Regulation of histone H3 lysine 9 methylation in oocytes and early pre-implantation embryos

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Summary

Epigenetic modifications of the genome, such as covalent modification of histone residues, ensure appropriate gene activation during pre-implantation development, and are probably involved in the asymmetric reprogramming of the parental genomes after fertilization. We investigated the methylation patterns of histone H3 at lysine 9 (H3/K9), and the regulatory mechanism involved in the asymmetric remodeling of parental genomes during early pre-implantation development in mice. Immunocytochemistry with an antibody that specifically recognizes methylated H3/K9 showed a very weak or absent methylation signal in the male pronucleus, whereas a distinct methylation signal was detected in the female pronucleus. This asymmetric H3/K9 methylation pattern in the different parental genomes persisted until the two-cell stage. However, de novo methylation of H3/K9 occurred and the asymmetry was lost during the four-cell stage. The unmethylated male pronucleus underwent de novo methylation when it was transferred into enucleated GV- or MII-stage oocytes, which suggests that histone H3 methylase is active before fertilization, but not afterwards, and that the asymmetric methylation pattern is generated by this change in methylase activity in the cytoplasm after fertilization. Thus, histone H3 is methylated only in the maternal chromosomes, which are present in the oocytes before fertilization, and is not methylated in the paternal chromosomes, which are absent. The maintenance of asymmetric H3/K9 methylation patterns in early embryos is an active process that depends on protein synthesis and zygotic transcription, as de novo methylation in the male pronucleus occurred when either protein synthesis or gene expression was inhibited by cycloheximide or α-amanitin, respectively. In addition, corresponding de novo methylation of H3/K9 and DNA occurred when the male pronucleus was transferred to an enucleated GV oocyte. Our results suggest that H3/K9 methylation is an epigenetic marker of parental genome origin during early pre-implantation development.

Supplemental data available online

Key words: Embryo, Oocyte, Histone H3, Lysine 9, Methylation, Nuclear transfer

Introduction

Mammalian genomes with different parental origins are asymmetric in terms of their functions. This asymmetry comprises two biologically important phenomena. First, genomic imprinting causes some genes to be expressed in a manner that is dependent on parental origin, i.e. some genes are expressed only from the genomes of paternal origin and others from the genomes of maternal origin (Reik and Walter, 2001). Second, one of the two X chromosomes is inactivated in female cells, which leads to dosage compensation for the X-linked gene products between XX females and XY males (Lyon, 1961). This inactivation is established during pre-implantation development (Singer-Sam et al., 1992; Costanzi et al., 2000; Huynh and Lee, 2003). In trophectoderm cells, inactivation always occurs in the X chromosome of paternal origin, whereas inactivation occurs randomly in one of two origins in the inner cells (Takagi and Sasaki, 1975; Takagi et al., 1978; Gardner et al., 1985). Therefore, the genomes of different parental origin contribute differently to certain aspects of cell regulation, especially development. Although the molecular mechanism underlying these phenomena has been elucidated, i.e. DNA methylation, the mechanism that distinguishes the genomes of different parental origin remains unknown.

After fertilization, the genomes from the two parents are separated in the embryos into male and female pronuclei, before being re-unified during the M phase at the one-cell stage. Although these pronuclei reside in the same cytoplasm, they are heterogeneous in many aspects. The temporal and spatial distribution of the sites of DNA replication are asynchronous between the two pronuclei, in that the female pronucleus requires a longer time to complete replication in the intranuclear region but not in the peripheral regions (Aoki and Schultz, 1999). Transcriptional regulation also differs; the male pronucleus supports a higher level of transcription than the female pronucleus (Bounial et al., 1995; Aoki et al., 1997), because the chromatin of the female pronucleus (but not that of the male pronucleus) is in a transcriptionally repressed state (Wiekowski et al., 1993; Majumder et al., 1997; Cho et al.,

2002). The two pronuclei also show asymmetric DNA methylation, which may be responsible for functional differences between the parental genomes during development. As early as 4 hours after fertilization, the male pronucleus is almost completely demethylated, whereas the female pronucleus remains methylated during the one-cell stage and undergoes gradual demethylation until the blastocyst stage (Mayer et al., 2000a; Reik et al., 2001). As the male and female pronuclei show different features in the same cytoplasmic environment, it appears that the differences in their chromosomes are distinguished by cytoplasmic factors after fertilization.

The differences in the properties of paternal and maternal genomes in early embryos may be attributable to differences in the epigenetic modifications to their genomes. Recent studies have shown that modifications of the chromatin-packaging proteins, histones, play important roles in the regulation of gene expression. Covalent modifications of histones, such as acetylation, methylation and phosphorylation, contribute to a mechanism that can alter chromatin structure, thereby causing inheritable differences in transcriptional 'on-off' states (Jenuwein and Allis, 2001; Goll and Bestor, 2002; Turner, 2002). It seems likely that histone modifications differ in the parental genomes in one-cell embryos, as the reconstruction of chromatin by protamine-histone exchange occurs in the paternal chromatin soon after fertilization. The chromosomal histones, which are acquired from the cytoplasmic pool of the oocyte, may undergo different modifications in the male pronucleus than in the female pronucleus, the latter of which is acquired from the oocyte chromosome. The different histone modifications are probably implicated in the differential properties of the parental genomes.

An interesting, recent finding suggests that histone H3 methylation at lysine 9 (H3/K9) is involved in the formation of the constitutive heterochromatin, as well as the facultative heterochromatin of the inactive X chromosome (Heard et al., 2001; Jacobs et al., 2001; Noma et al., 2001; Boggs et al., 2002; Peters et al., 2002). Methylation of H3/K9 has also been shown to be associated with the silencing of euchromatic genes (Hwang et al., 2001; Nielsen et al., 2001; Peters et al., 2002). In addition, H3/K9 methylation has been correlated with DNA cytosine methylation, and it has been suggested that DNA methylation acts downstream of H3/K9 methylation (Tamaru and Selker, 2001; Gendrel et al., 2002; Jackson et al., 2002).

In this study, we investigated H3/K9 methylation in oocytes and early pre-implantation embryos to understand the mechanism by which the genomes of different parental origin are distinguished. Our results show that the asymmetric H3/K9 methylation pattern between parental genomes is generated soon after fertilization, and persists during early pre-implantation development. The different methylation patterns are generated by changes in the properties of the cytoplasm after fertilization, and not by a specific chromatin structure. The mechanism that maintains the paternal genome in the undermethylated state is an active process, as inhibition of protein synthesis or gene expression increased methylation in the male pronucleus to a level that was similar to that of the female pronucleus. Finally, correspondent methylation of H3/K9 and DNA occurred in male pronuclei that were transplanted into oocytes, which suggests that asymmetric H3/K9 methylation is associated with asymmetric DNA methylation in genomes of different parental origin during pre-implantation development.

Materials and methods

In vitro fertilization and embryo cultures

Female ddY mice (SLC, Shizuoka, Japan), 21-23 days of age, were superovulated with 5 IU of pregnant mares' serum gonadotropin, followed 48 hours later with 5 IU of human chorionic gonadotropin (hCG). Unfertilized metaphase II-arrested oocytes were collected in Whitten's medium (Whitten, 1971), from the ampullae of oviducts, 14-15 hours after hCG injection. Sperm were collected from the caudal epididymis of adult ICR males (SLC, Shizuoka, Japan), and pre-incubated in Whitten's medium for 2 hours in an atmosphere of 5% CO_2, 95% air at 38°C. The oocytes were inseminated with capacitated sperm. Three hours after insemination, the fertilized oocytes were washed and cultured in CZB medium (Chatot et al., 1989).

