Changes in H3K79 methylation during preimplantation development in mice

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Abbreviations

- Bl: Blastocyst
- EX Bl: Expanded Blastocyst
- G1 phase: Gap 1 phase
- G2 phase: Gap 2 phase
- GV: Germinal Vesicle (at prophase on first meiosis)
- GVBD: Germinal Vesicle Break Down(=meiosis I stage)
- H3K79: 79th lysine from N terminus on Histone H3 polypeptide
- H3K79me2 :histone H3 lysine 79 dimethylation
- H3K79me3: histone H3 lysine 79 trimethylation
- H3K4: 4th lysine from N terminus on Histone H3 polypeptide
- H3K4me2: histone H3 lysine 4 dimethylation
- H3K4me3:histone H3 lysine 4 trimethylation
- H3K9: 9th lysine from N terminus on Histone H3 polypeptide
- H3K9me2: histone H3 lysine 9 dimethylation
- H3K9me3: histone H3 lysine 9 trimethylation
- H4K20: 20th lysine from N terminus on Histone H4 polypeptide
- H4K20me3: histone H4 lysine 20 trimethylation
- HP1β: Heterochromatin protein 1 β
- MII: meiosis II stage
- mDot1: mouse disrupter of teromere silencing 1
- Mo: Morula
- M phase: Mitosis phase

- NT: nuclear transfer
- Sir2: Silent information regulator 2
- S phase: DNA synthesis phase

Abstract

Before and/or after fertilization, differentiated oocytes are transformed into totipotent preimplantation embryos. In this process, gene expression pattern is reprogrammed. To elucidate the mechanism of genome reprogramming, we investigated histone H3 lysine 79 dimethylation (H3K79me2) and trimethylation (H3K79me3) in the oocytes and preimplantation embryos. H3K79me2 has been considered as an active gene marker which is maintained and inherited into the daughter cells after cell division, although the function of H3K79me3 has not been elucidated in mammalian cells. In somatic cells and oocytes, H3K79me2 were observed in the whole of the genome, while H3K79me3 was localized in the pericentromeric heterochromatin regions in which there is no active gene. Thus, H3K79 methylation seems to have the opposite functions dependently on number of the methyl groups added on same residues. Both of H3K79me2 and H3K79me3 disappeared soon after fertilization and the hypomethylated state was maintained at interphase before blastocyst stage except for the transient increase of H3K79me2 at M phase. However, H3K79me3 was not detected all through the preimplantation stage even at M phase. To investigate the involvement of H3K79me2 demethylation in the genome reprogramming, somatic nuclei were transplanted into the enucleated oocytes. The H3K79me2 in these nuclei were demethylated following parthenogenetic activation, although the nuclei which had been transplanted into the parthenogenetic embryos 7 h after activation were not demethylated. These results suggested that elimination of H3K79 methylation after fertilization would be involved in genomic reprogramming.

Introduction

Before and/or after fertilization, differentiated oocytes are transformed into totipotent preimplantation embryos (Fig. 1). In this process, gene expression pattern is reprogrammed. Growing oocytes actively express genes including oocyte-specific genes. However, they cease gene expression before they are fully grown and remain in a transcriptionally silent state during meiotic maturation (Fig. 1) (Latham and Schultz, 2001; Schultz, 1993; Worrad et al., 1994). After fertilization, the zygotes start gene expression in an embryo-specific pattern and then this pattern is dynamically altered during preimplantation development (Fig. 1) (Aoki et al., 1997; Hamatani et al., 2004; Wang et al., 2004; Zeng et al., 2004). Although the reprogramming of gene expression is thus an important event to create a new life, the mechanism regulating it has not been well elucidated.

Histone methylation plays important roles in regulating chromatin structure and gene expression. There are several lysine residues which have been known to be methylated in histones. Each of these lysine residues can be mono-, di-, or tri-methylated (Czermin et al., 2002; Kuzmichev et al., 2002; Santos-Rosa et al., 2002; Tamaru et al., 2003). Unlike acetylation, which is generally associated with transcriptional activation, methylation is involved in the activation and repression, depending on the lysine residues methylated (Martin and Zhang, 2005; Zhang and Reinberg, 2001). Furthermore, it is also important in transcriptional regulation how many methyl groups are added in a lysine residue (Martin and Zhang, 2005). For instance, histone H3 lysine 4 dimethylation (H3K4me2) and trimethylation (H3K4me3), which is known as active markers of gene expression, are localized on euchromatin, a

