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Dynamic Reprogramming of DNA Methylation in the Early Mouse Embryo

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Dynamic epigenetic modification of the genome occurs during early development of the mouse. Active demethylation of the paternal genome occurs in the zygote, followed by passive demethylation during cleavage stages, and *de novo* methylation, which is thought to happen after implantation. We have investigated these processes by using indirect immunofluoresence with an antibody to 5-methyl cytosine. In contrast to previous work, we show that demethylation of the male pronucleus is completed within 4 h of fertilisation. This activity is intricately linked with and not separable from pronucleus formation. In conditions permissive for polyspermy, up to five male pronuclei underwent demethylation in the same oocyte. Paternal demethylation in fertilised oocytes deficient for MBD2, the only candidate demethylase, occurred normally. Passive loss of methylation occurred in a stepwise fashion up to the morulae stage without any evidence of spatial compartmentalisation. *De novo* methylation was observed specifically in the inner cell mass (ICM) but not in the trophectoderm of the blastocyst and hence may have an important role in early lineage specification. This is the first complete and detailed analysis of the epigenetic reprogramming cycle during preimplantation development. The three phases of methylation reprogramming may have roles in imprinting, the control of gene expression, and the establishment of nuclear totipotency.

Key Words: **demethylation; active; passive; paternal; maternal resistance; epigenetic; mouse.**

INTRODUCTION

DNA methylation at CpG dinucleotides plays an important role in regulation of gene expression and is essential for normal mammalian development (Robertson and Wolffe, 2000). In general, DNA methylation functions in the regulation of specific genes through a transcriptional repression mechanism thought to be mediated in part by methyl-CpG binding proteins (MBDs) (Bird and Wolffe, 1999). Furthermore, DNA methylation is important in the sex-specific germline "marking" of parental alleles that contribute to appropriate expression of many imprinted genes (Latham *et al.*, 1995; Reik and Walter, 2001a). One specific function for DNA methylation includes the silencing of CpG-rich retrotransposons as part of a genome-wide defence system to silence their expression providing chromosome stability and structural integrity (Yoder *et al.*, 1997).

Genome-wide loss of DNA methylation is observed during early mouse development reaching a low point during the blastocyst stage (Monk *et al.*, 1987; Howlett and Reik, 1991; Kafri *et al.*, 1992). The earliest phase of this demethylation is confined to the paternal pronucleus, prior to DNA replication (Mayer *et al.*, 2000a; Oswald *et al.*, 2000), and has been described as "active" demethylation. After the completion of the first cell cycle, DNA methylation continues to decline due to the absence of the maintenance methylase, Dnmt1 (Carlson *et al.*, 1992; Howlett and Reik, 1991; Monk *et al.*, 1991; Rougier *et al.*, 1998). This is referred to as the passive phase of demethylation. During this time, methylation declines in housekeeping genes and repeat sequences throughout the genome. Remarkably, imprinted genes are largely exempt from this process and maintain their methylation. Not long after implantation, DNA methylation is restored and maintained thereafter in somatic lineages (Monk *et al.*, 1987). This restoration of methylation is carried out by Dnmt 3a and 3b, the so-called *de novo* methylases (Okano *et al.*, 1999), but the precise time point and lineage specificity for *de novo* methylation are not known.

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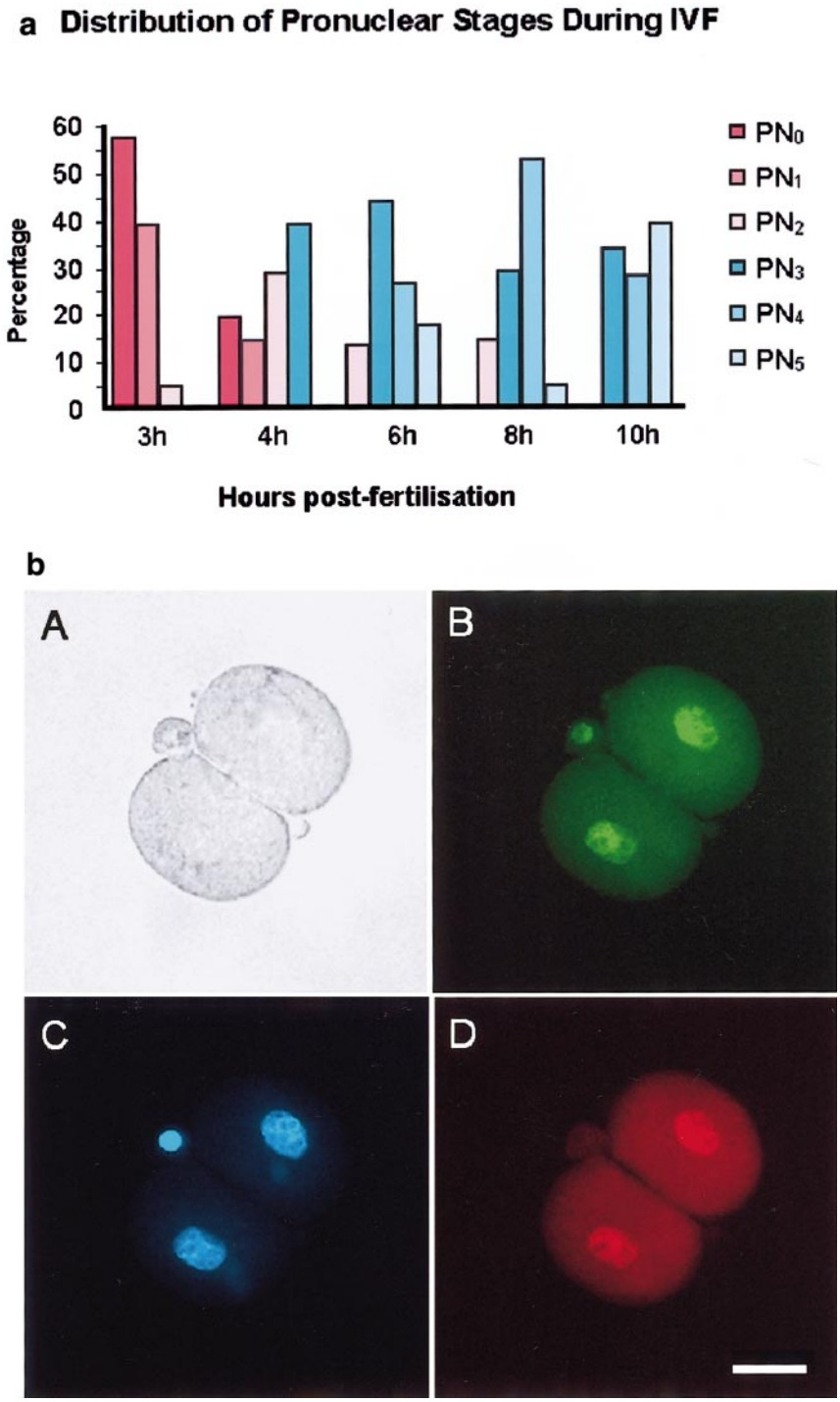