To inhibit the synthesis of protein or mRNA, 50 μg/ml cycloheximide or 25 μg/ml α-amanitin (Sigma, St Louis, MO, USA), respectively, was added to the culture medium at the time of insemination. These concentrations of the inhibitors were sufficient to inhibit completely the synthesis of protein and mRNA, as 20 μg/ml cycloheximide was previously demonstrated to abolish protein synthesis (Manejwala et al., 1991). Furthermore, we confirmed previously that 25 μg/ml α-amanitin completely inhibited transcription, which was detected by bromouridine (BrU) incorporation in embryos that were loaded with BrUTP (F.A., unpublished).

Parthenogenesis and androgenesis

Androgenetic embryos were prepared by fertilizing enucleated oocytes. Cumulus-oocyte complexes were collected from the ampullae of oviducts 14-15 hours after hCG injection, and placed in KSOM (Lawitts and Biggers, 1993) that contained 0.3 mg/ml bovine testicular hyaluronidase. After complete removal of the cumulus cells, the oocytes were transferred to a micromanipulation drop, which contained HEPES-buffered KSOM that was supplemented with 5 μg/ml cytochalasin B. The zona pellucida was cored by a Piezo-impact-driven micromanipulator (Prime Tech Ltd, Ibaraki, Japan), and the MII chromosomes were aspirated with a minimal volume of oocyte cytoplasm using a pipette. The enucleated oocytes were transferred to acidic MEMCO for a short time to dissolve the zona pellucida. Zona-free oocytes were cultured in Whitten's medium for 1 hour, to allow them to recover after zona pellucida removal, and were then subjected to in vitro fertilization. Thirty minutes after insemination, the oocytes were washed and cultured in CZB medium.

Parthenogenetically activated oocytes were prepared by exposure of MII-stage oocytes to 7% ethanol for 6 minutes or to 10 mM Sr^{2+} for 10 minutes in CZB. In order to obtain diploid parthenogenetic embryos, the oocytes were cultured in CZB that contained 5 μg/ml cytochalasin B for 6 hours, and then cultured in CZB.

Pronuclear transplantation

Enucleated germinal vesicle (GV) and MII-stage oocytes were used as recipients for nuclear transfer. Enucleation of MII oocytes was conducted as described above. The fully-grown GV oocytes were collected from 4-week-old ddY mice by puncturing the follicles with a sharp needle in Whitten's medium that was supplemented with 20 mM HEPES and 0.2 mM 3-isobutyl-1-methylxanthine (IBMX). Only those oocytes with a diameter >70 μm were sorted for further use. The cumulus cells were removed from cumulus-oocyte complexes by gentle pipetting through a narrow-bore glass pipette. The enucleation protocol for GV oocytes was similar to that for MII oocytes. GV-stage oocytes were cultured in HEPES-buffered KSOM that contained 5

μg/ml cytochalasin B and 0.2 mM IBMX for 30 minutes before GV aspiration. The zona pellucida was cored using a Piezo-impact-driven micromanipulator, and the GV, which was surrounded by a small amount of cytoplasm, was removed with a pipette of inner diameter 15 μm. To inhibit spontaneous meiosis resumption, 0.2 mM IBMX was added to the micromanipulation medium. The enucleated oocytes were cultured in CZB (for MII oocytes), or CZB that contained 0.2 mM IBMX (for GV oocytes), for 1 hour before use.

The male pronucleus at 6 hours after insemination was used as the nuclear donor. The zygotes were treated with 5 μg/ml cytochalasin B for 20 minutes before pronucleus aspiration. The zona pellucida was cored using a Piezo-impact-driven micromanipulator, and the male pronucleus, which was surrounded by a small amount of cytoplasm, was removed with a pipette of inner diameter 15 μm. The male pronucleus was then inserted into the perivitelline space of the enucleated oocyte. The fusion of donor-recipient pairs was induced by a DC pulse of 1500 V/cm for 20 μs in 300 mM mannitol that contained 0.1 mM $MgSO_4$, 0.1 mg/ml polyvinyl alcohol and 3 mg/ml bovine serum albumin. The oocytes were evaluated 30 minutes after application of the electropulses to ensure fusion. Successfully fused reconstructed oocytes were cultured for 3 hours in Whitten's medium with or without 0.2 mM IBMX. When MII-stage oocytes were used as recipients, the reconstructed oocytes were cultured with 0.5 μg/ml nocodazole to prevent spontaneous activation.

Immunocytochemistry

Oocytes and embryos were washed in PBS that contained 3 mg/ml polyvinylpyrrolidone (PBS/PVP), fixed for 1 hour in 3.7% paraformaldehyde in PBS, and permeabilized with 0.5% Triton X-100 in PBS for 20 minutes at room temperature. The cells were incubated for 1 hour with a 1:200 dilution of the rabbit polyclonal antibody that recognizes dimethyl-lysine 9 on histone H3 (Upstate Biotechnology, Lake Placid, NY, USA; catalogue number 07-212), and a secondary FITC-conjugated antibody (Jackson Immunoresearch, West Grove, PA, USA). For the detection of 5-methl-cytosine (5-MeC), the cells were treated with 2N HCl at room temperature for 30 minutes, and neutralized subsequently for 10 minutes with 100 mM Tris-HCl buffer (pH 8.5), after permeabilization. After extensive washing with 0.05% Tween-20 in PBS, the cells were incubated with anti-5-MeC antibodies (Eurogentec, Seraine, Belgium), followed by incubation with secondary FITC-conjugated antibody (Jackson Immunoresearch). Double-antibody staining was accomplished by successive incubation with the antibodies against 5-MeC and methylated H3/K9. DNA was stained with 3 μg/ml 4,6-diamidino-2-phenylindole (DAPI) for 20 minutes, and the cells were mounted on a glass slide in Vectashield anti-bleaching solution (Vector Laboratories, Burlingame, CA, USA). Fluorescence was detected using a Leica TCS SP2 laser-scanning confocal microscope.

Semi-quantitative analysis of the fluorescence signals was conducted using the NIH Image program (National Institute of Health, Bethesda, MD, USA), as described previously (Kim et al., 2002). Briefly, the pixel value/unit area was measured for the nucleus, and the value for the cytoplasm was subtracted as background. The derived value was multiplied by the nuclear area to yield the total amount of fluorescence in the nucleus.

Results

Differential H3/K9 methylation patterning between paternal and maternal genomes

The methylation of H3/K9 was examined in mouse oocytes and one-cell embryos. While our studies were in progress, reports appeared that described the asymmetric methylation of H3/K9 in male and female pronuclei (Arney et al., 2002; Cowell et al., 2002). In our studies, we confirmed that only the genomes of maternal origin were methylated at H3/K9. The signal from the antibody that specifically recognizes the dimethylated form of H3/K9 was detected in the germinal vesicles (GV) of the GV-stage oocytes, the chromosomes of MII-stage oocytes, and in the female pronuclei of the one-cell embryos (Fig. 1A). The signals were absent or faint in the male pronuclei. The specificity of the antibody for dimethylated H3K9 was confirmed by adsorption with various methylated peptides. The signal for the antibody was abolished by pre-incubation with histone H3 N-terminal peptides that were dimethylated on lysine 9, but not with peptides that were either trimethylated on lysine 9, dimethylated on lysine 4 or dimethylated lysine 27 (see Fig. S1 at http://dev.biologists.org/supplemental/).

To examine whether asymmetric methylation was inherent or due to different preferences for the histone methylase and demethylase in the pronuclei, parthenogenetic and androgenetic embryos were produced, and histone methylation examined when there was no competition between the nuclei of different origins. Intense methylation fluorescence appeared in the parthenogenetic embryos, but not in the androgenetic embryos (Fig. 1B), which indicates that the male pronucleus is intrinsically undermethylated. As early as 3 hours after fertilization, the sperm nucleus chromatin protein is changed from protamine to histone. At this time, asymmetric histone H3 methylation has already taken place, i.e. the male pronucleus is not methylated (Fig. 1A), which suggests that the cytoplasmic pool of histone H3 is not methylated at lysine 9.