transcriptionally active chromosomal domain (Miao and Natarajan, 2005; Ng et al., 2003b; Schneider et al., 2004; Schubeler et al., 2004; Sims et al., 2003). However, these different methylation states do not overlap completely. While the H3K4me2 is distributed across the whole body of active genes, H3K4me3 is localized specifically at 5' end of these genes (Bernstein et al., 2002; Martin and Zhang, 2005; Pokholok et al., 2005; Santos-Rosa et al., 2002). Histone H3 lysine 9 dimemethylation (H3K9me2) and histone H3 lysine 9 trimethylation (H3K9me3), which is known as repressive markers, are localized on heterochromatin, a transcriptional repressive chromosomal domain (Miao and Natarajan, 2005; Santos et al., 2005; Sims et al., 2003). Althouth H3K9me2 was detected on facultative heterochromatin which is a changeable condensed chromatic domain (Arney and Fisher, 2004; Martin and Zhang, 2005), H3K9me3 was observed in constitutive heterochromatin which comprise of highly condensed pericentormeric heterochromatin (Arney and Fisher, 2004; Peters et al., 2003; Rice et al., 2003). It would be involved in these differences that heterochromatin protein 1 (HP1) which associates with methylated H3K9 to form heterochromatin binds to H3K9me3 2-fold stronger than H3K9me2 (Fischle et al., 2003). Thus, not only which lysine residue is methylated but also how many methyl groups are added in a lysine residue is important in the regulation of chromatin structure and gene expression (Fischle et al., 2003; Jenuwein, 2006; Santos-Rosa et al., 2002).

Among several methylated lysines, H3K79 methylation has unique characteristics. First, although most of other methylated lysine residues locate near N terminus, H3K79 locate in globular domain (Ng et al., 2002). Although the globular domain exists on the center of Histone H3 polypeptide chain, it is localized on the surface in nucleosomal structure so that it could be accessed by any transcriptional

factors (van Leeuwen et al., 2002). Second, H3K79 methylation is catalyzed by disrupter of telomere silencing like protein (Dot1) which was originally identified as a gene whose mutation caused the disruption of telomere silencing in *S.cervisiae* (Lacoste et al., 2002; Ng et al., 2002). Dot1 does not contain set domain, which is highly evolutionally conserved characteristic motif in other all histone methyltransferases (Feng et al., 2002; Min et al., 2003). Therefore, H3K79 methylation may be regulated by different pathway from other histone methylation.

It has been suggested that H3K79 methylation play roles as a marker of euchromatin (Im et al., 2003; Miao and Natarajan, 2005; Ng et al., 2003a; Schubeler et al., 2004; Sims et al., 2003). In *S.cervisiae*, Sir2, which is involved in heterochromatin formation, is not recruited in the region of chromosome where H3K79 is methylated (Ng et al., 2002; Sims et al., 2003). On the contrary, H3K79me2 does not exist on particular regions, i.e. telomere, mating type loci and, in which Sir2 is associated with chromatin, leading to the heterochromatin formation (Ng et al., 2003a; Sims et al., 2003; van Leeuwen et al., 2002). In mammalian cells, H3K79me2 has been also suggested as an active gene marker (Im et al., 2003; Martin and Zhang, 2005; Miao and Natarajan, 2005). It exists on the promoter and 5' regions within coding region of transcriptionally active genes (Kouskouti and Talianidis, 2005). After the first round of transcription, H3K79 is dimethylated to plays a role as an active gene marker (Im et al., 2003; Martin and Zhang, 2005; Miao and Natarajan, 2005; Ng et al., 2003a; Schubeler et al., 2004; Sims et al., 2003). Similarly to H3K79me2, H3K79me3 has been suggested as an active gene marker in *S.cervisiae* (Pokholok et al., 2005). However, there is no knowledge about the function of H3K79me3 in mammalian cells.

In this study, we investigated H3K79 methylation states in the oocytes and

preimplantation embryos to elucidate the mechanism regulating the reprogaramming of gene expression pattern during oogenesis and preimplantation development. We found that H3K79me2 and H3K79me3 exist in the different regions of chromosomes from each other in the oocytes: H3K79me2 was detected in whole genome, whereas H3K79me3 was in pericentromeric region. This result suggests that these two modifications have different functions: H3K79me2 and H3K79me3 may be an active and repressive marker of gene expression, respectively. Interestingly, both of these modifications disappeared after fertilization when embryos acquire totipotency. These results suggest that the alteration of H3K79 methylation state is involved in the reprogramming of gene expression in the embryos.

Materials and methods

Collection and culture of oocytes and embryos

Oocytes at the germinal vesicle stage were collected from 3-week-old B6CDF1mice that had been injected with 5 IU of pregnant mare's serum gonadotropin (PMSG; Sankyo Co., Ltd., Tokyo). The cells were incubated in KSOM (Erbach et al., 1994) containing 0.2 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma Chemical CO., St Louis, MO), as described previously (Choi et al., 1991), and were subsequently cultured without IBMX to allow meiotic maturation in a humidified 5% CO_2/95% air atmosphere at 38°C.

The unfertilized MII stage oocytes were collected in KSOM from 3-week-old B6CDF1mice (CLEA Japan, Inc., Tokyo, Japan) that had been superovulated by injection with 5 IU of PMSG followed 48 h later by 5 IU of human chorionic gonadotropin (hCG; Sankyo Co., Ltd., Tokyo). Spermatozoa were collected in HTF medium (Quinn and Begley, 1984) supplemented with 10 mg/ml BSA from the cauda epididymis of mature male ICR mice (SLC Shizuoka, Japan). The oocytes were inseminated with spermatozoa that had been incubated for 2 h at 38°C. The embryos were washed with KSOM containing 3 mg/ml BSA at 5 h after insemination and then cultured in a humidified 5% CO_2/95% air atmosphere at 38°C.

