 2000). Furthermore, recent evidence shows that despite demethylation of the *Xist* promoter in PGCs, the *Xist* transcript decreases progressively and is extinguished in most PGCs by 13.5 dpc, despite re-activation of the inactive X in female PGCs (Nesterova et al., 2002). It seems therefore that re-activation of the inactive X chromosome is accompanied by epigenetic mechanisms other than DNA methylation. A similar suggestion has been made for the control of *Xist* expression during early embryogenesis where methylation at the promoter is apparently absent (McDonald et al., 1998)

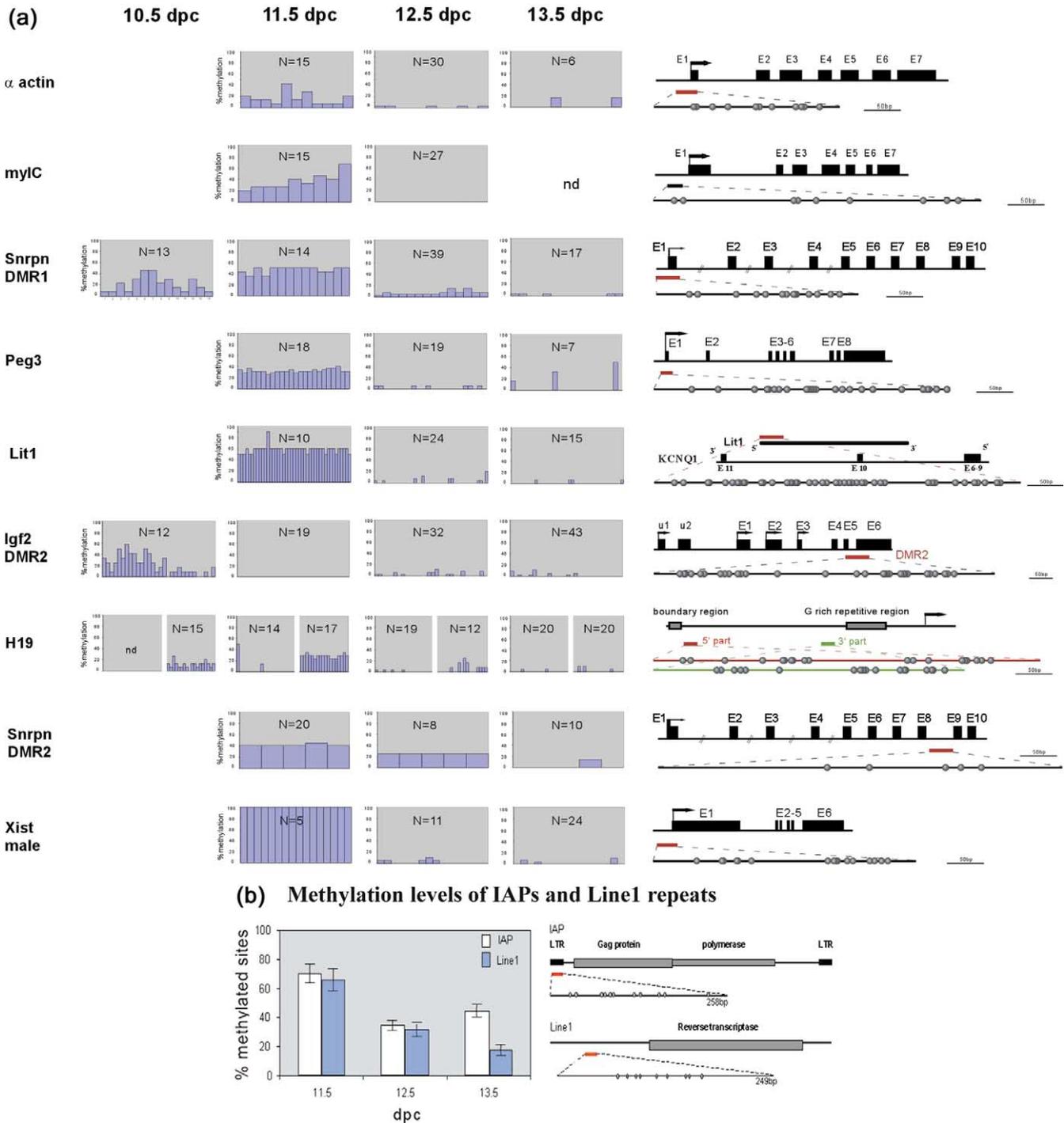


Fig. 1. Dynamics of DNA methylation changes in PGCs. The figure shows a summary of methylation at imprinted, non-imprinted (a) and repetitive sequences (b). (a) Graphical representation of relative methylation levels (in %) at individual CpG dinucleotides in the various genes. Each bar represents the sum of methylation of individually sequenced clones at single CpG positions. The number of unique clones (based on the polymorphism in bisulphite converted Cs) analysed for each individual fragment is given below the graphs. Each data set represents the sum of at least two independent bisulphite and PCR experiments. Note that the *H19* graph consists of two different parts of the *H19* upstream promoter region (left: 5' part (Olek and Walter, 1997) and right: the 3' part (Ueda et al., 2000)) which behave differently. The 3' part representing the imprinting box as defined previously (Olek and Walter, 1997). (b) Summary of methylation changes at CpGs in Line1 and IAP elements. The regions analysed by bisulphite sequencing are shown. At least two experiments were carried out for each time point (minimum number of clones analysed 27), and the error bars show standard error of the mean. Because of the variable content of CpGs in individually sequenced repetitive elements, only the sum of the total CpG methylation is given; however, methylation in most individual elements followed the population kinetics shown.