During the first M phase, the maternal and paternal chromosomes move together but remain compartmentalized. Even after cleavage to form the two-cell stage, the chromosomes are still separated topologically, which suggests that differential epigenetic reprogramming occurs in the parental genomes (Mayer et al., 2000a; Mayer et al., 2000b; Haaf, 2001). If H3/K9 methylation is an epigenetic marker, it should also be localized within the compartmentalized areas of the nuclei of the two-cell embryos. Immunocytochemistry revealed methylated H3/K9 fluorescence present in only half of the nuclear area (Fig. 2A). This topological separation was due to the different genome origins, as uniform fluorescence was observed in the whole nuclei of two-cell embryos that were produced by parthenogenesis, and no fluorescence was seen in two-cell embryos that were produced by androgenesis (Fig. 2B). Thus, asymmetric methylation of H3/K9 in parents of different origin was maintained until the late two-cell stage.

This asymmetric methylation seems to be maintained by the absence of de novo methylation. The methylation level of H3/K9 was assessed semi-quantitatively by measuring the intensity of fluorescence of the anti-H3/K9 antibody. In this assay, the relative level of immunofluorescence reflected the level of H3K9 methylation, as the fluorescence intensities of diploid and haploid parthenogenetic two-cell embryos were similar when calculated on the basis of DNA content (see Fig. S2 at http://dev.biologists.org/supplemental/). The methylation level, which was expressed as total fluorescence based on DNA content, decreased by almost 50% following each DNA replication during the first and second cell cycles (Fig. 3). The level of methylation at the G2 phase was almost half that at the G1 phase in both one- and two-cell embryos, and the levels were similar in embryos at the G2 phase of the one-cell stage and G1 phase of the two-cell stage, during which period no DNA replication occurred. These results suggest that de novo methylation does not occur, and that the methylation level

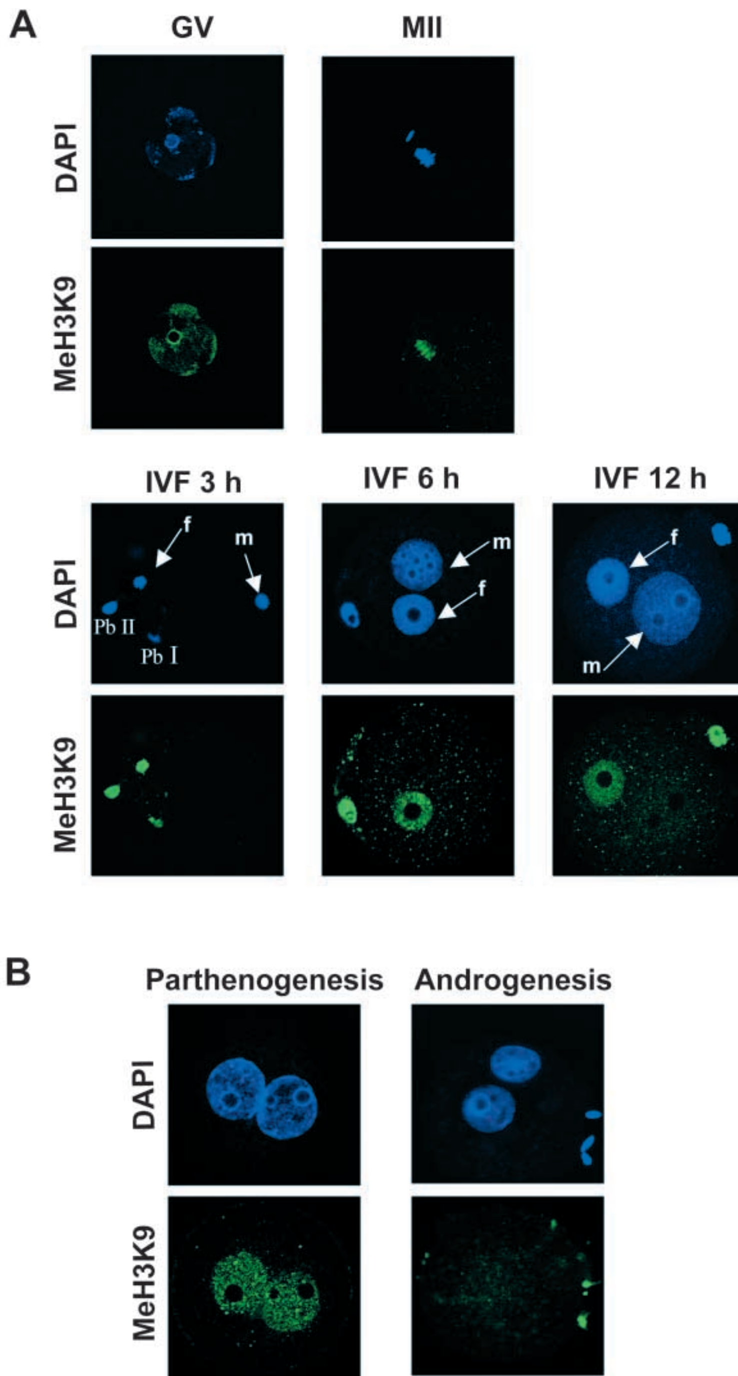

Fig. 1. Asymmetric histone H3 methylation of lysine 9 in the parental genomes of one-cell mouse embryos. Mouse GV-stage (GV) and MII-stage (MII) oocytes, one-cell embryos collected 3, 6 and 12 hours after in vitro fertilization (IVF 3, 6, 12 h) (A), and parthenogenetic and androgenetic one-cell embryos (B), were subjected to immunocytochemistry with antibody to methylated histone H3 Lys 9 (MeH3/K9). The antibody was localized with FITC-conjugated secondary antibodies (green), and DNA was stained with DAPI (blue). The parthenogenetic embryos were prepared by exposing MII-stage oocytes to 7% ethanol for 6 minutes, and collected for immunocytochemistry 6 hours later. The androgenetic embryos were prepared by fertilizing enucleated oocytes in vitro, and collected for immunocytochemistry 6 hours after fertilization. Arrows indicate the male (m) and female (f) pronuclei. pb, polar body. The experiments were conducted at least three times and similar results were obtained. More than 25 images were obtained for each type of embryo and representative examples are shown.

decreases passively during DNA replication, until the two-cell stage.

However, the asymmetry of H3/K9 methylation was lost at the four-cell stage. Uniform methylation fluorescence was observed in the nuclei of four-cell stage embryos (Fig. 4A), although genomes of paternal origin are still compartmentalized in the nucleus at this stage (Mayer et al., 2000b). This change appears to be caused by de novo methylation. Semi-quantification of the methylation fluorescence revealed that the methylation level increased after the two-cell stage, and that this increase occurred in genomes originating from both parents (Fig. 4B). At the four-cell stage, the methylation levels in the parthenogenetic and androgenetic embryos were almost the same as those in the fertilized embryos. At the eight-cell stage, the methylation level increased further, and was similar in all three types of embryo.

The oocyte cytoplasm, but not that of the embryo, methylates H3/K9

Asymmetric H3/K9 methylation in the parental genomes persisted until the two-cell stage. This difference may be due to differences in chromatin structure if the chromatin of paternal origin is scarcely methylated, or to differences in the cytoplasm before and after fertilization if the cytoplasm can methylate H3/K9 before but not after fertilization. Pronuclear transplantation experiments were carried out to investigate these alternatives. Male pronuclei were transferred to the enucleated cytoplasm of fully grown GV oocytes or MII-stage oocytes, and H3/K9 methylation was examined 3 hours after nuclear transfer. Intense signals for H3/K9 methylation appeared in the male pronuclei that were transferred to enucleated GV (Fig. 5A) and MII oocytes (Fig. 5B), which indicated that the oocyte cytoplasm methylates H3/K9 in a manner that is independent of genome origin. In order to exclude the possibility that female pronuclei constituted the donor pronuclei, the remaining zygotes, from which the male pronuclei had been removed, were immunostained for H3/K9 methylation. Intense fluorescence was observed in all of these embryos (data not shown), which confirmed that all of the donor pronuclei were male. Thus, the oocyte cytoplasm can methylate H3/K9 efficiently in both male and female pronuclei, whereas the embryonic cytoplasm does not have this activity. These results suggest that the properties of the cytoplasm change after fertilization, so as to maintain the male pronucleus in the undermethylated state.