Immunocytochemistry

Oocytes and embryos were washed in PBS that contained 1 mg/ml bovine serum albumin (PBS/BSA), fixed for an hour at room temperature or overnight at 3°C in 3.7% paraformaldehyde in PBS, and permeabilized with 0.5% Triton X-100 in PBS for 15 min at room temperature. The cells were incubated for over night with a 1:100 dilution of the primary antibody against H3K79me2 (Abcam, Lake Placid, NY, USA; catalogue number ab3594), H3K79me3 (Abcam; catalogue number 2621), centromere proteins (CREST) (Antibodies Incorporated, CA, USA catalog number 15-235), or HP1-β (Abcam; catalogue number ab11164). The first antibodies adsorbed with the cells were probed by the incubation for 1 h with a 1:50 dilution of the secondary antibodies.

The secondary antibodies used were purchased from Jackson Immunoresearch (West Grove, PA, USA) as follows: FITC-conjugated anti-rabbit IgG (catalogue number 111-095-003), Cy5-conjugated anti-rabbit IgG (catalogue number 111-175-003), FITC-conjugated anti-goat IgG (catalogue number 705-095-147), Cy5-conjugated anti-goat IgG (catalogue number 705-175-147), Cy5-conjugated ant--antibody human IgG (catalogue number 109-175-003), TRITC-conjugated anti-human IgG (catalogue number 109-025-003) or TRITC-conjugated anti-human IgG (catalogue number 705-025-149). DNA was stained with 100 μg/ml propidium iodide (PI; Sigma-Aldrich, Inc, St. Louis, MO, USA) under RNase existing condition for 20 minutes, and the cells were mounted on a glass slide in Vectashield anti-bleaching solution (Vector Laboratories, Burlingame, CA, USA). In some experiments, the cells treated with RNase were mounted on glass slide in vectashield anti-bleaching solution containing 100 μg/ml propidium iodide. Fluorescence was detected using a Carl Zeiss 510 Laser-scanning confocal microscope.

When the chromosomes of MII stage oocytes were triple-stained with anti-H3K79me3 antibody, CREST and PI, the samples were prepared according to the procedure as described previously (Hodges and Hunt, 2002).

Parthenogenesis

MII stage oocytes were collected from ovaries in KSOM containing 0.3 mg/ml bovine testicular hyaluronidase (Sigma chemical co. MO, USA). After culture for 1 h in KSOM medium, the oocytes were treated with 10 mM Sr^{2+} for 4 h in Ca^{2+}-free KSOM. In order to obtain diploid parthenogenetic embryos, cytochalasin B (1% DMSO) was included in KSOM/ Sr^{2+}.

Nuclear transfer into unfertilized oocytes

The enucleation of oocytes was conducted as described previously (Kim et al., 2002). The nuclei of NIH 3T3 cells were introduced into the enucleated oocytes by electrofusion, using a DC pulse of 1,500 V/cm for 30-50 μs in 300 mM mannitol that contained 0.1 mM $MgSO_4$, 0.1 mg/ml polyvinyl alcohol, and 3 mg bovine serum albumin.

Nuclear transfer into parthenogenetically activated embryos

Parthenogenetically activated embryos were prepared as described above. Seven hours after activation, they were enucleated and transplanted with the nuclei of NIH3T3 cells by electrofusion, using a DC pulse of 1,500 V/cm for 30-60 μs in 300 mM mannnitol containing 0.1 mM $MgSO_4$, 0.1 mg/ml polyvinyl alcohol, and 3 mg bovine serum albumin.

Results

Sub-nuclear localization of H3K79 methylation in somatic cells

Since the localization of H3K79me3 has not been reported in any types of mammalian cells, we firstly examined the sub-nuclear localization of H3K79me2 and H3K79me3 in mouse somatic cells, *i.e.*, NIH3T3 cells and fibroblasts from tail-tip, at interphase and M phase. Immunocytochemistry with specific antibody against H3K79me2 and H3K79me3 revealed that the pattern of localization was always similar between these two types of cells. The intense fluorescence signals of H3K79me2 were observed on the whole of the genome in these cells at interphase (Fig. 2A and B; image a and g) and M phase (Fig. 2A and B; image b, c and h). However, the signals of the antibody against H3K79me3 were detected as several foci in the nucleus at interphase. These foci seemed to be the constitutive heterochromatin such as pericentoromere or telomere, since they were completely superimposed with the highly condensed regions of DNA in the images of propidium iodide staining (Fig. 2A and B; image d and i). At metaphase, H3K79me3 were observed in many small foci, which were localized on the edge of chromosomes (Fig. 2A and B; image e and j). These foci gathered in the outside area of the bundles of chromosomes when the two sets of chromosomes were separated at anaphase (Fig. 2A; image f), suggesting that H3K79me3 is localized on or near centromere region. Interestingly, the signal of H3K79me3 was not detected in all cells. It was detected in only one-third of NIH3T3 cells and fibroblasts examined but not in others at interphase, although it was detected in all cells at M phase. To clarify the localization of H3K79me3, NIH3T3 cells were double-stained with anti-centoromere protein antibody, CREST, and anti-H3K79me3. The result showed that the signals of H3K79me3 and CREST were not overlapped but always localized adjacent to each other (Fig. 2C), suggesting that H3K79me3 is localized on pericentoromeric heterochromatin. It should be noted that although it has been suggested that H3K79me2 is a marker of euchromatin (Miao and Natarajan, 2005; Sims et al., 2003), H3K79me3 seems to be a marker of heterochromatin. Thus, the lysine 79 methylation has opposite functions when the number of methyl group is different.