In spite of the combined observations described above, we did note that demethylation of DMR2 of *Snrpn* (Shemer et al., 1997) occurred more gradually than expected during 12.5–13.5 dpc. When examining the sequence of this DMR in more detail we found that unlike other imprinted DMRs, it does not represent a single copy sequence, because all its CpGs are contained within a repetitive Line1-like element. This prompted us to follow the fate of demethylation of other repetitive elements. Using primers against CpG-rich regions within IAPs and Line1 elements, we randomly cloned and sequenced elements of the developmental stages 11.5–13.5 dpc (at least two experiments were performed at each stage, and a minimum of 27 clones were sequenced per stage and experiment; Fig. 1b). We found that these

elements were as highly methylated in PGCs as in somatic cells from 11.5 dpc. While the overall DNA methylation diminished protractively between 11.5 and 13.5 dpc in these collectively analysed repetitive elements, and in Line1 element in particular, a substantial proportion of CpGs in IAPs remained methylated even at 13.5 dpc (Fig. 1b). This finding contrasts with a previous study (Walsh and Bestor, 1999), which suggested that IAP elements are almost completely demethylated by 13.5 dpc. The difference may be explained by our use of the more sensitive bisulphite technique for the analysis of DNA methylation. Furthermore, to assess the methylation changes at a global level, we performed immunohistochemical staining using a monoclonal antibody against 5-mC. We observed a high