Protein synthesis and gene expression are essential for the maintenance of asymmetric H3/K9 methylation in the parental genomes

As described above, the cytoplasm is dramatically changed after fertilization, so as not to methylate H3/K9. This change may be caused by de novo protein synthesis after fertilization, as it has been reported that the synthesis from maternal mRNA of some proteins begins after fertilization (Xu et al., 1997; Oh et al., 2000; Fuchimoto et al., 2001). These newly synthesized proteins could change the properties of the embryonic cytoplasm

dramatically. To address this possibility, protein synthesis was inhibited in one-cell embryos. The embryos were treated with cycloheximide from the time of insemination, and H3/K9 methylation was examined 12 hours after insemination. The intensity of H3/K9 methylation fluorescence was almost the same for male and female pronuclei in the cycloheximide-treated embryos (Fig. 6A). The treatment with cycloheximide did not impair embryonic viability, as the embryos developed

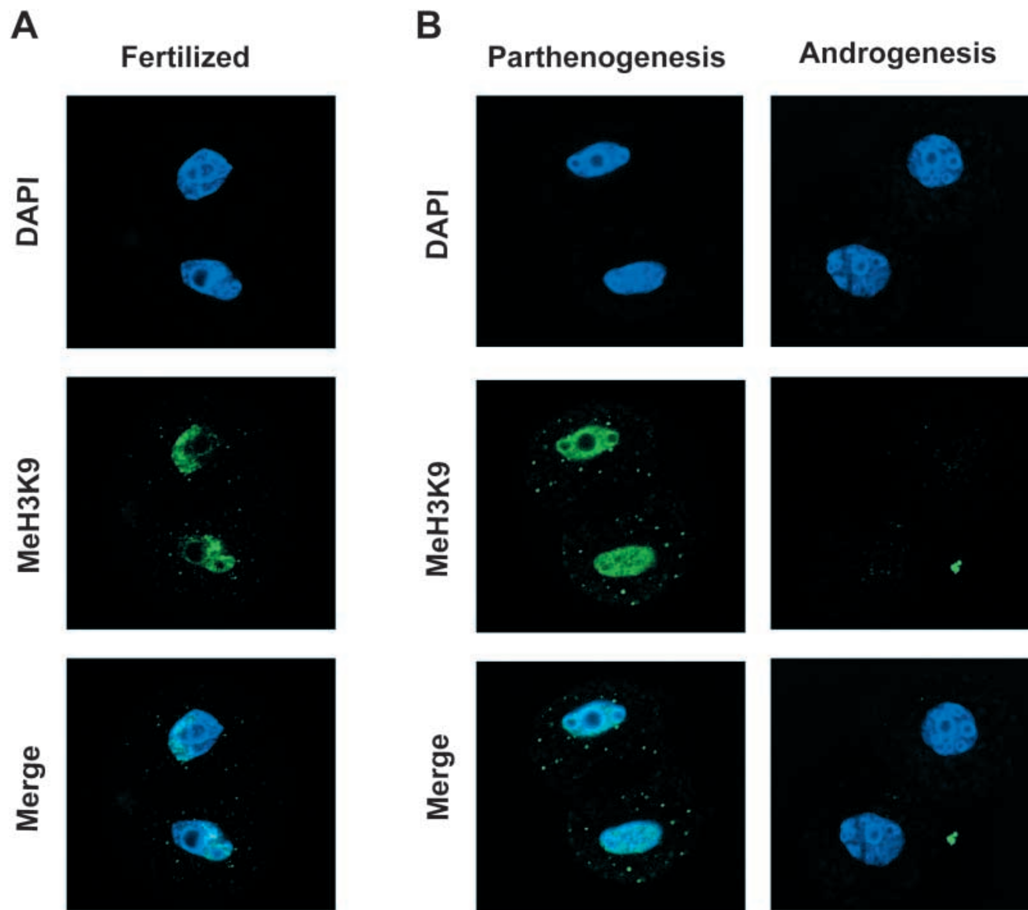

Fig. 2. Histone H3 lysine 9 (H3/K9) methylation patterns in two-cell mouse embryos that were produced by fertilization, parthenogenesis and androgenesis. Mouse two-cell embryos that were collected 32 hours after in vitro fertilization (A), and parthenogenetic and androgenetic two-cell embryos (B), were subjected to immunocytochemistry with the antibody to methylated H3/K9 (MeH3K9). The antibody was localized with FITC-conjugated secondary antibodies (green), and DNA was stained with DAPI (blue). The parthenogenetic embryos were prepared by exposing MII-stage oocytes to 7% ethanol for 6 minutes, and collected for immunocytochemistry 32 hours later. The androgenetic embryos were prepared by fertilizing enucleated oocytes in vitro, and collected for immunocytochemistry 32 hours after fertilization. The experiments were conducted four times and similar results were obtained. More than 25 images were obtained for each type of embryo and representative examples are shown.

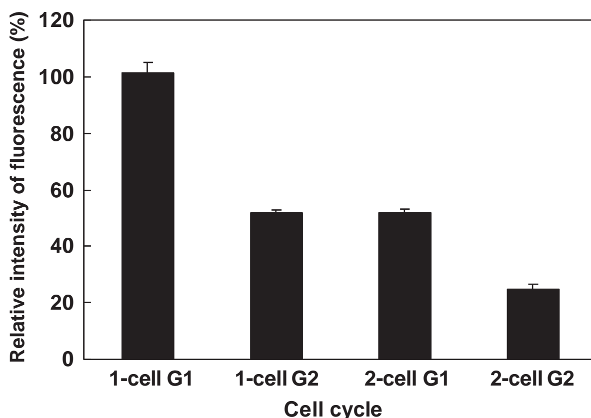

Fig. 3. Changes in the methylation levels of H3/K9 during the first and second cell cycles of mouse embryos. The embryos were collected at 6, 12, 20 and 30 hours after insemination, designated as samples one-cell G1, one-cell G2, two-cell G1 and two-cell G2, respectively, and subjected to immunocytochemistry using the antibody against methylated H3/K9. The intensity of fluorescence was analyzed semi-quantitatively, as described in Materials and methods. The nuclei of diploid parthenogenetic two-cell G2 embryos were used as controls in each experiment, and the averaged value of the fluorescence intensity in the controls was arbitrarily set at 50%. The fluorescence intensity of each sample was expressed relative to this value. Data were accumulated from two independent experiments, and were expressed on the basis of DNA content. The numbers of blastomeres examined were: 14, 53, 90 and 38 for the one-cell G1, one-cell G2, two-cell G1 and two-cell G2 stages, respectively. The columns and bars represent mean±s.e.m.