Dynamics of H3K79 methylation during meiotic maturation and preimplantation development

The oocytes and preimplantation embryos showed the essentially same nuclear localization of H3K79 methylaion as the somatic cells. However, their methylation levels dynamically changed during preimplantation development. Although H3K79me2 was always detected in the whole of genome in the oocytes at GV, GVBD and MII stages, it disappeared after fertilization (Fig. 3). After that, H3K79me2 was not detected or detected in a marginal level at interphase before 4-cell stage. Its level slightly increased but it still remained in a low level at 4-cell and morula stages. However, it prominently increased at blastocyst stage and detected at the level comparable to that in the oocytes. At M phase, H3K79me2 was always detected during preimplantation development. Thus, H3K79me2 was not detected or detected in a low level at interphase, but it transiently appeared at M phase, before blastocyst stage during preimplantation development. At blastocyst stage, it was detected both at interphase and M phase. Similarly to H3K79me2, H3K79me3 was detected in the oocytes and disappeared after fertilization (Fig. 4). However, it remained to be undetectable all through the preimplantation development even at M phase.

In the MII stage oocytes, the localization of H3K79me3 on pericentromeric region was confirmed again by double-staining with the antibody against H3K79me3 and CREST (Fig. 5A). Furthermore, since it has been reported that HP1β is localized in pericentromeric region in GV stage oocytes (Chang et al., 2005), the oocytes were double-stained with the antibodies against H3K79me3 and HP1β to confirm the pericentromeric localization of H3K79me3 in the GV stage oocytes. The results showed that H3K79me3 and HP1β were co-localized on the foci with dense DNA (Fig. 5B).

In somatic cells, H3K79me2 is regulated in a cell cycle dependent manner. H3K79me2 level decreases during S phase, reaches it's lowest at G2 phase, increases during M phase, and maintains at high level during G1 phase (Feng et al., 2002). To examine whether the decrease in H3K79 methylation also depends on DNA synthesis after fertilization, the methylation level was examined 4, 8 and 12 h after fertilization, which correspond to G1, S and G2 phase, respectively (Aoki and Schultz, 1999). The results showed that both of H3K79me2 and H3K79me3 disappeared as early as 4 h after fertilization at which DNA synthesis had not yet occurred (Fig. 6A). The independency

of H3K79 demethylation on DNA synthesis was confirmed by the experiment in which DNA synthesis was inhibited in the 1-cell embryos by the treatment with aphidicolin: demethylation of H3K79me2 occurred in these embryos (Fig 6B). Thus, the demethylation of H3K79 occurs independently of DNA synthesis but seems to occur following the signal of fertilization. To address this hypothesis, the unfertilized oocytes were parthenogenetically activated: stimulation of parthenogenesis mimic the signal of fertilization. In these oocytes, both of H3K79me2 and H3K79me3 disappeared (Fig. 7), suggesting that the signal of fertilization triggers the demetylation of H3K79.

Taken together, the demethylation of H3K79 after fertilization has unique characteristics which have not been observed in the somatic cells. First, it occurs independently on DNA synthesis. Second, H3K79 is almost completely demethylated after fertilization: in somatic cells, although H3K79me2 is demethylated during S phase, an appreciable level of H3K79me2 still remained at G2 phase at which H3K79 level is the lowest (Feng et al., 2002).

Involvement of H3K79 demethylation in genome reprogramming in transplanted somatic nucleus

The rapid demethylation of H3K79 after fertilization led us to the hypothesis that H3K79 demethylation is involved in genome reprogramming. It has been suggested that the program of gene expression in the differentiated oocytes is altered into that in the totipotent embryos before and/or after fertilization. In this process, epigenetic modifications marked on active and/or inactive genes would be deleted in the oocytes. H3K79me2 has been suggested to be the marker of active genes in the somatic cells and that H3K79me2 marked on parental nucleosomes were retained during M phase and inherited to the daughter cells after cell division (Kouskouti and Talianidis, 2005). Therefore, it is possible that demethylation of H3K79 is required for genome reprogramming. To address this hypothesis, H3K79me2 was examined for the somatic nucleus transplanted into the enucleated unfertilized oocyte. A number of recent reports demonstrated that the transplanted somatic nuclei were successfully reprogrammed after they were transplanted into unfertilized oocytes (Rideout et al., 2001; Wakayama et al., 1998; Wilmut et al., 1997), which suggests that the epigenetic modifications marked on active genes would be lost in those nuclei. The results showed that H3K79me2

disappeared in the transplanted nucleus from a NIH3T3 cell 5 h after parthenogenetic activation, while it remained in the embedded nucleus which lay between the plasma membrane and zona pellucida of oocyte and was not exposed to the cytoplasm of the oocyte (Fig. 8B). These results suggested that the cytoplasm of the early 1-cell embryos possess the ability to demetylate H3K79 and that the demthylation of H3K79 is involved in the genome reprogramming after fertilization.