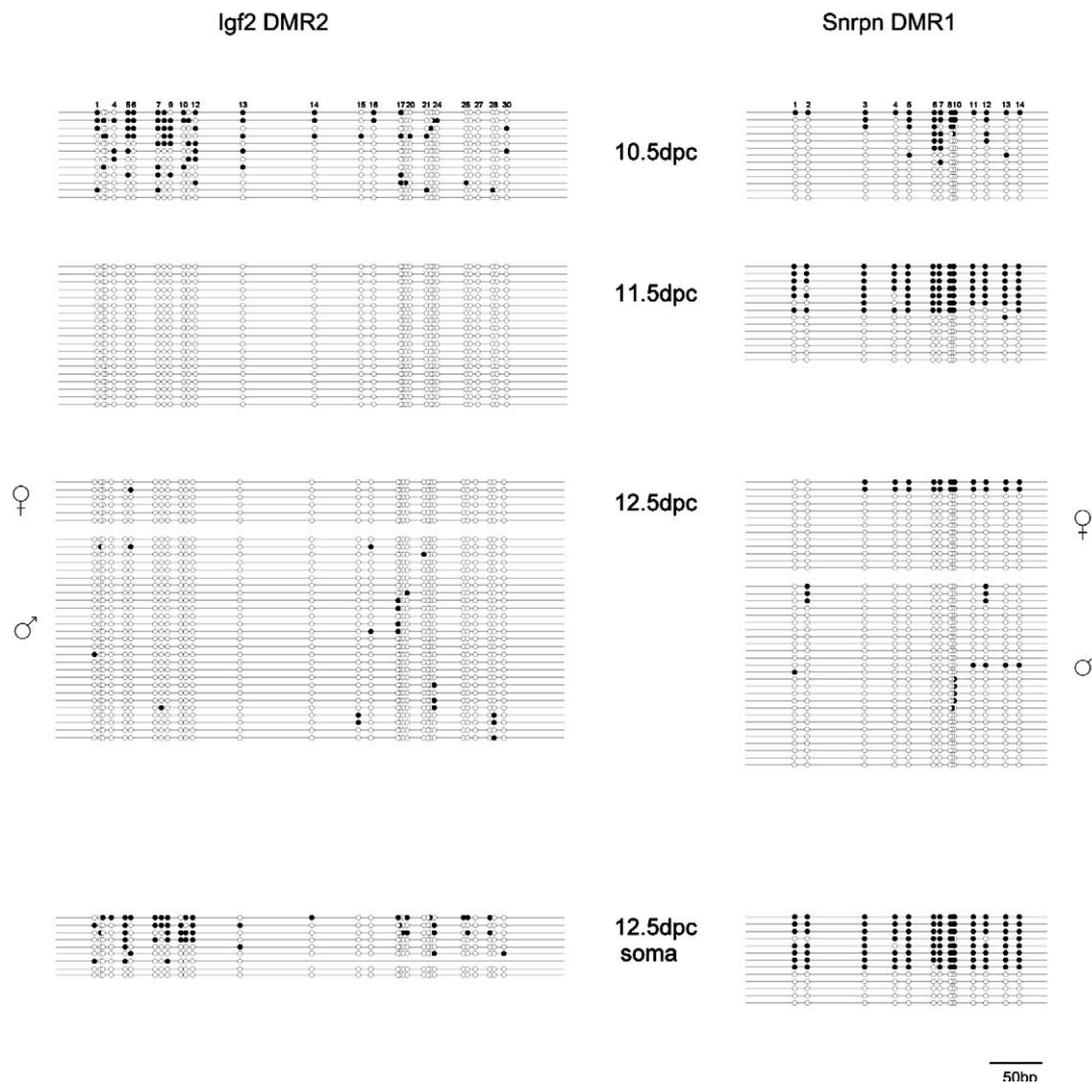


Fig. 2. Bisulphite sequencing profiles of the *Igf2* and *Snrpn* genes. The individual profiles of sequences of the imprinted *Igf2* and *Snrpn* genes with every CpG dinucleotide represented by a circle. Filled circles represent the methylated CpGs, open circles non-methylated CpGs. The parental alleles could not be distinguished in this experiment. All PCR conditions were tested for an unbiased amplification of methylated and unmethylated alleles (data not shown, see also Oswald et al., 2000; El-Maarri et al., 2001). The presence of an equal distribution of methylated and unmethylated clones in the samples from somatic cells of the genital ridges at 12.5 dpc, shows that the observed demethylation in PGCs is specific. PGCs from both male and female embryos showed similar changes in DNA methylation observed in male and female PGC, and therefore combined in the graphs of Fig. 1a.

frequency of mC-positive foci in PGCs at 11.5 dpc comparable to somatic cells, and a considerable reduction of mC positive foci at 13.5 dpc (data not shown). This result confirms that early PGCs possess levels of DNA methylation that are similar to somatic cells, which gradually diminishes from 11.5 dpc onwards.

2.2. Localization of Dnmts in PGCs

The different dynamics of demethylation of single copy genes and repetitive elements led us to investigate the status and localization of the three major DNA methyltransferases (Dnmts) (Li et al., 1992; Okano et al., 1999). We found Dnmt1 to be highly expressed and present in the nuclei of more than 95% of PGCs between 10.5 and 13.5 dpc. The level of Dnmt1 signal in somatic cells appeared to be less intense than in PGCs. In contrast, the de novo methyltransferase, Dnmt3a, was absent in PGCs. While Dnmt3b was highly expressed in PGCs at all the stages examined, it was located predominantly in the cytoplasm (Fig. 3).