FIG. 1. (a) Distribution of pronuclear stages as a result of IVF procedures. In order to describe the kinetic pattern of demethylation, fertilised oocytes were sampled at intervals that permitted a profile of pronuclear maturation stages as a function of time to be constructed. These stages are described in terms of methylation status in Fig. 2a. These results establish sampling windows for descriptive and mechanistic evaluations. (b) Antibody accessibility was evaluated in two-cell embryos fixed and prepared after labelling with BrdU. (A) Pseudo-DIC optical image of an embryo after the standard fixation procedure. (B) A two-cell embryo stained for 5-MeC distribution using an FITC-conjugated secondary antibody. Note that the polar body is positive for methylation signal. (C) Embryos were counterstained with DAPI to identify the nuclear compartment. (D) Immunofluorescent detection of BrdU-labelled nuclei confirmed antibody accessibility. Note that the polar body is negative in this micrograph consistent with the absence of replication. Scale bar, 20 μm.

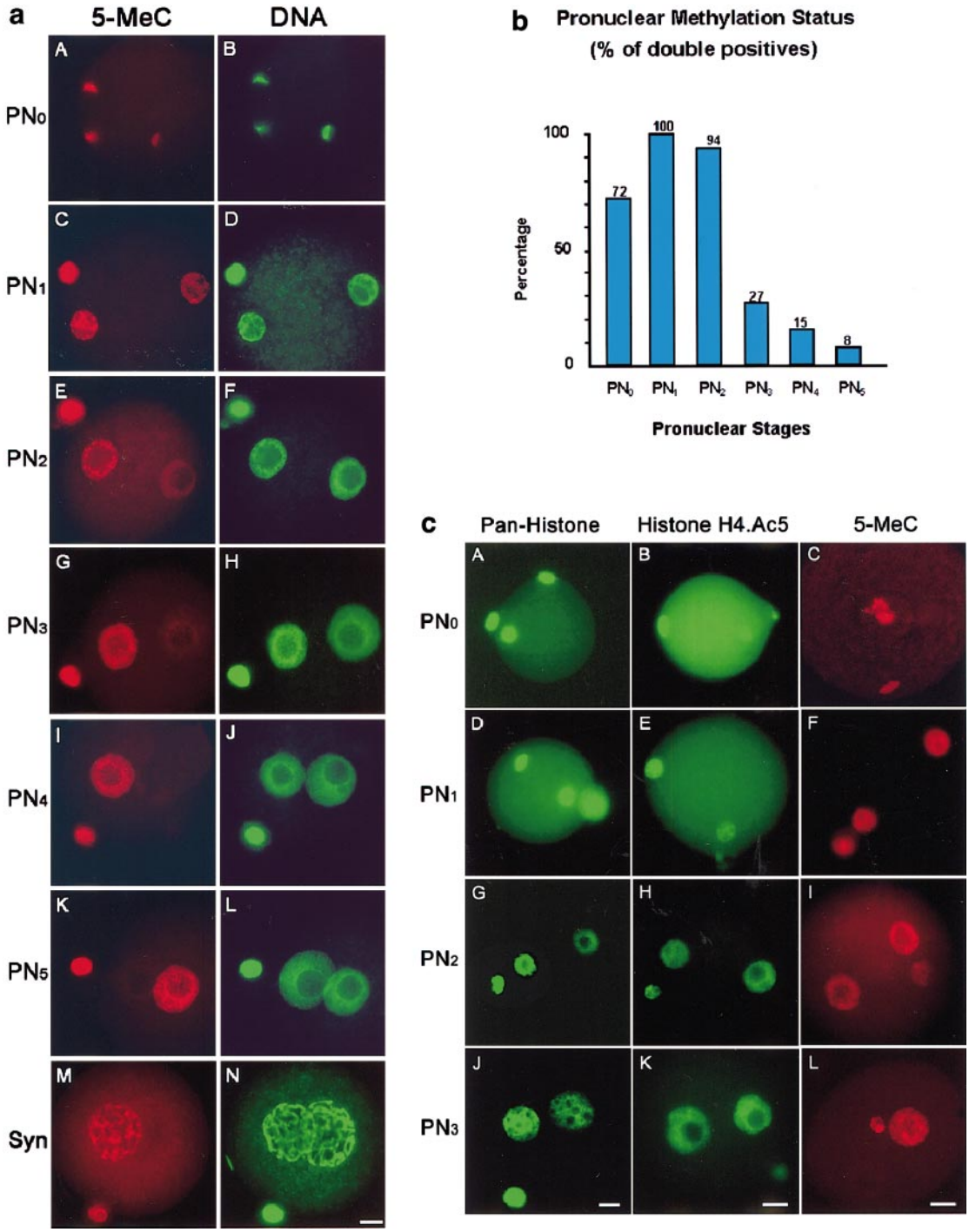

FIG. 2. (a) Distribution of CpG methylation during pronuclear stages in the mouse fertilised oocyte. Indirect immunofluoresence of dynamic methylation changes are portrayed (red; A, C, E, G, I, K), while the pronuclei and polar body were resolved by using YOYO-1 (green; B, D, F, H, J, L). In the mouse, the identity of the pronuclei can be easily assigned as the female is the smaller of the two and resides most often in close proximity to the polar body. In contrast, the male pronucleus is the larger of the two. (A, B) Immediately on sperm entry, the completion of meiosis is triggered and respective haploid pronuclei begin to form. Decondensation of the sperm (right-hand side) and the elimination of the second polar body proceeds quickly. At this stage, all three haploid genomes are highly condensed. (C, D) Amongst the most advanced samples collected within three hours of fertilisation, the pronuclei appear very similar in size, yet the differences in intensity of methylation are already evident. (E, F) Within 4 h post fertilisation, the male pronucleus has undergone considerable demethylation while the female appears resistant to this activity. (G, H) Within 6 h, the pronuclei have reached their maximum size and