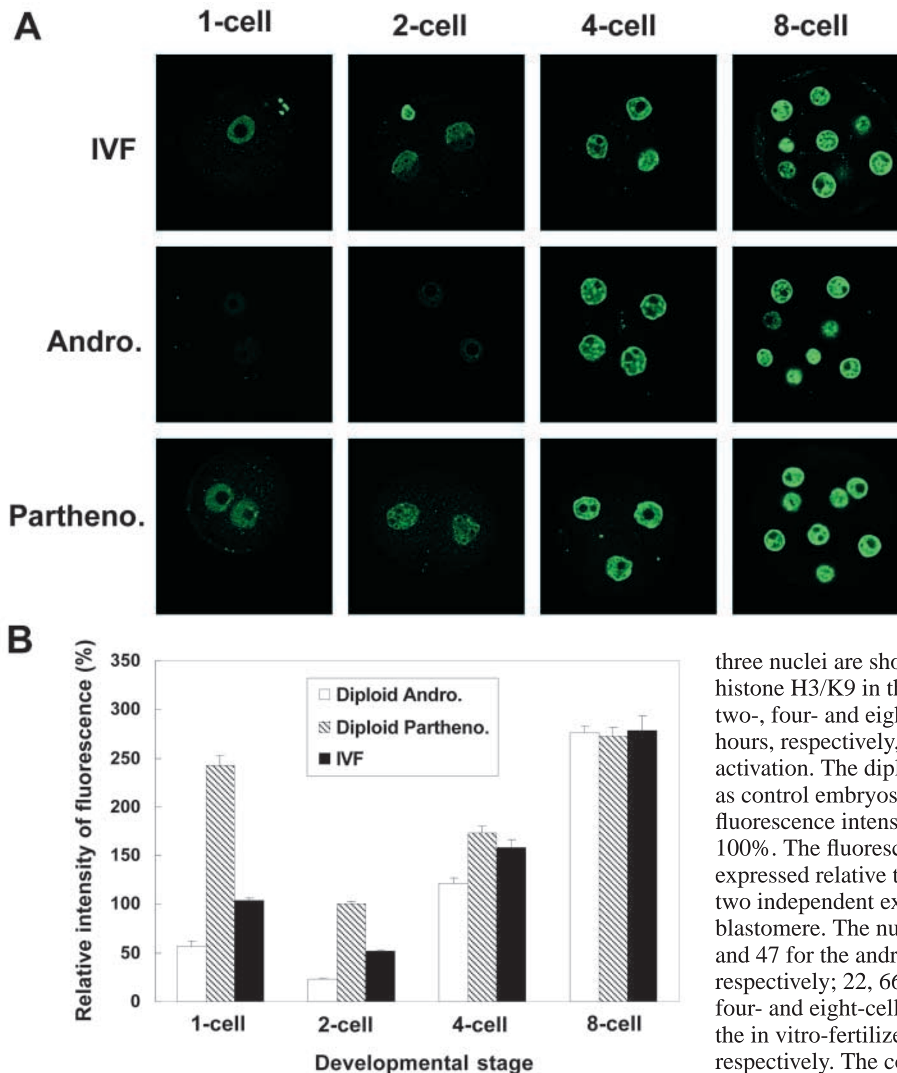

Fig. 4. Changes in the methylation levels of H3/K9 during early pre-implantation development in the mouse. Embryos that were prepared by in vitro fertilization (IVF), parthenogenesis (Partheno) and androgenesis (Andro) were examined for H3/K9 methylation using immunocytochemistry. The diploid parthenogenetic embryos were prepared by activating MII-stage oocytes by treatment with 10 mM Sr^{2+} in CZB for 10 minutes, followed by an additional 6-hour incubation in CZB that contained 5 μg/ml cytochalasin B to prevent extrusion of the polar bodies from the oocytes. The diploid androgenetic embryos were prepared by collecting the one-cell embryos with two pronuclei after in vitro fertilization of the enucleated oocytes. (A) Immunofluorescent confocal micrographs of mouse embryos. The two-, four- and eight-cell embryos were collected 30, 40 and 50 hours, respectively, after in vitro fertilization or parthenogenetic activation. As not all of the nuclei in some of the four-cell embryos could be aligned, only three nuclei are shown. (B) Semi-quantification of methylated histone H3/K9 in the pre-implantation mouse embryos. Mouse one-, two-, four- and eight-cell embryos were collected 12, 20, 40 and 50 hours, respectively, after in vitro fertilization or parthenogenetic activation. The diploid parthenogenetic two-cell embryos were used as control embryos in each experiment, and the averaged value of the fluorescence intensity in the control embryos was arbitrarily set at 100%. The fluorescence intensity observed for each sample was expressed relative to this value. Data were accumulated from at least two independent experiments, and were expressed on the basis of the blastomere. The numbers of blastomeres examined were: 8, 18, 32 and 47 for the androgenetic one-, two-, four- and eight-cell embryos, respectively; 22, 66, 47 and 70 for the parthenogenetic one-, two-, four- and eight-cell embryos, respectively; and 53, 90, 56 and 76 for the in vitro-fertilized one-, two-, four- and eight-cell embryos, respectively. The columns and bars represent mean±s.e.m.

to the two-cell stage with the same frequency as the control group following the removal of cycloheximide (data not shown). Thus, these results suggest that newly synthesized proteins are required to maintain asymmetric methylation through the inhibition of de novo methylation after fertilization.

In mice, zygotic gene activation (ZGA) starts at the S-G2 phase in one-cell embryos, and a burst of ZGA occurs late in the two-cell stage. The degradation of maternally-derived transcripts progresses until the late two-cell stage, and the newly synthesized zygote-derived transcripts compensate for the lost transcripts (Schultz, 1993; Aoki et al., 1997). Therefore, we investigated the involvement of ZGA in the maintenance of asymmetric H3/K9 methylation at the two-cell stage. When the androgenetic embryos were treated with α-amanitin, which is an inhibitor of RNA polymerase II, the intensity of the fluorescence signal increased prominently compared with that of the untreated androgenetic embryos at the late two-cell stage (Fig. 6B). Thus, the de novo H3/K9 methylation of paternal genomes is the result of transcription inhibition, and ZGA is involved in the maintenance of asymmetric H3/K9 methylation between paternal and maternal genomes.

Methylation of DNA cytosine in the male pronucleus after transfer to an enucleated oocyte

DNA methylation of CpG dinucleotides, which is a major epigenetic modification of the genome, plays an important role in the regulation of gene expression and is essential for mammalian embryogenesis (Ferguson-Smith and Surani, 2001; Reik et al., 2001). Studies using fungi and plants have revealed a relationship between DNA methylation and H3/K9 methylation, and have suggested that DNA methylation acts downstream of H3/K9 methylation (Tamaru and Selker, 2001; Gendrel et al., 2002; Jackson et al., 2002). However, there is little evidence to support a similar scenario in mammals. To investigate the relationship between DNA and H3/K9 methylation, male pronuclei were transferred into enucleated oocytes and changes in the methylation patterns were examined. Before nuclear transfer, we examined the DNA methylation patterns of the donor pronuclei and verified the previous observation that active DNA demethylation was confined to the paternal pronucleus (Mayer et al., 2000a). No cytosine methylation fluorescence signals were detected in the male pronuclei 6 hours after insemination (data not shown). Reconstructed oocytes were produced by transferring the male