3. Discussion

Our study documents for the first time and comprehensively, the process of erasure of DNA methylation in mouse primordial germ cells. We show that PGCs before they colonize gonads are substantially methylated, which corre-

sponds to the pattern in somatic cells. This includes a normal pattern of methylation associated with imprinted genes. These observations prove conclusively that PGCs are not exempt from genome wide de novo methylation which begins in inner cell mass cells of the blastocyst (Santos et al., 2002). The analysis also shows that methylation imprints are initially inherited and maintained in PGCs. Rapid demethylation then occurs around the time of entry into the gonads. This rapid reprogramming is apparently selective and only affects single copy imprinted and non-imprinted genes, whereas the reprogramming of repetitive elements is more protracted and incomplete. While there was considerable variability between individual Line1 and IAP copies, most of the individual elements followed the general pattern shown in Fig. 1b. It will be important to follow the fate of individual elements in future experiments, to see whether a specific subset/class of repetitive elements remains refractory to reprogramming. The protracted demethylation of (some) repetitive elements may be necessary to prevent transcriptional activation of the transposable elements, since this would increase the risk of germline mutations through dysregulation of adjacent genes and through transposition. This contention is supported by the observation that there is no substantial transcription of IAP proviruses observed in PGCs at any stage (Walsh et al., 1998).

It has previously been postulated that a major function of

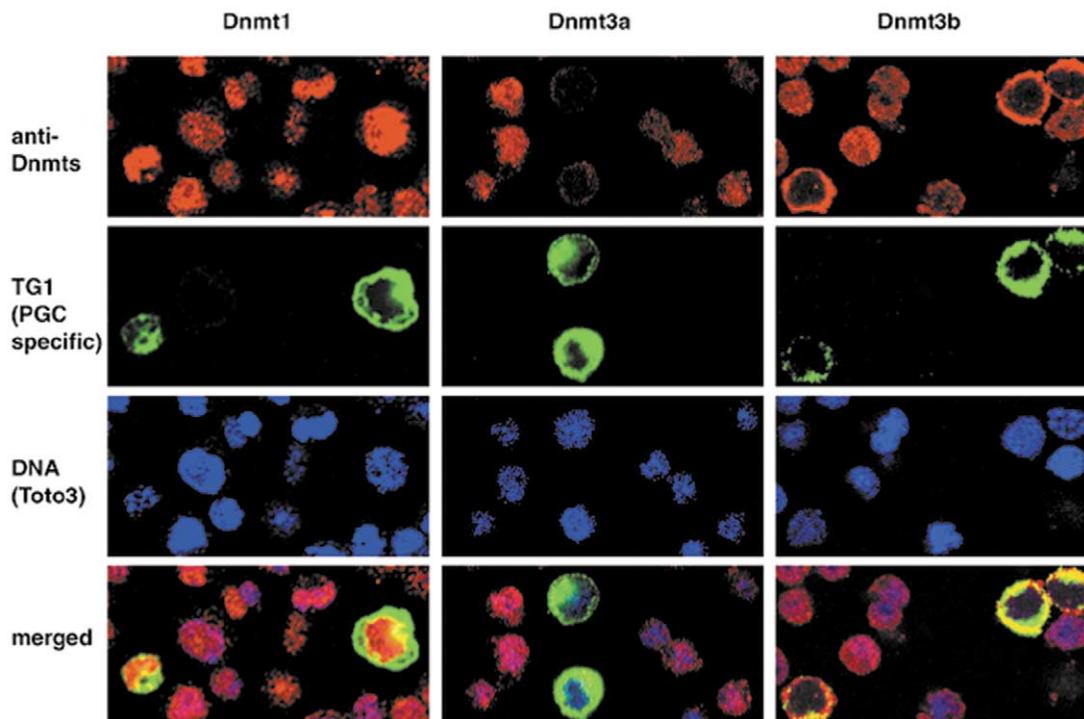


Fig. 3. Anti-DNA-methyltransferase stainings of cell suspensions prepared from whole genital ridges at 12.5 dpc (Donovan et al., 1986). Anti-Dnmt1, -Dnmt3a and -Dnmt3b staining (in red) is shown in conjunction with anti SSEA1 (TG1, a germ cell-specific marker, in green) and DNA staining (in blue). Dnmt1 is highly expressed in most (>95%) PGCs and expressed in lower levels in surrounding somatic cells. Dnmt3a is expressed in somatic cells. The very weak staining of Dnmt3a in PGCs is probably due to non-specific cross-reaction, or it represents very low expression of Dnmt3a in PGCs compared to the expression in somatic cells. Dnmt3b is expressed in all cells, but in contrast to the nuclear localization in somatic cells, it is predominantly located in the cytoplasm.