Perhaps the most intriguing feature of methylation reprogramming is the asymmetric loss of methylation by the male pronucleus after fertilisation. An enzyme responsible for this active demethylation in newly fertilised oocytes has not been isolated, but recently a candidate for a demethylase *in vitro*, MBD2, has been identified (Bhattacharya *et al.*, 1999). In an attempt to understand this process and to gain mechanistic insights, we were interested in characterising the precise timing of this active demethylation in the mouse, and to investigate the involvement of MBD2 *in vivo*. Here we present evidence that this active process is extremely rapid as measured in *in vitro* fertilised (IVF) embryos. Examination of metaphase chromosomes from the respective male and female pronucleus suggests that some sequences in the male pronucleus resist active demethylation. We were also interested in establishing the outcome of the passive phase of demethylation with respect to the spatial distribution in the interphase nucleus. We find that during passive demethylation the signal is not compartmentalised as previously reported (Mayer *et al.*, 2000b), but rather that it is random from the two-cell stage onwards. Contrary to previous expectations, we find that *de novo* methylation completes the cycle of methylation reprogramming during preimplantation development, since it clearly occurs in the inner cell mass (ICM) but not the trophectoderm at the blastocyst stage. These results represent the first complete description of the temporal and spatial alterations of methylation during preimplantation development in the mouse.

MATERIALS AND METHODS

Collection of Mouse Oocytes and Embryos

Mouse embryos were collected from superovulated females on appropriate days for cleavage-stage embryos according to standard procedures (Hogan *et al.*, 1994). Embryos used throughout this study were derived from a cross of (C57BL/6 × CBA/Ca) F_1 females, hereafter referred to as $B6CBAF_1$, mated to $B6CBAF_1$ males. The day after mating is termed day 1. Embryos were also obtained from natural matings between *Mbd2* (−/−) females and *Mbd2* (+/+) males, and between *Mbd2* (+/+) females and *Mbd2* (−/−) males. Mice used for these experiments were derived from a (C57BL/6 × 129/Ola) hybrid line.

Precise timing of progression of the pronuclei through stages was made possible by fertilising them *in vitro*. Unfertilised oocytes used in IVF were collected from B6CBA F_1 mice approximately 18 h post human chorionic gonadotropin (hCG) injection. For IVF generation of fertilised oocytes, the procedure followed was that described in Summers *et al.* (1995) with the exception that sperm capacitation was carried out in modified KSOM (Lawitts and Biggers, 1993) containing 5.56 mM glucose; initially, in the absence of BSA, and then in the presence of 15 mg/ml embryo tested BSA (Sigma). Two-, four-, eight-celled embryos, morulae, and blastocysts were collected following IVF fertilisation and culture in KSOM under standard conditions (Hogan *et al.*, 1994). The data represented herein comprise the collective results of 10 independent IVF experiments.

In order to generate polyspermic fertilisation of oocytes by IVF, oocytes were collected at 18 h post hCG and the zonae removed. These oocytes were incubated for 15–30 min, according to the sperm concentration, in a standard IVF drop. Oocytes were washed extensively and cultured for approximately 4, 6.5, and 9.5 h after exposure to sperm. In this way, conditions were established which permitted a highly controlled degree of polyspermy, ranging from normal single sperm penetration up to five extra sperm.

Fertilised oocytes were collected from $B6CBAF_1$ mice on day 1 and placed into culture until 32 h post hCG in order to sample embryos at syngamy.

BrdU Labelling

Fertilised oocytes and early preimplantation mouse embryos were pulse-labelled *in vitro* with 5-bromo-deoxyuridine (BrdU) in order to evaluate antibody accessibility. The zygotes were incubated in KSOM medium supplemented with 100 μM BrdU (Sigma) for 1 h at 37°C in a 5% CO_2 in air. After incubation, the zygotes were rinsed in PBS, fixed for 15 min in 4% paraformaldehyde in PBS, and permeabilized with 0.2% Triton X-100 in PBS for 15 min at room temperature.

Immunofluorescence Staining

Fertilised oocytes and early preimplantation embryos were washed in PBS, fixed for 15 min in 4% paraformaldehyde in PBS, and permeabilized with 0.2% Triton X-100 in PBS for 15 min at room temperature. The zygotes were blocked overnight at 4°C in 1% BSA, 0.05% Tween 20 in PBS. Cells were incubated for 1 h at room temperature with either a monoclonal antibody anti-pan histone (Roche), or with a rabbit polyclonal anti-H4Ac5 (R41; Stein *et al.*, 1997) and a secondary antibody coupled with fluorescein (Jackson Immunoresearch/Dako). For the detection of either BrdU-

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the male appears completely demethylated. (I, J) The pronuclei move closer together and (K, L) oppose one another immediately prior to syngamy, 8 h. (M, N) At syngamy, the methylation status of the male (negative) and female (positive) pronuclei appear clear. Scale bar, 10 μm. (b) Methylation distribution as a function of pronuclear maturation. Fertilised oocytes generated by IVF were scored according to stages, assessing size and morphology of pronuclei. Following staining with 5-MeC antibody, fertilised oocytes were scored for positive signals in both pronuclei (double positives). The total number of oocytes scored to produce this profile was $PN_0 = 36$, $PN_1 = 26$, $PN_2 = 31$, $PN_3 = 56$, $PN_4 = 26$, $PN_5 = 13$. (c) Chromatin remodelling immediately following fertilisation and during pronuclear maturation. Fertilised oocytes were collected at regular intervals and evaluated for the extent of chromatin remodelling according to pronuclear maturation stages. (A) Immediately following fertilisation, sperm complexed with protamine acquires histones from the maternal cytoplasm (B). These histones are already acetylated when newly acquired by the decondensing sperm nucleus. (C) All nuclear compartments are positive for methylation at this early stage. In subsequent stages, pan histone (D, G, J) and acetylated histone H4 (E, H, K) remain constant in each pronucleus. In contrast is the diminishing methylation signal in the male pronucleus (F, I, L).

labelled DNA or 5-methyl-cytosine (5-MeC), zygotes were treated with 2 M HCl at room temperature for 30 min and subsequently neutralised for 10 min with 100 mM Tris/HCl buffer, pH 8.5, after permeabilisation. After extensive washing with 0.05% Tween 20 in PBS, zygotes were blocked overnight at 4°C in 1% BSA, 0.05% Tween 20 in PBS. Anti-5-MeC antibodies (Reynauld *et al.,* 1991) and anti-BrdU antibodies (Sigma) were detected by a secondary antibody coupled with either Cy3, or Texas Red and fluorescein, respectively (Jackson Immunoresearch). Double antibody staining (BrdU and 5-MeC) was accomplished by sequential incubation with first and second round primary and secondary antibodies. DNA was stained with either the intercalating dye YOYO-1 iodide (Molecular Probes, Inc.) at 100 nM or 3 μg/ml 4,6-diamidino-2-phenylindole (DAPI) and zygotes were mounted in 50% glycerol in PBS (Sigma).