Since it has been reported that the ability of the embryos to reprogram the genome of transplanted somatic nucleus had been lost as early as 5-6 h after fertilization (Wakayama et al., 2000), we sought to examine whether or not the ability to demethylate H3K79 would be also lost in the mid 1-cell stage embryos. The nucleus from a NIH-3T3 cell was transplanted into the enucleated oocyte 7 h after activation. The H3K79 methylation still remained at the level comparable with the embedded nucleus after the incubation for 5 h (Fig. 8C), indicating that the ability to demetylate H3K79me2 was lost before mid 1-cell stage.

H3K79me2 as an active marker of gene expression is not deleted in the 2-cell embryos

It has been suggested that in somatic cells, H3K79me2 is an active gene marker which persists through M phase and inherited into the daughter cells after cell division (Kouskouti and Talianidis, 2005). On the other hand, it was reported that the level of H3K79me2 increased at M phase during which genes are not activated, that this increased level of H3K79 was maintained during G1 phase and that it decreased to the original level during S phase (Feng et al., 2002). Thus, it seems that H3K79me2 functioning as an active gene marker is maintained all though the cell cycle and the additional H3K79me2 occur at M phase to play a role in some M pahse-specific events. In the preimplantation embryos, however, H3K79me2 that had appeared at M phase decreased into an undetectable or very low level during interphase, before blastocyst stage (Fig. 3). Therefore, we considered that H3K79me2 as an active gene marker does not occur, and H3K79me2 that appeared at M phase is completely deleted soon after cleavage in the preimplantation embryos: G1 phase is very short in these embryos (Chisholm, 1988; Moore et al., 1996; Smith and Johnson, 1985). To address this hypothesis, somatic nuclei which had H3K79me2 as an active gene marker were transplanted into the late 1-cell stage embryos and were examined for the changes in

H3K79 level at M phase and after cleavage. The nuclei from NIH3T3 cells were transplanted into the enucleated embryos 10 h after parthenogenetic activation and then observed for H3K79me2 5 and 12 h later. Five hours after transplantation, the nuclei in the embryos which had proceeded into M phase prominently increased their H3K79me2 level (Fig. 9A). In the cleaved embryos 12 h after transplantation, the H3K79me2 decreased but still remained at the level comparable to the original one (Fig. 9A). Thus, H3K79me2 in the interphase somatic nuclei, which would be an active gene marker, was not deleted in the 2-cell embryos. When the female pronulei in which no H3K79me2 was observed were transplanted into parthenogenetically activated embryos, H3K79me2 also increased at M phase, but was completely lost after cleavage into the 2-cell stage (Fig. 9B). Taken together, the H3K79 which had been added only at M phase but not interphase seems to be deleted in the 2-cell embryos.

Discussion

In the present study, we examined the localization of H3K79me2 and H3K79me3 in the somatic cells and oocytes, and found that the sub-nuclear localization was different between these modifications. H3K79me2 were observed in whole of the genome, while H3K79me3 were localized in the pericentromeric regions (Fig. 2-5). Both of these modifications were lost in the oocytes soon after fertilization and remained in a low level before blastocyst stage except that H3K79me2 level transiently increased at M phase (Fig. 3, 4 and 6). The deletion of H3K79 methylation was also observed in the somatic nucleus which had been transplanted into the enucleated oocytes followed by parthenogenetic activation (Fig. 8).

The difference in sub-nuclear localization between H3K79me2 and H3K79me3 suggests that they have different functions from each other. Generally, as more methyl groups are added, the lysine residue increases its hydrophobic nature. Therefore, the addition of the third methyl group on dimethylated lysine seems to make its function secure. Indeed, in H3K4 methylation, which has been known as the marker of active genes, H3K4me2 was detected mainly on active genes but also on some inactive genes, although H3K4me3 was detected only on active genes (Santos-Rosa et al., 2002). In H3K9 methylation, which is associated with gene silencing, H3K9me2 is observed on facultative heterochromatin which is a changeable condensed chromatic domain (Arney and Fisher, 2004), while H3K9me3 is observed in constitutive heterochromatin which comprise of highly condensed pericentormeric heterochromatin (Arney and Fisher, 2004; Peters et al., 2003; Rice et al., 2003). We found that H3K79me3 is localized on pericentromeric heterochromatin (Fig. 2 and 5), in which there is no active gene, although H3K79me2 has been suggested as a marker of active genes (Im et al., 2003; Miao and Natarajan, 2005; Ng et al., 2003a; Schubeler et al., 2004; Sims et al., 2003). It is thus interesting that these two modifications seem to have opposite functions. It is possible that H3K79 methylation act as a switch of gene expression which is regulated by the number of methyl group. When an active gene with H3K79me2 is added with a methyl group, it might get into repressed state and be recruited into pericentoromeric region. It has been reported that some genes, *e.g. β-globin* and *Sox1*, were repressed by being recruited on pericentromeric heterochromatin (Brown et al.,

2001).