DNA methylation, besides its role in gene regulation, is to safeguard genome integrity by preventing expression and activity of transposable elements in the germ line (Yoder et al., 1997). The insertion of a Line1 element into the DMR2 of *Snrpn* shows that retrotransposable sequences elements may be more resistant to demethylation even in the environment of a single copy gene. These highly discriminatory mechanisms might sometimes lead to aberrant reprogramming, as in the case of the Agouti locus, where normal demethylation is apparently perturbed by the insertion of the IAP element into a single copy gene control region (Morgan et al., 1999). Some demethylation however occurs in these elements despite the presence of Dnmt1, perhaps because of the absence of the de novo DNA methyltransferases, Dnmt3a and Dnmt3b, from the nuclei of PGCs. Indeed, it has been shown recently that Line 1 elements may require Dnmt1, as well as Dnmt3a and Dnmt3b, for the maintenance of high levels of methylation (Liang et al., 2002).

The kinetics of synchronous demethylation of most single copy loci in PGCs between 11.5 and 12.5 dpc may be even more rapid than the 24-h sampling interval we have used. Another recent study also demonstrates that demethylation of imprinted genes is complete by 12.5 dpc and that major changes occur between 11.5 and 12.5 dpc (Lee et al., 2002). In contrast to our work the authors found that the demethylation event is more heterogeneous, with some PGCs losing methylation as early as 10.5 or 11.5 dpc. These differences might be due to the differences in the mouse strains between our studies, or more likely, due to a different approach involving transplantation of PGC nuclei into oocytes used in their studies. There is a possibility of some additional epigenetic modifications following transplantation of PGC nuclei into oocytes in these experiments. Nevertheless, the consistent observation of completeness of reprogramming within 1 day of development, suggests a rapid and probably active process of demethylation. Two observations support this notion. First, the massive loss of DNA methylation cannot be a consequence of several rounds of DNA replication since the cell cycle time of PGCs is 16 h (Tam and Snow, 1981). Second, demethylation occurred despite the presence of Dnmt1 in the nucleus, which also strongly argues against a simple passive loss of DNA methylation. In light of our data, we furthermore postulate that demethylation of the somatic nuclei fused with embryonic germ cells (Tada et al., 1997), may also be due to a dominant active process. Active demethylation has also been observed predominantly of the paternal genome in the zygote, which is apparently unique to mammals (Oswald et al., 2000; Mayer et al., 2000; Dean et al., 2001). However, in contrast to the PGCs, active demethylation in the zygote does not affect DNA methylation of the control regions of imprinted genes (Oswald et al., 2000; Mayer et al., 2000). These differences could be due to the variations in the accessibility of the chromosomal regions in PGCs and zygotes. Alternatively, the demethylating activities might be different as a result of distinct enzymatic complexes. It is there-

fore important to discover the role of demethylation as well as the nature of this key modifier, which is of wider interest in the context of genomic plasticity, pluripotency of stem cells, and cloning (Reik et al., 2001; Surani, 2001).

The reprogramming of imprints and the *Xist* promoter in PGCs is evidently crucial for mammalian development, which might not apply to the non-mammalian species, which do not exhibit genomic imprinting. The timing of demethylation in PGCs upon their entry into the genital ridge is also particularly noteworthy. This reprogramming may be initiated in response to an intrinsic developmental clock, or in response to a signal from the somatic cells in the genital ridge. The former is supported by the finding that in EG cells, demethylation and X-reactivation occur precociously (Tada et al., 1998), although this could be in response to some unknown in vitro culture conditions involving potential signals and factors emanating from feeder cells. In contrast, the finding that female PGCs that fail to enter the gonad anlagen do not reactivate their X chromosome, supports the notion that the entry of germ cells into the gonads may be required for epigenetic reprogramming (Tam et al., 1994). We speculate that demethylation in vivo might be initiated in response to signal(s) from somatic cells of the undifferentiated bipotential gonadal anlagen, when PGCs enter the genital ridge (Donovan et al., 1986). Such an exquisite control over the timing of genomic reprogramming would be an advantage, since it would ensure that PGCs have an equivalent epigenetic state prior to the development of definitive gonads, and the subsequent mitotic or meiotic arrest of germ cells in male and female gonads, respectively (Fig. 4). Further studies are needed to distinguish between the intrinsic clock versus the somatic signal involved in the onset of the crucial epigenetic reprogramming in PGCs.