Digital Imaging Microscopy

Observations were performed with an Olympus BX40 epifluorescence microscope. Images were recorded digitally with a high-resolution CCD camera (F-View) by using the analySIS 3.0 image analysis software (SIS GmbH). Greyscale images were pseudo-coloured after capture by using separate filter sets for FITC/YOYO-1, CY3/Texas Red, and DAPI and merged with Adobe Photoshop 5.0 software.

Confocal Microscopy

Digital optical sections from both fertilised oocytes and early preimplantation embryos were recorded with a confocal laser-scanning microscope Ultraview (Perkin-Elmer). For each wavelength, a z-series of 0.2-μm slices were scanned and exported as 8-bit TIFF files. The images were later projected by using ImageJ 1.19z and pseudo-coloured by using Adobe Photoshop 5.0.

RESULTS

Timing of Active Demethylation

In order to provide a precisely defined time point for fertilisation and monitor the rate of active demethylation, mouse oocytes were fertilised *in vitro*. Genomic methylation was visualised by using indirect immunofluoresence with an antibody to 5-methyl cytosine. The reliability of this technique has been firmly established by a number of independent studies. The antibody has been well characterised and used with success in several laboratories (Reynaud et al.,1991; Miniou *et al.,* 1994; Niveleau *et al.,* 1994; Tweedie *et al.,* 1997).

The progression of the decondensation of the fertilising sperm can be clearly followed. The overall pattern of change in the pronuclei has been assigned staged terms by using the nomenclature of Adenot *et al.* (1997). We have extended this terminology to include the very recently fertilised oocyte that is referred to as PN_0. *In vitro* fertilised oocytes were scored from groups taken between 3 and 10 h post fertilisation and assigned to one of the six groups, PN_0–PN_5 (Fig. 1a).

Previous reports employing a similar approach failed to establish that negative results could not be attributed to inaccessibility of the antibody for the 5-methyl C moiety (Mayer *et al.,* 2000a) as the anti-DNA antibody does not work under conditions compatible with 5-methyl C detection. The compatibility of the fixation procedure for the detection of another nuclear antigen, in addition to 5-methyl cytosine, within the same embryo was therefore an important requirement for this study. The most useful and compatible antigen identified was bromodeoxyuridine, a nucleotide analogue (BrdU), which sits in the major groove (as does the methyl group). However, as the fertilised zygote does not enter S phase until after demethylation has taken place, the two-cell embryo was elected as an ideal stage in which to demonstrate antibody access to the nucleus (Fig. 1b). Accessibility of the nucleus in the two-cell embryo was demonstrated by the signal provided after indirect immunofluoresence with an antibody for 5-methyl cytosine (Fig. 1b, B) and BrdU (Fig. 1b, D). The nucleus was identified on the basis of its staining with DAPI (Fig. 1b, C). Note that the polar body acts as a reliable positive control as it stains for 5-methyl C but not for BrdU incorporation. Another useful feature of this fixation procedure is that the spatial relationships of the embryo and its compartments are retained. Although the absence of DNA methylation from the male pronucleus has been established at specific loci by using bisulphite sequencing (Oswald *et al.,* 2000), the possibility remains that some aspects of the genome-wide loss might also be explained as a consequence of specific masking of the methylation moiety within the male pronucleus.

Decondensation of the fertilising sperm takes approximately 45–60 min in the mouse (Fig. 2a, A and B). Thus, during the initial stages of the loss of protamine and the reacquisition of nucleosomal histones, the methylation signal was weak owing to the inaccessibility of the antibody during this transition. Immediately after this phase, the male and female pronuclei stained for 5-methyl cytosine (Fig. 2a, C and D). The male pronucleus was already less intensely stained than the female (C and D, and E and F), suggesting that the loss of paternal methylation begins very quickly after the removal of protamine. As early as 4 h after initiating IVF, a substantial proportion of oocytes display a PN_3 (Fig. 2a, G and H) pattern, indicating that the process of active demethylation takes place very rapidly, resulting in almost complete loss of methylation from the male pronucleus (Fig. 2a, PN_0–PN_3). This process takes 90–120 min after decondensation as measured by IVF, suggesting that *in vivo* the process is likely to take place in less than 90 min. Maturation of the pronuclei continues (Fig. 2a, I and J, and K and L). As the fertilised embryo enters syngamy (Fig. 2a, M and N), the only apparent remaining methylation can be attributed to the female pronucleus, which is the smaller of the two pronuclei, and to the polar body.

Using the classification PN_0–PN_5, we characterised the time point and stage when the demethylation of the male pronucleus occurred and thus defined a window for active demethylation. Embryos were collected at fixed time points during IVF and assigned to the stages corresponding to the changes in size, shape, and positioning of the pronuclei and evaluated for methylation status with an antibody for