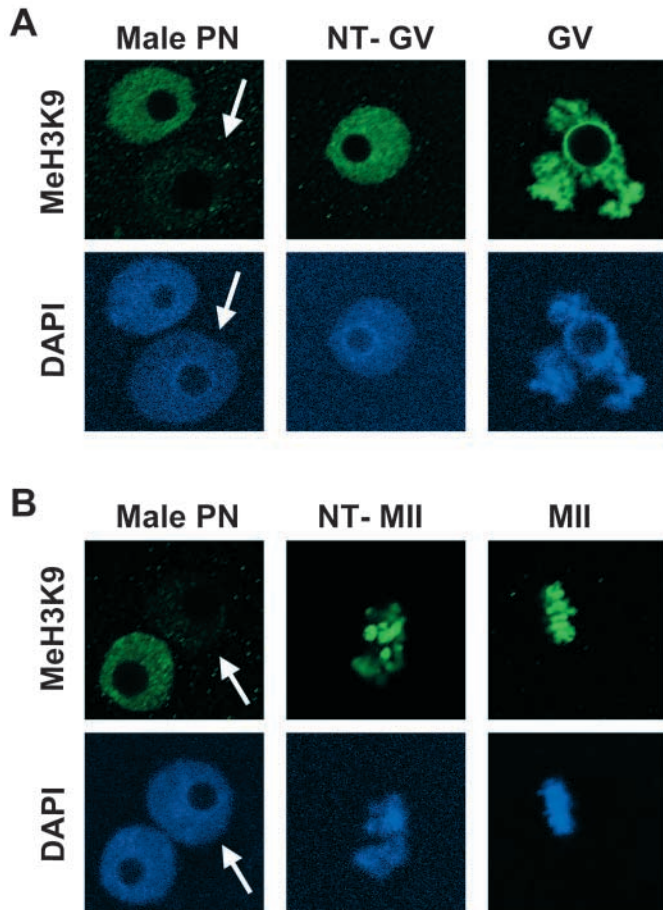

Fig. 5. H3/K9 methylation in male pronuclei that were transplanted into enucleated GV- or MII-stage oocytes. The reconstructed oocytes were produced by transferring the male pronucleus 6 hours after insemination into enucleated oocytes at (A) the GV stage (NT-GV) or (B) the MII stage (NT-MII), and were used for immunocytochemistry following culture for 3 hours with (for NT-GV) or without (for NT-MII) 0.2 mM IBMX. The antibody that recognizes the methylated lysine 9 on histone H3 (MeH3K9) was localized with FITC-conjugated secondary antibodies (green), and DNA was stained with DAPI (blue). Intact oocytes at the GV stage (GV) and MII stage (MII) that had not been enucleated are shown as controls. The reconstruction experiments for the NT-GV and NT-MII oocytes were conducted four and three times, respectively. In total, 23 NT-GV and 18 NT-MII oocytes were reconstructed successfully and examined for histone methylation. Similar patterns were observed for each type of reconstructed oocyte; representative examples are shown.

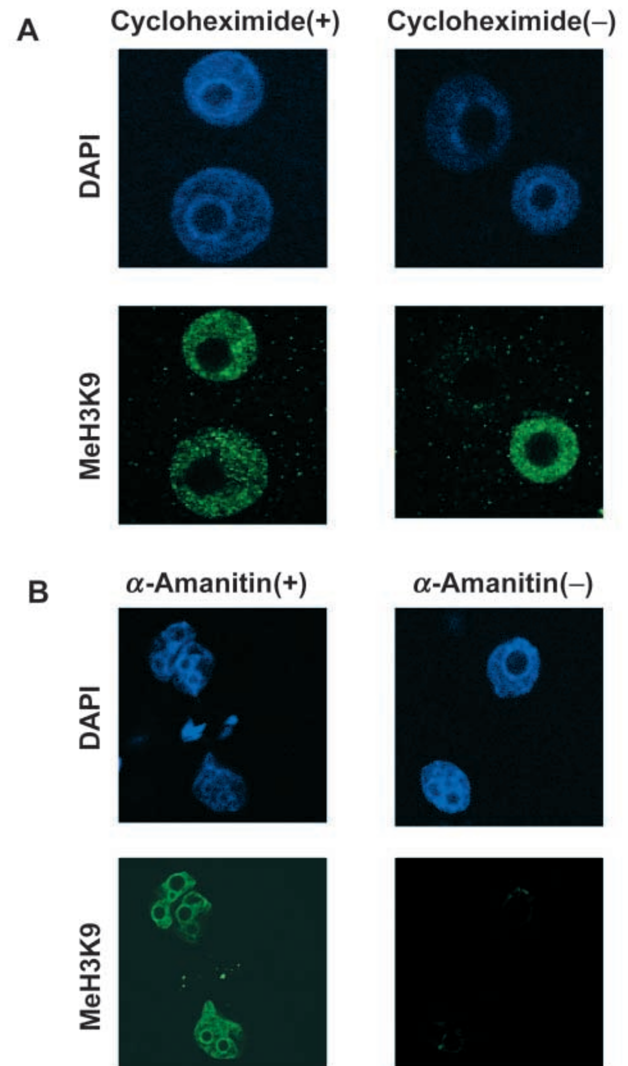

Fig. 6. Involvement of protein synthesis and gene expression in H3/K9 methylation in mouse embryos. (A) Cycloheximide (50 μg/ml) was added at the time of insemination, and H3/K9 methylation (MeH3K9) was examined 12 hours post-insemination. (B) α-Amanitin (25 μg/ml) was added 4 hours after activation of the androgenetic embryos, and H3/K9 methylation was examined 32 hours post-activation. The polyclonal antibody that recognizes methylated H3/K9 was localized with FITC-conjugated secondary antibodies (green), and the DNA was stained with DAPI (blue). The experiments using cycloheximide and α-amanitin were conducted three and two times, respectively, and similar results were obtained. 21, 12, 18 and 11 embryos were examined in the cycloheximide (+), cycloheximide (–), α-amanitin (+) and α-amanitin (–) groups, respectively, and representative examples are shown.

pronuclei to enucleated GV-stage or MII-stage oocytes. When GV-stage oocytes were used as recipients, the reconstructed oocytes were arrested at prophase of the first meiosis in the presence of IBMX, which is an inhibitor of meiotic resumption, whereas they spontaneously resumed meiosis and underwent germinal vesicle breakdown (GVB) 3 hours after nuclear transfer in the absence of IBMX. Regardless of the occurrence of GVB, de novo DNA methylation occurred in the transplanted nuclei, and was accompanied by global histone H3/K9 methylation (Fig. 7). By contrast, DNA methylation did not occur in the male pronuclei that were transferred into MII-stage oocytes, although H3/K9 methylation did occur in this case.

Discussion

This study investigated the changes in H3/K9 methylation patterns and the mechanism regulating those changes during early pre-implantation development. Before fertilization, the maternal genome was methylated at H3/K9 in oocytes and was

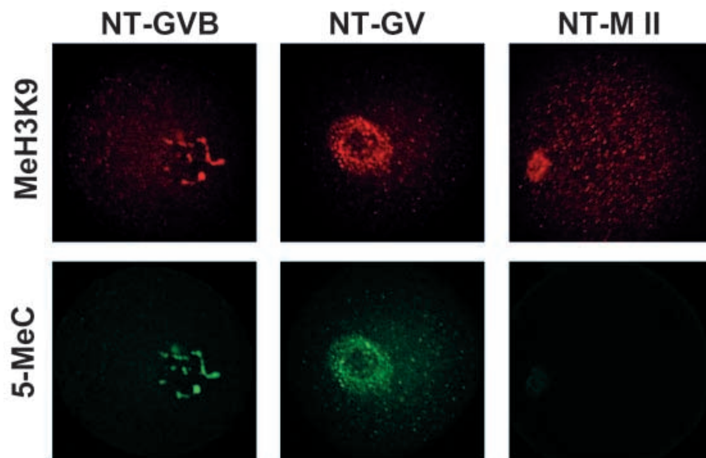

Fig. 7. Relationship between H3/K9 and DNA cytosine methylation in the male pronucleus following transplantation into GV-stage or MII-stage oocytes. Reconstructed oocytes were produced by transferring male pronuclei into enucleated GV-stage oocytes, followed by a 3-hour culture to retrieve meiosis (NT-GVB) or to inhibit meiotic resumption in the presence of IBMX (NT-GV). Alternatively, the male pronuclei were transferred into enucleated MII-stage oocytes (NT-MII), followed by culture for 3 hours in the presence of nocodazole to prevent spontaneous activation. The antibodies directed against H3/K9 methylation (MeH3K9) and DNA cytosine methylation (5-MeC) were detected using the cy3-conjugated secondary antibody (red) and the FITC-conjugated secondary antibody (green), respectively. The reconstruction experiments for the NT-GVB, NT-GV and NT-MII oocytes were conducted three times. In total, 18 NT-GVB, 20 NT-GV and 18 NT-MII oocytes were reconstructed successfully and examined for histone methylation. Similar patterns were observed for each type of reconstructed oocytes; representative examples are shown.

maintained in a methylated state after fertilization, whereas the paternal genome was undermethylated (Fig. 1A). The undermethylation of the paternal genome seems to be caused by inactivation of the H3/K9 methylase in the cytoplasm after fertilization, and not by the structural properties of chromatin itself, as it was methylated after the male pronucleus was transferred into an unfertilized oocyte (Fig. 5B). Thus, the methylase probably acts before fertilization and not afterwards, which leads to asymmetric H3/K9 methylation, whereby only the genome present in the oocyte before fertilization is methylated, and that absent from the oocyte is not methylated. Asymmetric methylation was maintained until the two-cell stage, and the methylation levels were increased symmetrically in the genomes of both parental origins at the four-cell stage (Figs 2, 4), which suggests that the methylase begins to function at this stage.