4. Experimental procedures

4.1. Germ cell preparation

Primordial germ cells were isolated from whole embryonic genital ridges at different stages of development. Embryos were obtained from crosses between MF1 females and Oct4-GFP transgenic males (F1-129 background) (Oct4-GFP construct was a kind gift of H. Schoeler) (Yeom et al., 1996). In this mixed genetic background (MF1 × F1-129), the number of PGCs recovered were between 500 and 10 000 per embryo in 10.5–13.5 dpc, respectively, depending on the developmental stage. Embryos were staged by following the detection of the vaginal plug (0.5 dpc), and by morphological appearance of embryos. PGCs were sorted in batches of 200 cells, spun down and frozen immediately in dry ice. PGCs were sorted by virtue of the expression of Oct4-GFP reporter transgene in germ cells, using a MoFlo (Cytomation Bioinstruments GmbH, Freiburg im Breisgau, Germany). The purity of PGCs was verified by tissue non-specific alkaline phosphatase staining (Sigma Diagnostics

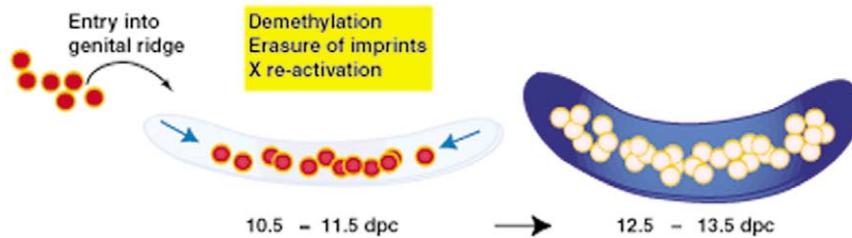


Fig. 4. Demethylation of PGCs in vivo. At 10.5 dpc, the PGCs are migrating towards the forming genital ridges (blue). Imprinted and non-imprinted genes as well as DNA repeats show the same methylation pattern (indicated by red nuclei) in PGCs as in somatic cells. At 11.5 dpc, most PGCs have reached their final destination and are undergoing reprogramming, perhaps in response to a specific signal(s) from the genital ridge (indicated in blue). By 12.5 dpc, methylation of single copy genes is erased (faint red nuclei) and only some methylation on repetitive elements remains by the time PGCs enter mitotic/meiotic arrest at 13.5 dpc.

R86), which was found to be always in excess of 95%. Embryos of 12.5–13.5 dpc were sexed according to the distinct morphology of the gonads, and by the use of a polymerase chain reaction (PCR)-based assay for gonads obtained at 11.5 dpc. (Chuma and Nakatsuji, 2001).

4.2. Bisulphite treatment

The isolated PGC cells (batches of < 200 cells) were embedded in agarose, lysed and the chromosomal DNA subjected to the bisulphite treatment followed by gene specific PCR amplifications (Oswald et al., 2000). The PCR products were gel purified using QiaexII (Qiagen), ligated into pGEM cloning vector (pGEM-T vector cloning system I, Promega), or pCR-TOPO 2.1 cloning vector (TOPO cloning kit, Invitrogen) for the repeat elements, and transformed into TOP10 (Invitrogen) *Escherichia coli* cells. Positive clones were verified by colony PCR or restriction analysis and the products sequenced using standard methods. Primers and conditions used for the gene specific amplifications: The primer and conditions used for *Mylc*; *Igf2*, *Snrpn* DMR1 and the *H19* 5' and 3' region were described previously (Oswald et al., 2000; El-Maarri et al., 2001; Olek and Walter, 1997; Ueda et al., 2000).

4.2.1. Amplification of α -actin

Amplification of α -actin was carried out as described previously with the use of the additional primer F2 ggttttagt-tattgggttagggt for a semi-nested PCR (Oswald et al., 2000).