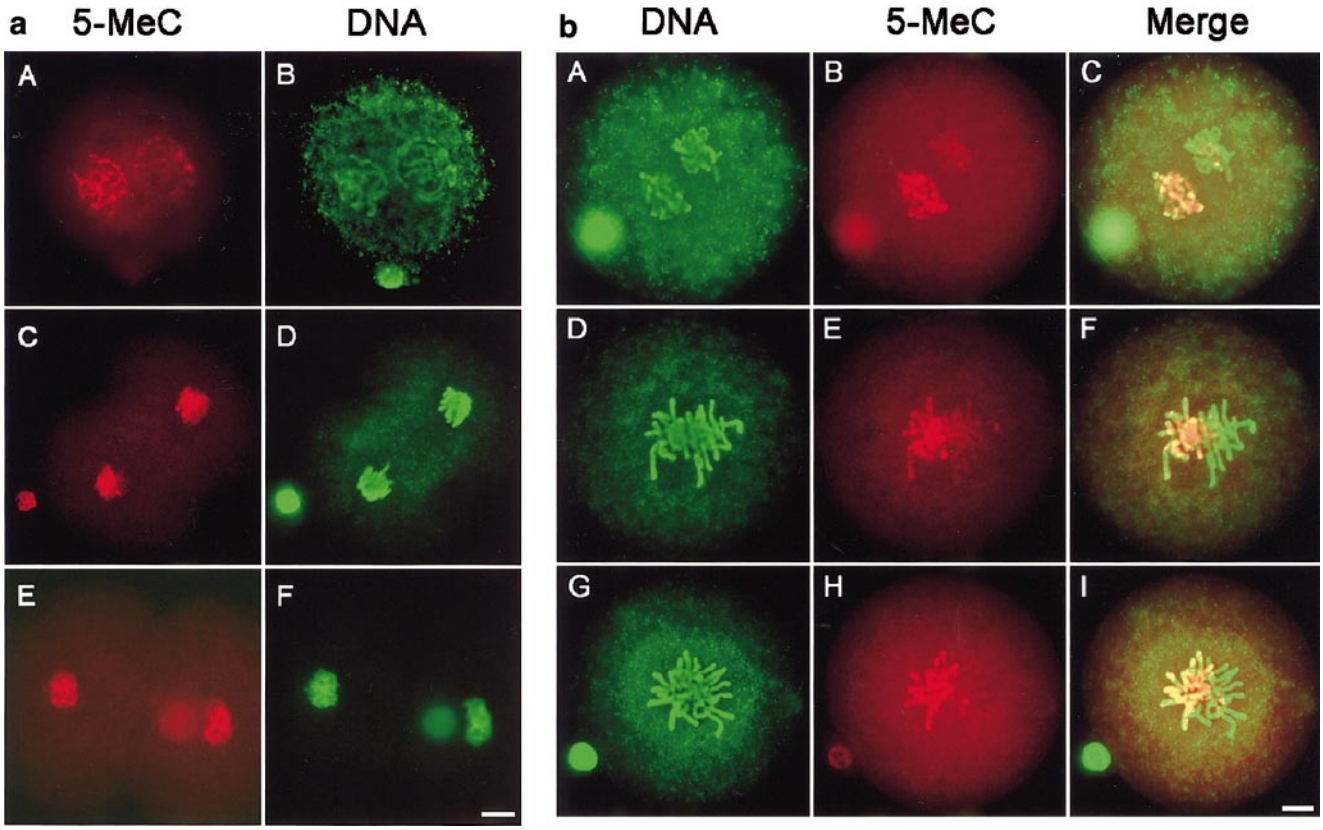

FIG. 3. (a) Distribution of CpG methylation during the latter portion of the first cell cycle. (A, B) Upon entering mitosis during early metaphase, the condensation of the respective genomes reveals the separation of the maternal (positively stained) and paternal components. As the chromosomes approach each other and form a metaphase plate, their positions are maintained with respect to each other. (C, D) Anaphase chromosomes have mixed maternal and paternal staining patterns, which are reflected in the differences in the pattern of fluorescent staining between the 5-MeC (C) and the DNA (D). On completion of mitosis, the positive methylation signal of the telophase nuclei of the two-cell embryo (E, F) appears less intense compared to earlier stages. This is attributed to the absence of the maintenance methylase activity in the cleavage stages. Scale bar, 10 μm. (b) Spatial distribution of residual 5-methyl cytosine signal in maternal and paternal compartments. Upon condensation of the respective genomes, a residual positive signal is revealed, though weak compared to maternal, attributable to the paternal component of the zygote (A–C). Note that the intensity of the staining with 5-MeC antibody corresponds to an area of diminished intensity of YOYO-1 staining (see maternal haploid genome). As the chromosomes approach each other (D–F), their positions are maintained on the metaphase plate (G–I). Banding of methylation-positive areas can be seen in these examples. Note that the high degree of cytoplasmic staining is due to the tendency of YOYO-1 to stain the DNA of the mitochondria. Scale bar, 10 μm.

5-methyl cytosine (Fig. 2a). The histogram shows the percentage of embryos showing staining of both pronuclei as a function of the stage of the pronucleus (Fig. 2b). This identifies the stage at which demethylation is initiated. As 100% of all embryos evaluated were positive for both pronuclei on staining with 5-methyl C antibody at PN_1, this suggests that demethylation is initiated during or immediately after this stage (Fig. 2b).

Chromatin Remodelling during Pronuclear Maturation

On fertilisation, sperm chromatin is remodelled with protamines being replaced by histones. Previous studies have investigated the process of the acquisition of histones (McLay and Clarke, 1997) or acetylated histones (Adenot *et al.*, 1997) following loss of protamines during this early period of pronuclear maturation. Ideally, we were interested in describing the simultaneous loss of protamines and the incorporation of histones and the methylation status of the paternal genome in the same embryos. Unfortunately, the technical requirements for the staining of basic proteins and the DNA were not compatible with the fixation processes. Thus, groups of embryos were selected at specific times after fertilisation and fixed for evaluation of either histones or cytosine methylation independently.

As soon as the removal of protamines takes place, the decondensing sperm becomes associated with histones (Fig.