H3K79me3 was localized adjacent to centromere protein and co-localized with HP1β in mouse somatic cells and oocytes (Fig. 2 and 5), suggesting that H3K79me3, as well as H3K9me3 and H4K20me3 (Kourmouli et al., 2004; Peters et al., 2003; Schotta et al., 2004), is hallmark of pericentoromeric heterochromatin. Histone code hypothesis suggests that various histone modifications interact with each other to play roles in the formations of various chromatin domains (Jenuwein and Allis, 2001; Turner, 2002). Schotta et al. (2004) suggested that H4K20me3 is associated with H3K9me3 to participate in the formation of pericentromeric heterochromatin. H3K79me3 would be also involved in this process by interacting with these modifications. In yeast, a previous work using chromatin immunoprecipitation (ChIp) showed that H3K79me3 was detected on the active genes (Pokholok et al., 2005), suggesting that the localization of H3K79me3 differs between mammals and fungi. However, it cannot be excluded that H3K79me3 is localized in pericentoromeric region in yeast, since the previous work investigated active genes, but not pericentoromeric domain in ChIp experiments (Pokholok et al., 2005).

H3K79me2 was not detected or at a marginal level in the embryos before blastocyst stage except that it transiently appeared only at M phase, suggesting that it does not function at interphase and play a role specific for M phase during preimplantation development (Fig. 3). It has been reported that in the somatic cells, H3K79me2 is detected at interphase and its level increases at M phase (Feng et al., 2002). It occurs in both of promoters and coding regions after the first round transcription in activated genes at interphase and persisted during M phase at which transcription is suppressed (Kouskouti and Talianidis, 2005; Martin and Zhang, 2005). Therefore, it has been suggested that H3K79me2 on active genes is inherited in daughter cells after mitosis and acts as an epigenetic mark of active gene expression (Kouskouti and Talianidis, 2005). However, H3K79me2 level increases at M phase, although there is no transcription during M phase, which suggests that there is a M phase-specific function which is different from that at interphase (Feng et al., 2002; Kouskouti and Talianidis, 2005). In the embryos, H3K79me2 level also increased at M phase (Fig. 3), which may also play a role specific for M phase. However, it was not detected at interphase before blastocyst stage (Fig. 3), which suggested that it does not

function as an epigenetic mark of active gene expression in the early preimplantaion embryos: if it does, it would be left after cleavage and detected at interhpase. Indeed, H3K79me2 in the transplanted somatic nucleus, which would contain an epigenetic mark, was left after cleavage into 2-cell embryos (Fig. 9). The schematic view of this hypothesis is shown in Fig. 10. Before blastocyst stage, the embryos are not differentiated and seem to have totipotency. The absence of the epigenetic mark would be involved in the genome plasticity to maintain the totipotency in these embryos.

The gene expression pattern of differentiated oocytes is reprogrammed when they are transformed into totipotent embryos after fertilization. During this process, epigenetic marks which had sustained the oocyte-specific gene expression pattern would be erased to play a role in the genome reprogramming. In the somatic nucleus transplanted into the enucleated oocyte, the epigenetic marks would be also deleted, since gene expression pattern in the differentiated somatic cells should be reprogrammed. In the present study, we showed that both of H3K79me2 and H3K79me3, which are involved in the active gene expression and gene silencing, respectively, disappeared in the oocytes soon after fertilization and H3K79me2 also disappeared from the nucleus transplanted into the enucleated oocytes after activation (Fig. 8B). These results suggest that the disappearance of these modifications would be involved in the genome reprogramming. Furthermore, our results suggest that a phenomenon involved in genome reprogramming occurs soon after fertilization. Although active transcription occurs in the growing oocytes, it ceases when the oocytes are fully grown. Then, they remain in transcriptionally silent state until zygotic gene is activated after fertilization: in mice, the expression of zygotic gene start as early as mid/late 1-cell stage (Aoki et al., 1997). Therefore, the deletion of epigenetic marks is likely to occur during this transcriptionally silent period. However, it has not been known when this phenomenon occurs during this period, *e.g.* at germinal vesicle stage, during meiotic maturation, or after fertilization, etc. Our results suggest that the deletion of epigenetic marks, at least a part of it, occurs soon after fertilization. These results are consistent with the reports that transplanted somatic nuclei were efficiently reprogrammed when they were transplanted into the enucleated bovine oocytes soon after the oocytes were activated (Schurmann et al., 2006; Sung et al., 2006). It has been reported that the ability to reprogram the transplanted somatic nucleus decrease with the

time after the activation of enucleated oocytes: the percentages of the transplanted oocytes which developed into blastocyst stage gradually decreased with the time at which the somatic nucleus was transplanted, and no embryos which had been subjected to nuclear transplantation 5-6 h after fertilization developed into blastocyst stage (Wakayama et al., 2000). Consistent with this report is that in our experiments, the somatic nucleus was not demethylated on H3K79 when they were transplanted into the enucleated embryos 7 h after insemination (Fig. 8C). Thus, we suggest that global H3K79 demethylation is involved in the genome reprogramming and that the ability to delete H3K79 methylation seems to be lost soon after fertilization.