4.2.2. Amplification of *Lit1* (AJ271885, pos. 141 776–141 223)

Primers and conditions: F1: tattatttgggtgtggttatatcgggta, R1: atttttctcaacacccttctttccct, F2: ggggtataaagttagggttttaggatt, R2: aaactttctatcaactaattcccaac; 1st PCR: F1/R1; 2nd PCR: F2/R2. PCR conditions: 95 °C 5 min, 95 °C 1 min, 59 °C 90 s (2nd PCR at 58 °C), 72 °C 90 s, 72 °C 10 min (30 cycles for each PCR).

4.2.3. Amplification of *Snrpn* DMR2

Primers and conditions: F1: gtgtaagttggtataatattat, R1: aattaaaaaaataaaccaacaataaca, F2: aaaaaataattcttatacta-

taaac; 1st PCR (35 cycles): F1/R1; 2nd PCR (35 cycles): F2/R1. PCR conditions: 95 °C 5 min, 95 °C 60 s, 59 °C 90 s (2nd PCR at 57 °C), 72 °C 90 s, 72 °C 10 min.

4.2.4. Amplification of *Peg3* (AF105262, pos. 2597–3125)

Primers and conditions: F1: ttttagatttgggtttttaaata, R1: aatccctatcacctaaataacatccctaca, F2: ttgataatagtagttgattggtagggtgt, R2: atcacaaccttcaattacccttaaaaa; 1st PCR (30 cycles): F1/R1; 2nd PCR (30 cycles): F2/R2. PCR conditions: 95 °C 5 min, 95 °C 1 min, 61 °C 90sec (2.PCR at 59 °C), 72 °C 60 s, 72 °C 10 min.

4.2.5. Amplification of *Xist* promoter region (U29341, pos. 823–1282)

Primers and conditions: F1: tggttgtttaagtagaagatatattg, R1: aaaaactctacaaacatatcaaac; F2: gtatagataggtgtgtgatttaagt, R2: ttaatatattttctaaataaac; 1st PCR (35 cycles): F1/R1; 2nd PCR (35 cycles): F2/R2. PCR conditions: 95 °C 5 min, 95 °C 1 min, 61 °C 90 s (2nd PCR at 59 °C), 72 °C 90 s, 72 °C 10 min.

4.2.6. Amplification of *IAP* LTRs (M17551, pos. 41–315)

Primers and conditions: F1: ttgatagttgttttaagtggtataataa, R1: caaaaaaacacacaacaaaat, F2: ttgtgtttaagtggtataataaatttg, R2: aaaacaccacaacaaaactctctac; 1st PCR (30 cycles): F1/R1; 2nd PCR (30 cycles): F2/R2. PCR conditions: 94 °C 3 min, 94 °C 1 min, 53 °C 1 min (2nd PCR at 53 °C), 72 °C 1 min, 72 °C 5 min.

4.2.7. Amplification of *Line1* (D84391, 5' end pos. 975–1155)

Primers and conditions: F1: taggaaattagttgaataggtgagaggt, R1: caaaacaaaacctttctcaaacatatat, F2: gttagagaatttgatagtttttggaatagg; R2: tcaaacactatattactttaacaattccca; 1st PCR (30 cycles): F1/R1; 2nd PCR (30 cycles): F2/R2. PCR conditions: 94 °C 3 min, 94 °C 1 min, 53 °C 1 min (2nd PCR at 53 °C), 72 °C 1 min, 72 °C 5 min.

4.3. Methyltransferase staining

Whole embryonal genital ridges were treated with trypsin to prepare single cell suspension. The cells were allowed to

settle on poly-L-lysine coated slides, fixed with 4% PFA, washed three times with PBS and permeabilised in AB buffer (1% Triton X-100, 0.2% SDS, 10 mg/ml BSA in PBS) for 30 min. The following incubation with methyltransferase specific antibody Dnmt1 (a kind gift of T. Bestor), and Dnmt3a and anti Dnmt3b (kind gifts of E. Li) was carried out at 4 °C overnight. PGCs were identified by specific TG1 antibody staining against the germ cell-specific surface marker SSEA1. Slides were subsequently washed with AB buffer, incubated with secondary antibodies (goat anti-rabbit Alexa 564, goat anti-mouse Alexa 488, Molecular probes) for 1 h. The slides were washed three times in PBS for 5 min with 100 µg/ml RNaseA in the last wash and overlaid with Vectashield mounting medium (Vector Laboratories) containing TOTO3 DNA staining (1:2500, Molecular probes). Immunofluorescence was visualized on a BioRad Radiance 2000 confocal microscope.

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