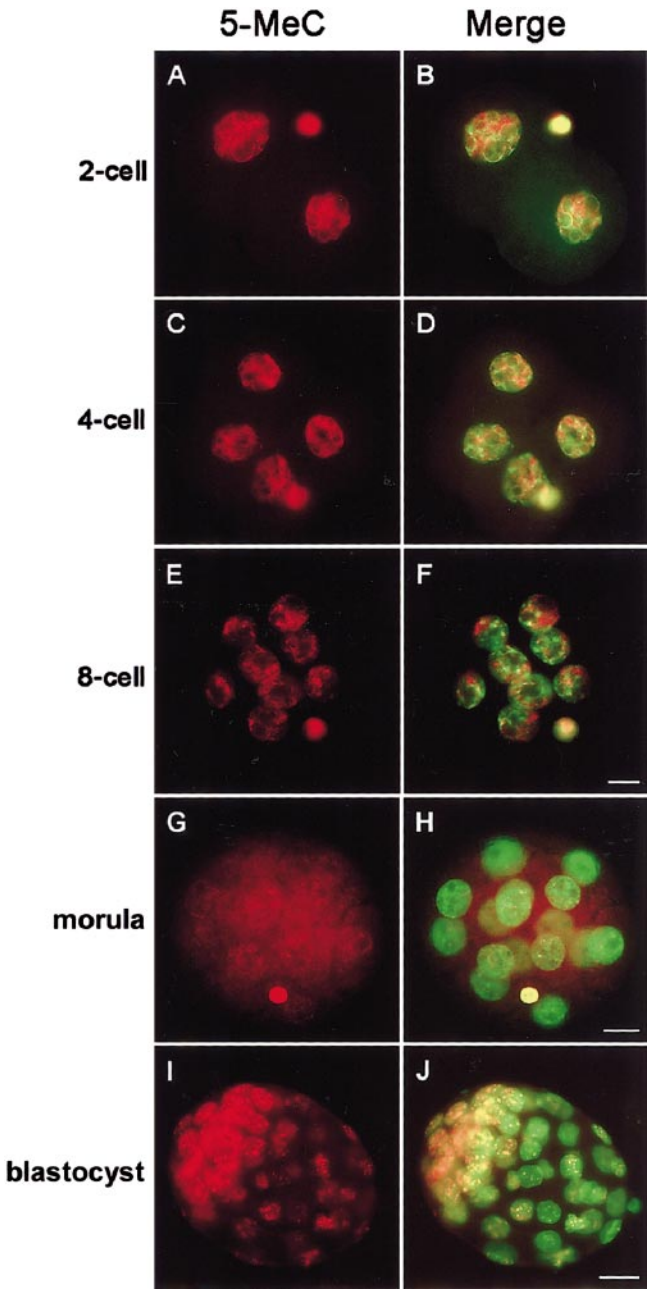

FIG. 4. Spatial distribution of 5-methyl cytosine during the passive phase of demethylation. Embryos were collected at the two- (A, B), four- (C, D), eight-cell (E, F), morula (G, H), and blastocyst (I, J) stage, fixed immediately, and prepared for immunofluorescence evaluation. Embryos were stained for DNA with DAPI and Cy3 secondary antibody for 5-MeC and images captured by using the Olympus Epifluorescence microscope. Images were pseudocoloured and merged by using Adobe Photoshop 5.0. Scale bar, 20 μm.

2c, A–C). Moreover, there is a bias towards the acquisition of acetylated histones by the male pronucleus (Fig. 2c, E and H). These results are in close agreement with those reported by Adenot *et al.* (1997). Thus, despite the presence of histones and nucleosomal organisation, demethylation takes place preferentially in the male pronucleus while the female pronucleus remains highly methylated and apparently unaffected (Fig. 2c, L).

Spatial Distribution of DNA Methylation

On entering mitosis and aligning properly at the equator, the maternal and paternal components are discernible by their methylation signal (Fig. 3a, A and B). By anaphase, the two genomes have mixed and equal contributions of maternal and paternal chromosomes move to opposite poles of the zygote (Fig. 3a, C and D). The two-cell embryo is the product of this cell cycle and the typical pattern of the telophase nucleus is depicted (Fig. 3a, E and F).

Through careful observation of the population of zygotes moving through syngamy and into the latter phase of the cell cycle, the stepwise features of the separate parental genomes and the newly emergent zygotic genome become apparent (Fig. 3b, A–C). The separate diploid genomes are condensing and begin to reform the chromosomes. The maternal (lower) is more highly methylated than the paternal (upper); however, the paternal genome does have a low but significant positive signal (Fig. 3b: D–F and G–I). The extending arms of the chromosomes from the respective genomes approach the metaphase plate with their distinctive methylation patterns evident (maternal on the left). This confirms the methylation patterns of the respective genomes prior to division.

Demethylation and de Novo Methylation in Cleavage-Stage Embryos

A spatial separation between the maternal and paternal genomes during early preimplantation stages in the mouse had been suggested (Mayer *et al.*, 2000b). However, our observations, using two different methods of fixation (data not shown), did not show this separation of the genomes. To clarify this discrepancy, we evaluated two-, four-, eight-cell, morula, and blastocyst-stage embryos after staining with 5-methyl C antibody. In order to strictly compare and evaluate the methylation status of individual embryos during this phase, it was important that all stages were treated and stained together on the same slide. These results show that patterns of methylation staining throughout the nucleus are random at the two-, four-, and eight-cell stage with no apparent separation or spatially uneven distribution (Fig. 4).

By the morulae stage (Figs. 4G and 4H), very little methylation was detected within the nuclei, although the polar body remained strongly staining. However, by the blastocyst stage, an intense signal was clearly visible within the ICM while the trophectoderm was mostly negative (Figs. 4I and 4J), demonstrating that *de novo* methylation begins in preimplantation embryos and is lineage-specific. It is interesting to ask whether the residual positive cells of