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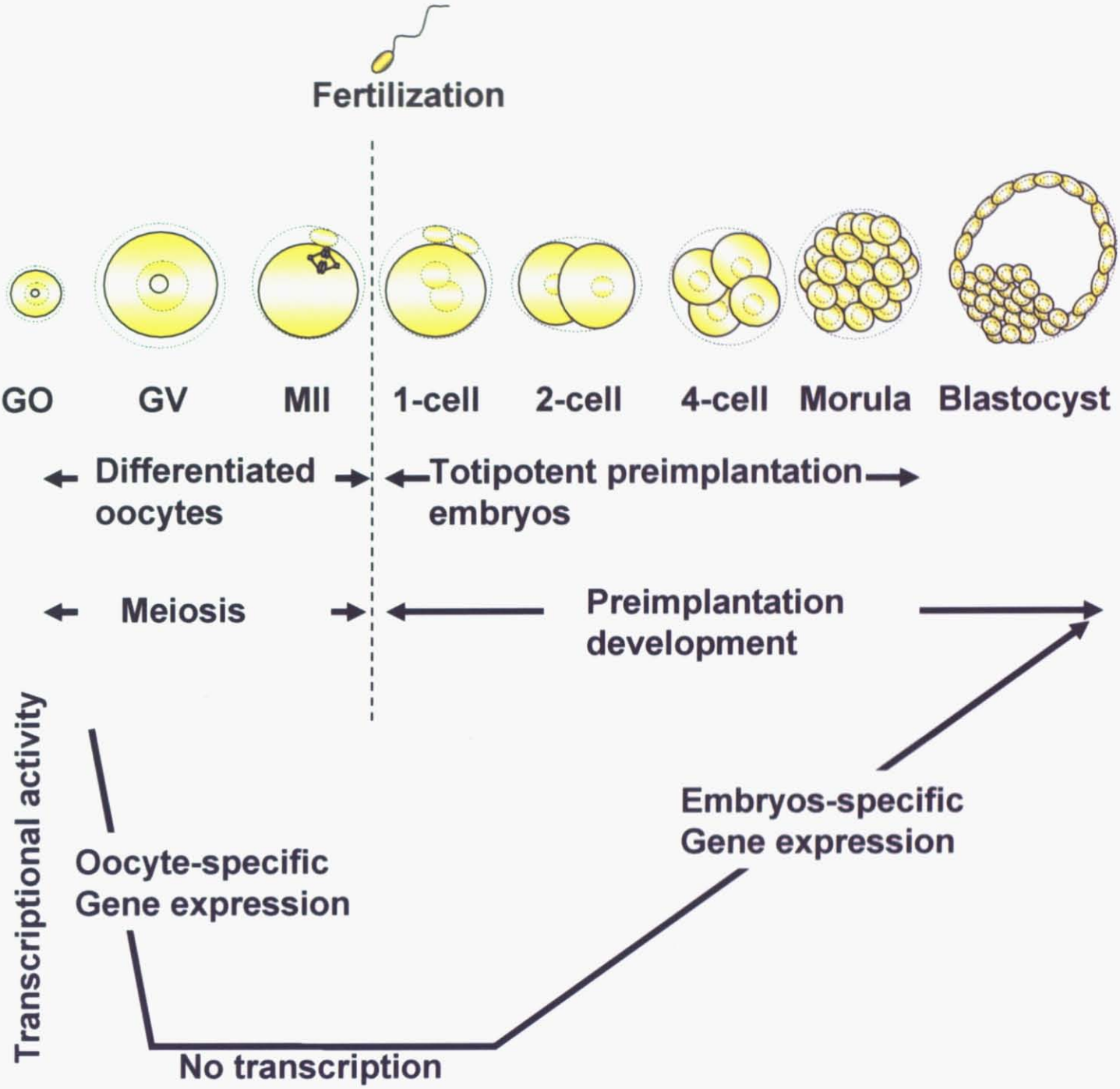


Fig. 1. Diagram of the change in gene expression during oogenesis and preimplantation development. During growth, oocytes actively express their genes including oocyte-specific ones. When oocytes were fully grown, the gene expression is silenced. This transcriptionally silent state is maintained during meiotic maturation. After fertilization, the transcription from zygotic genome starts in an embryo-specific pattern at the mid/late 1-cell stage. GO: growing oocytes. GV: the oocytes at germinal vesicle stage; MII: the unfertilized oocytes at MII stage; 1-cell: the embryos at 1-cell stage. 2-cell: the embryos at 2-cell stage; 4-cell: the embryos at 4-cell stage: Morula: the embryos at morula stage; Blastocyst: the embryos at blastocyst stages.