Histones in the oocyte cytoplasmic pool vary in terms of modifications with respect to different histones and different lysine residues. For instance, histone H4 is acetylated at lysines 5 and 12 by the cytoplasmic acetyltransferase, transported to the nucleus, and assembled into chromatin (Sobel et al., 1995; Verreault et al., 1996; Adenot et al., 1997). After assembly, H4 is deacetylated, and then acetylated in an appropriate manner by the nuclear deacetylases and acetyltransferases. In one-cell embryos, the male pronuclei exhibit higher levels of hyperacetylated H4 than the female pronuclei at G1 phase, but the levels are similar in both pronuclei during the S and G2 phases (Adenot et al., 1997). Thus, the H4 acetylation pattern changes dramatically during the one-cell stage. In contrast to histone H4 acetylation, the asymmetric methylation pattern of H3 in the parent genomes was stably maintained until the two-cell stage. H3 was methylated in the chromatin, but undermethylated in the oocyte cytoplasmic pool. After fertilization, the sperm chromatin exchanged protamines for histones. Thus, the paternal chromatin incorporated hypomethylated H3, and could be discriminated from the maternal genome, which consisted of methylated H3. This difference in methylation pattern was maintained stably, as the H3 methylase did not function after fertilization. These results suggest that H3/K9 methylation serves as an inherited epigenetic code to distinguish parental genomes during early pre-implantation development.

Asymmetric H3/K9 methylation may function as a precursor for asymmetric DNA methylation, which is involved in genomic imprinting or asymmetric X chromosome inactivation in trophectoderm cells. Studies have shown that parent-specific imprints are established on some endogenous genes and exogenous transgenes during or after fertilization (El-Maarri et al., 2001; Pickard et al., 2001). After fertilization, however, the DNA methylation level decreases until the blastocyst stage, and de novo DNA methylation occurs around the time of implantation (Reik et al., 2001). For instance, the imprinting control region of the *Lit1* gene is completely demethylated until the two-cell stage and then is methylated only in the genome of maternal origin at the blastocyst stage (Yatsuki et al., 2002). Furthermore, we have shown that the DNA methyltransferase does not function in unfertilized oocytes (Fig. 7), which indicates that DNA methylation is not sufficient for epigenetic marking during and after fertilization. Therefore, the epigenetic marking appears to involve an epigenetic modification other than DNA methylation. This suggests the existence of an epigenetic marking process that occurs in the cytoplasm of oocytes and targets the DNA for methylation at later stages of development. The de novo cytosine methyltransferase seems to take cues from this type of epigenetic marker (El-Maarri et al., 2001; Pickard et al., 2001). It is unclear how the two parental genomes are distinguished in embryos. The asymmetric H3/K9 methylation we observed, in which only the histone that was taken over from the oocyte chromosome was methylated, could be a simple yet secure mechanism for distinguishing parental genomes in embryos.

Studies with *Neurospora* and *Arabidopsis* suggest that DNA methylation takes its cue from histone H3/K9 methylation (Bartee et al., 2001; Tamaru and Selker, 2001; Gendrel et al., 2002; Jackson et al., 2002; Richards and Elgin, 2002). In *Neurospora*, mutation of the histone H3 methyltransferase appears to abolish all cytosine methylation (Tamaru and Selker, 2001). In *Arabidopsis*, recent research has shown that chromomethylase 3 (CMT3) interacts with the *Arabidopsis* homologue of HP1 (Cbx5 – Mouse Genome Informatics), which, in turn, interacts with methylated histones, suggesting that CpNpG DNA methylation is controlled by histone H3/K9 methylation via the interaction of CMT3 with methylated chromatin (Jackson et al., 2002). Furthermore, the Suv39h histone methylase in mammalian cells is required for DNA methylation (Lehnertz et al., 2003). Thus, H3/K9 methylation

appears to direct DNA methylation, although the opposing view that CpG methylation directs H3/K9 methylation has also been suggested for *Arabidopsis* (Soppe et al., 2002). In mouse zygotes, the methylation states of DNA cytosine are consistent with those of H3/K9 (Arney et al., 2002). The absence of H3/K9 methylation in the paternal pronucleus is consistent with the preferential demethylation of paternal DNA, which occurs within 4 hours of fertilization (Santos et al., 2002). In bovine embryos, the methylation level of H3/K9 has been found to increase in parallel with that of DNA after the eight-cell stage (Santos et al., 2003). Our results also reveal corresponding de novo methylation of H3/K9 and DNA in paternal pronuclei that were transferred into enucleated GV-stage oocytes (Fig. 7). Furthermore, our finding that the levels of H3/K9 methylation, but not those of DNA methylation, increased in pronuclei that were transferred into MII-stage oocytes (Fig. 7) suggests that H3/K9 methylation does not depend on DNA methylation, as these results would not have been obtained if DNA methylation directed H3/K9 methylation. Similarly, the level of DNA methylation does not increase in embryos treated with α-amanitin or cycloheximide (F.A. and J.-M.K., unpublished), although the level of H3K9 methylation increased (Fig. 6). The DNA methyltransferase would be inactivated during meiotic maturation, resulting in unchanged levels of DNA methylation in the nuclei of two-cell embryos treated with α-amanitin or cycloheximide, or in pronuclei transferred into MII-stage oocytes, despite increased H3/K9 methylation. Our finding that the levels of DNA methylation were unchanged in paternal pronuclei that were transferred into MII-stage oocytes is consistent with the successful production of cloned animals by the transfer of somatic nuclei, and the observation that genome imprinting is inherited faithfully by cloned mice (Inoue et al., 2002).

A recent report has suggested that trimethyl-, but not dimethyl-, H3/K9, is involved in DNA methylation (Tamaru et al., 2003). We have examined the changes in the levels of H3/K9 trimethylation during meiotic maturation and early pre-implantation development. Immunocytochemistry using an antibody that specifically recognizes trimethyl-H3/K9 revealed that the level of trimethylation changes in a manner similar to that observed for dimethylation in this study (F.A. and J.-M.K., unpublished). As the anti-dimethyl-H3/K9 antibody used in our study did not cross-react with trimethyl-H3/K9 (see Fig. S1 at http://dev.biologists.org/supplemental/), both the dimethylated and trimethylated forms of H3/K9 are present, and their levels appear to be under the control of a single mechanism. It remains to be clarified whether these two epigenetic modifications play different roles in the regulation of genome function.

In mouse embryos, transcription is initiated during the one-cell stage, at which stage transcriptional activity is much higher in the male pronucleus than in the female pronucleus (Aoki et al., 1997). Studies have suggested that this difference is caused by differences in the repressive states of chromatin; the chromatin in the male pronucleus is not repressed, whereas that in the female pronucleus is partially repressed (Wiekowski et al., 1993; Majumder et al., 1997). However, the mechanism regulating this differential repression is not clear. It has been suggested that H3/K9 methylation is involved in the repression of gene expression in both euchromatic and heterochromatic regions (Hwang et al., 2001; Nielsen et al., 2001; Peters et al., 2002; Saccani and Natoli, 2002). It is possible that the asymmetric methylation of H3/K9 is involved in the different repressive states of the two parental genomes.