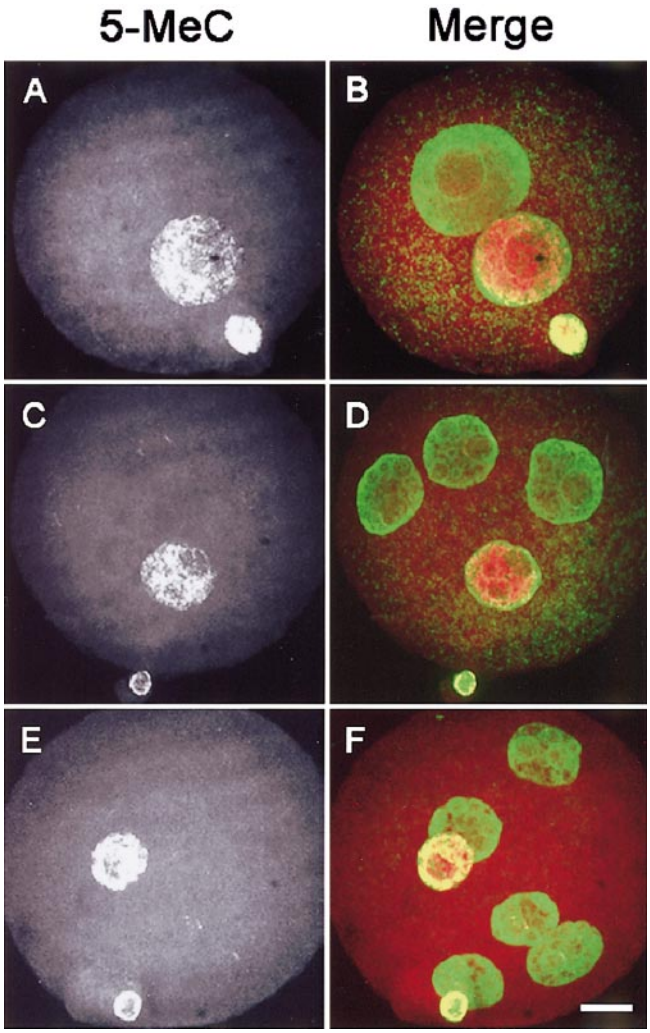

FIG. 5. Polyspermic fertilisation of dezonated mouse oocytes. To evaluate whether the demethylation of the paternal allele was titratable, oocytes were exposed to capacitated sperm after removal of the zonae and incubated for 9.5 h prior to fixation. This procedure produced fertilised oocytes containing multiple extra male pronuclei. The results show some of these polyspermic fertilised embryos with (A, B) one male pronucleus, (C, D) three, and (E, F) five male pronuclei. Fertilised oocytes were evaluated using the Ultraview (Perkin-Elmer) laser scanning confocal microscope and two-dimensional projections of 0.2-μm optical sections were produced. Scale bar, 20 μm.

the trophectoderm represent a specific population of cells, which might be identified on the basis of their nuclear complement (i.e., diploid vs. giant cell).

Functional Evaluation of Demethylating Activity

Titration of demethylation activity in polyspermic oocytes. The oocyte has a rich resource of proteins, enzymes, and RNAs that support the maturation and growth of the pronuclei following fertilisation. This is achieved by the incorporation of lamins from the cytoplasm into the maturing pronuclei. It has been suggested that the oocyte has the capacity to support the decondensation of up to six sperm (Perrault and Zirkin, 1982). We were interested in the possibility that the capacity for demethylation might be titrated out by the addition of supernumerary sperm. In order to investigate this hypothesis, oocytes were subjected to IVF conditions that selected for polyspermy. Ten hours after a 15-min exposure to capacitated sperm, zone free oocytes were fixed and prepared for staining to evaluate the methylation status of the respective pronuclei. These oocytes were observed to have up to four supernumerary male pronuclei (Figs. 5E and 5F). Representative embryos from this experimental group are shown. Oocytes containing a single male pronucleus were able to mature to PN_5 stage (Figs. 5A and 5B), while those with three (Figs. 5C and 5D) or more closely resembled PN_2–PN_3 stages. The pronuclei were variable in their extent of maturation under these conditions. However, irrespective of the extent of nuclear swelling and hence maturation, the demethylation of all male pronuclei had occurred. These results suggest either that under these conditions the demethylation activity is not limiting in the fertilised oocyte or that the activity is intimately associated with the sperm. The fact that the maternal pronucleus does not become sensitive to the demethylating activity is notable.

Active demethylation in fertilised oocytes lacking MBD2. To begin to investigate the mechanistic basis of the active demethylation observed in the fertilised oocyte, we made use of mice deficient in a candidate demethylase enzyme (Bhattacharya *et al.*, 1999; Hendrich *et al.*, 2001). This protein had been isolated independently and identified as a methyl CpG-binding protein (MBD2) (Hendrich and Bird, 1998). Reciprocal crosses with the Mbd2 null mice (Hendrich *et al.*, 2001) resulted in a pattern of 5-MeC staining identical to the wild-type embryos (Fig. 6). These results show that MBD2 is not the active demethylase responsible for removal of methylation in the paternal pronucleus of the fertilised oocyte.

DISCUSSION

In this study, we have provided the first complete description of the cycle of methylation reprogramming in mouse preimplantation embryos. This cycle is initiated shortly after sperm decondensation. To fully characterise this phase of selective demethylation of the paternal genome, we have used IVF to create a precisely timed profile of pronuclear maturation and chromatin remodelling in the mouse zygote. Using a sensitive immunofluorescence assay with a well-characterised antibody to 5-methyl cytosine (Reynaud *et al.*, 1991; Miniou *et al.*, 1994; Niveleau *et al.*, 1994; Tweedie *et al.*, 1997), we have identified the earliest stage when this activity becomes apparent. The paternal genome is selectively demethylated immediately following

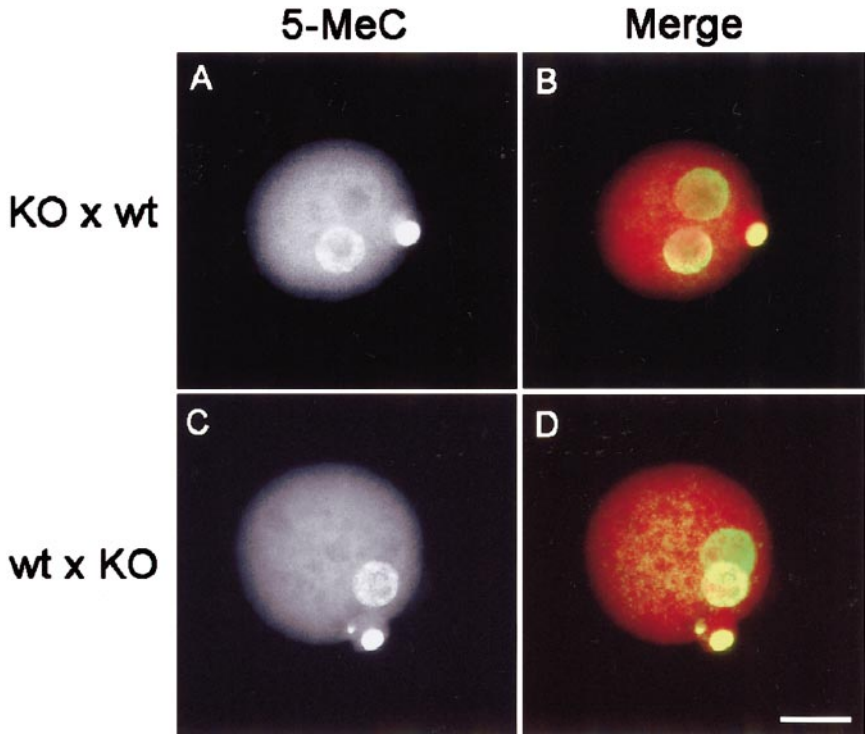

FIG. 6. Distribution of CpG methylation during pronuclear stages in the MBD2 null mouse fertilised oocyte. Fertilised oocytes were collected from reciprocal crosses of mice null for MBD2 inherited the maternally (A, B) or paternally (C, D). Fertilised oocytes were stained for DNA with YOYO-1 and Cy3 as secondary antibody (5-MeC) and images captured by using the Olympus Epifluorescence microscope. Images were pseudocoloured and merged by using Adobe Photoshop 5.0. Scale bar, 25 μm.

sperm decondensation and by 4 h postfertilisation maternal and paternal genomes are unequally methylated. Demethylation is complete within 90–120 min of sperm decondensation. These results contradict a previous report that suggested that both paternal and maternal chromosomes stained equally intensely 6 h after fertilisation (Mayer *et al.*, 2000a). This discrepancy may have arisen, in part, from the use of oocytes derived from naturally timed matings rather than IVF. A parallel evaluation of naturally fertilised embryos, however, confirmed that unequal methylation was pronounced as early as 5 h post fertilisation with the majority showing complete demethylation of the paternal pronucleus (data not shown).

At syngamy, there was a small but significant residual methylation signal in some but not all of the paternally derived chromosomes. Candidate sequences for this residual methylation may include B1 repeats (Rougier *et al.*, 1998). The exact nature of their epigenetic status is of interest as they may share some common features with imprinted genes, which remain methylated during preimplantation (Olek and Walter, 1997; Warnecke *et al.*, 1998). Using bisulphite sequencing, we have indeed identified a specific family that resists active demethylation (Lane *et al.*, unpublished observations).

The oocyte possesses a limited capacity to decondense and remodel sperm into functional pronuclei (Perreault and Zirkin, 1982). Demethylation and the maturation of pronuclei were coincident events as attempts to titrate this activity on polyspermic fertilisation were unsuccessful as all male pronuclei were demethylated; yet the methylation status of the female pronucleus was unaffected.

Although protamine removal and histone deposition are tightly coupled, at least one study reports the occurrence of naked sperm DNA after protamine removal and before histone acquisition (Perreault, 1992), such that the male but not the female pronuclei might experience a brief period of susceptibility to demethylating activity. However, the continued presence of naked DNA is clearly not required for the completion of demethylation. It is reasonable to speculate that the demethylation activity may target the male pronucleus during the histone–protamine exchange. Thus, chromatin remodelling may be required to trigger the demethylation reaction, and the absence of chromatin remodelling in the female pronucleus may explain the persistence of methylation during this phase.

The nature of this *in vivo* demethylating activity is unknown. Previous work has shown that microinjected methylated DNA fragments may undergo active demethylation in early mouse embryos (Kafri *et al.*, 1993) and various assays have demonstrated demethylating activities in

nuclear extracts from cultured cells or embryos (Weiss *et al.*, 1996; Jost *et al.*, 1995; Bhattacharya *et al.*, 1999). Bhattacharya *et al.* (1999) reported that an activity had been isolated which removed the methyl group *in vitro* and produced methanol in the reaction. This activity was identified as a short form of the methyl-binding domain protein-2 (MBD2), and was proposed as a candidate for the demethylase activity in the early embryo. Although other studies failed to reproduce these *in vitro* results (Wade *et al.*, 1999; Ng *et al.*, 1999), it was important to assess whether *in vivo* MBD2 was required for demethylation in the zygote. Evaluation of fertilised oocytes homozygous for a MBD2 knockout allele gave results that were indistinguishable from the wild-type controls. Therefore, MBD2 is not required for the active demethylation observed in the mouse zygote.

The methylation asymmetry between the maternal and paternal pronucleus has led to the speculation that there may be some functional importance in this spatial organisation within the nucleus beyond the one-cell stage (Mayer *et al.*, 2000b). A recent report suggesting a transient segregation of parental genomes up to the four-cell stage (Mayer *et al.*, 2000b) was not supported by our results on methylation distribution during cleavage stages. These differences may be due to the preparative techniques which employed a harsh fixation protocol that results in significant embryo loss (W.D. *et al.*, unpublished observation). By the morula stage, very little genomic methylation remains. In contrast to earlier reports (Monk *et al.*, 1987; Howlett and Reik, 1991; Kafri *et al.*, 1992), which used DNA extracted from blastocysts, we show that *de novo* methylation occurs by the blastocyst stage but is restricted to the ICM, while the trophectoderm is virtually devoid of methylation. Thus, lineage-based methylation differences are established at the time of the differentiation of embryonic and extraembryonic lineages. The observation that ES cells are substantially methylated (Okano *et al.*, 1999) is therefore consistent with their origins in the ICM. The early *de novo* methylation in the ICM is essential for early postimplantation development, as embryos deficient for the *de novo* methylases Dnmt3a and 3b show abnormalities in all embryonic lineages but no effect of the extraembryonic tissue (Okano *et al.*, 1999).

The functional importance of the temporal and quantitative features of the loss of methylation prior to implantation is at present unclear. While demethylation is widely observed in mammalian embryos (W.D. *et al.*, unpublished observations), it is not observed in the zebrafish (Macleod *et al.*, 1999). Roles in both imprinting (Reik and Walter, 2001b) and germ-line reprogramming, ensuring totipotency, have been suggested (Monk *et al.*, 1987; Howlett and Reik, 1991). Indeed, there is a critical requirement for the oocyte form of Dnmt1 to maintain methylation at imprinted loci (Howell *et al.*, 2001). This raises the interesting question of the outcome of placing a somatic nucleus into the environment of the oocyte cytoplasm. During the cloning of somatic nuclei, a highly methylated donor cell must become transcriptionally reeducated at the same time methylation reprogramming events normally take place. Whether the consistently low efficiency of somatic nuclear cloning is related to the inability of a somatic nucleus to undergo the normal changes in methylation is an attractive question to pose (Reik *et al.*, 2001; Rideout *et al.*, 2001). We are interested in evaluating this possibility to better identify somatic nuclear donors or to improve reprogramming efficiency leading to improved rates of cloning. Resolving some of these questions will make cloning a powerful technique to investigate fundamental questions of early mammalian embryogenesis and development, especially genomic imprinting.

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