During the one-cell stage, DNA replication occurs asynchronously between the two pronuclei, in that the female pronucleus requires a longer time to complete replication (Aoki and Schultz, 1999). This suggests that heterochromatin is asymmetrically constituted in the two pronuclei, as H3K9 methylation is involved in the formation of heterochromatin, which is late-replicating (O'Keefe et al., 1992; Spector, 1993). A higher level of H3K9 methylation would promote the formation of heterochromatin in the female pronucleus, resulting in late DNA replication. Supporting this hypothesis are the facts that methylated H3K9 recruits heterochromatin protein 1 (HP1), an essential protein constituting heterochromatin (Bannister et al., 2001; Schultz et al., 2002), and that this protein is accumulated only in the female pronucleus before S phase of the one-cell stage (Arney et al., 2002). The biological significance of this asymmetric heterochromatin formation is unclear. It does not directly involve asymmetric X chromosome inactivation, as HP1 does not accumulate with the inactive X chromosome (Peters et al., 2002).

The maintenance of differential H3/K9 methylation patterns between the paternal and maternal genomes is an active process that depends on de novo protein synthesis and gene expression. De novo H3/K9 methylation occurred in the male pronuclei after transfer into enucleated oocytes (Fig. 5), which indicates that H3/K9 methylase activity exists during meiosis. The absence of histone methylase activity after fertilization was not due to degradation of the enzyme, as the enzyme functioned when protein synthesis was inhibited by cycloheximide (Fig. 6A). Although the histone methylase exists in both oocytes and embryos after fertilization, the activity of the enzyme is inhibited by its inhibitor(s), which may be synthesized as early as male pronucleus formation, i.e. 4 hours after insemination. Fertilization of oocytes is accompanied by changes in the pattern of protein synthesis (Xu et al., 1997). These changes occur within 4 hours, at which time gene expression has not yet been initiated (Bounial et al., 1995; Aoki et al., 1997); thus, these changes are due to the recruitment of maternal mRNAs. It has been reported that several maternal mRNAs are recruited for protein synthesis after fertilization, including α-catenin, Ptp4a1, Spin (Oh et al., 2000) and cyclin A2 (Fuchimoto et al., 2001). The maternal mRNA for the inhibitor of methylase may also be recruited for translation after fertilization. Alternatively, it is possible that the inhibitor protein is already present, albeit in an inactive form, in the oocyte cytoplasm before fertilization. After fertilization, this protein is activated and functions as an inhibitor of H3/K9 methylase. When the inhibitor is labile, the inhibition of protein synthesis by cycloheximide brings about the abolition of the inhibitor protein, which results in increased levels of H3/K9 methylation in the male pronuclei. In any case, it seems likely that there is an inhibitor protein(s) and that inhibition of its synthesis results in the increase in the H3/K9 level in the male pronucleus after fertilization. This suggests that the inhibitor protein(s) inactivates H3/K9 methylase, thereby generating an asymmetric H3/K9 methylation pattern after fertilization.

The inhibition of zygotic transcription resulted in de novo histone methylation of the paternal genome (Fig. 6B). The

transition from maternal to embryonic control of development occurs in the two-cell stage of mouse embryos. During the two-cell stage, a burst of transcriptional activation occurs in the embryonic genome. The transcripts from the embryonic genome are used soon after their synthesis, and most of the maternal transcripts used before this timepoint are degraded (Flach, 1982; Schultz, 1993). The asymmetric H3/K9 methylation pattern between parental genomes persists during the transition from maternal to embryonic control, and gene expression from the embryonic genomes is required for the maintenance of the asymmetric pattern. After the degradation of maternal mRNA, the inhibitor of histone methylase is probably produced via a transcript from the embryonic genome.

Asymmetric H3/K9 methylation may function as the mechanism that distinguishes the genomes of different parental origin. The genomes are simply identified as being marked or unmarked, based on whether they are in oocytes before fertilization; only maternal genomes are present in the oocytes before fertilization. This mechanism does not require differences between the genomes themselves, as genomic distinction is maintained because the H3/K9 methylase does not function, and de novo methylation does not occur after fertilization. In addition, active H3/K9 demethylation does not seem to occur, as no histone demethylase has been found in any organism. However, the methylation level of the maternal genome decreases without de novo methylation. During embryonic development, the chromatin is replicated, and methylated H3 is diluted with unmethylated H3 from the cytoplasmic or nucleoplasmic pools. The H3/K9 methylase is activated at the four-cell stage, and the genomes of both parental origins are methylated symmetrically. Thus, after the two-cell stage, some other mechanism takes over from H3/K9 methylation to maintain distinction of the parental genomes. Indeed, Xist begins to be expressed from the two-cell stage and is associated with the X chromosome of paternal origin (Huynh and Lee, 2003). In the mechanism of paternal X chromosome inactivation during pre-implantation development, Xist may take-over from H3/K9 methylation as the mechanism for discriminating paternal genomes. Our hypothesis for the mechanism that generates different H3/K9 methylation patterns, and thereby distinguishes the different parental origins of genomes, is summarized in Fig. 8.

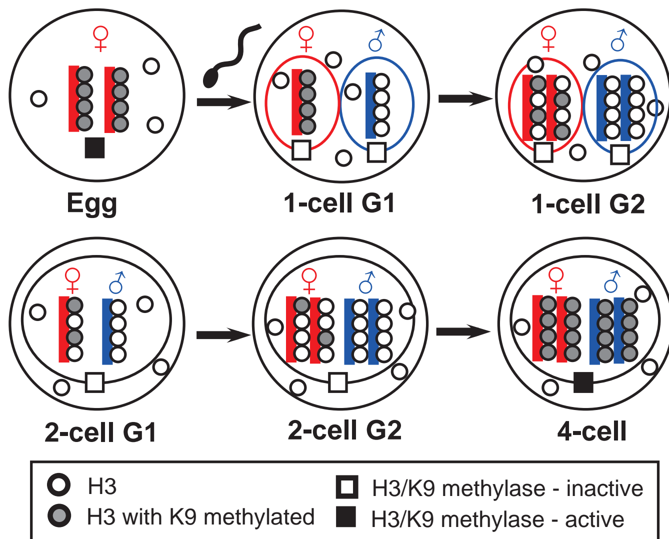

Fig. 8. Schematic of the hypothetical mechanism that generates different H3/K9 methylation patterns, so that the different parental origins of genomes are distinguished during early pre-implantation development. In oocytes, H3/K9 methylase actively catalyzes the protein in the maternal genomes, but not in the cytoplasmic pool. After fertilization, the methylase is inactivated, and H3/K9 in the paternal genome, which is incorporated from the cytoplasmic pool, remains undermethylated. The methylase is still present but inactivated by proteins that are newly synthesized after fertilization. Thus, asymmetric H3/K9 methylation is established in the parent genomes. This asymmetric methylation is maintained until the two-cell stage, because of the lack of methylase and demethylase activities. However, the methylation level decreases gradually in a passive fashion after each DNA replication. When the DNA is replicated, the pre-existing nucleosomal structure is disrupted at the replication fork, and the core histones are dissociated from the DNA. The histones from the nucleoplasmic pool are sequestered together with the histones from the pre-existing nucleosomes, and amalgamate into a single structure. Thus, in the nascent chromatin, pre-existing histones are diluted with histones that are incorporated from the nucleoplasm after DNA replication (Wolffe, 1998). At the four-cell stage, H3/K9 methylase is activated, and catalyzes the proteins in the genomes of both parental origins.

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