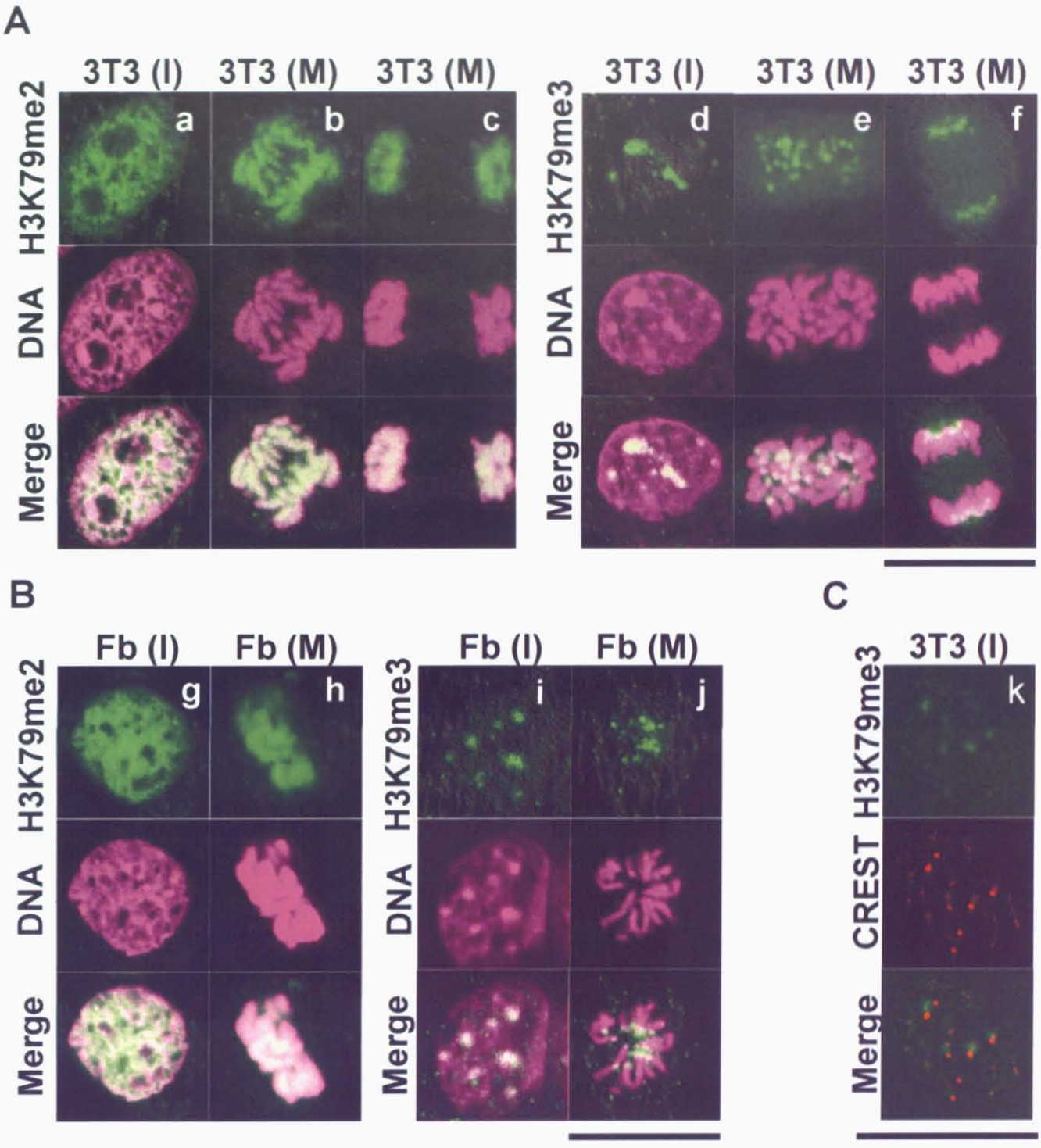


Fig. 2. Intra-nuclear localization of H3K79me2 and H3K79me3 in somatic cells. NIH 3T3 cells (A) and fibroblast cells from mouse tail tip (B) were immunostained with antibodies against H3K79me2 or H3K79me3. The antibodies were localized with a FITC-conjugated secondary antibody (green). DNA was stained with propidium iodide (purple). In the merged images, the areas in which H3K79 methylation and DNA are co-localized look white. 3T3 (I), 3T3 (M), Fb (I) and Fb (M) indicate NIH3T3 cells at interphase, NIH3T3 cells at M phase, fibroblast cells at interphase and fibroblast cells at M phase, respectively. (C): Double staining of NIH3T3 cells at interphase using antibodies against H3K79me3 and centromere proteins (CREST) which were probed with FITC-conjugated (green) and rhodamin-conjugated (red) secondary antibodies, respectively. The scale bars indicate 20 um.

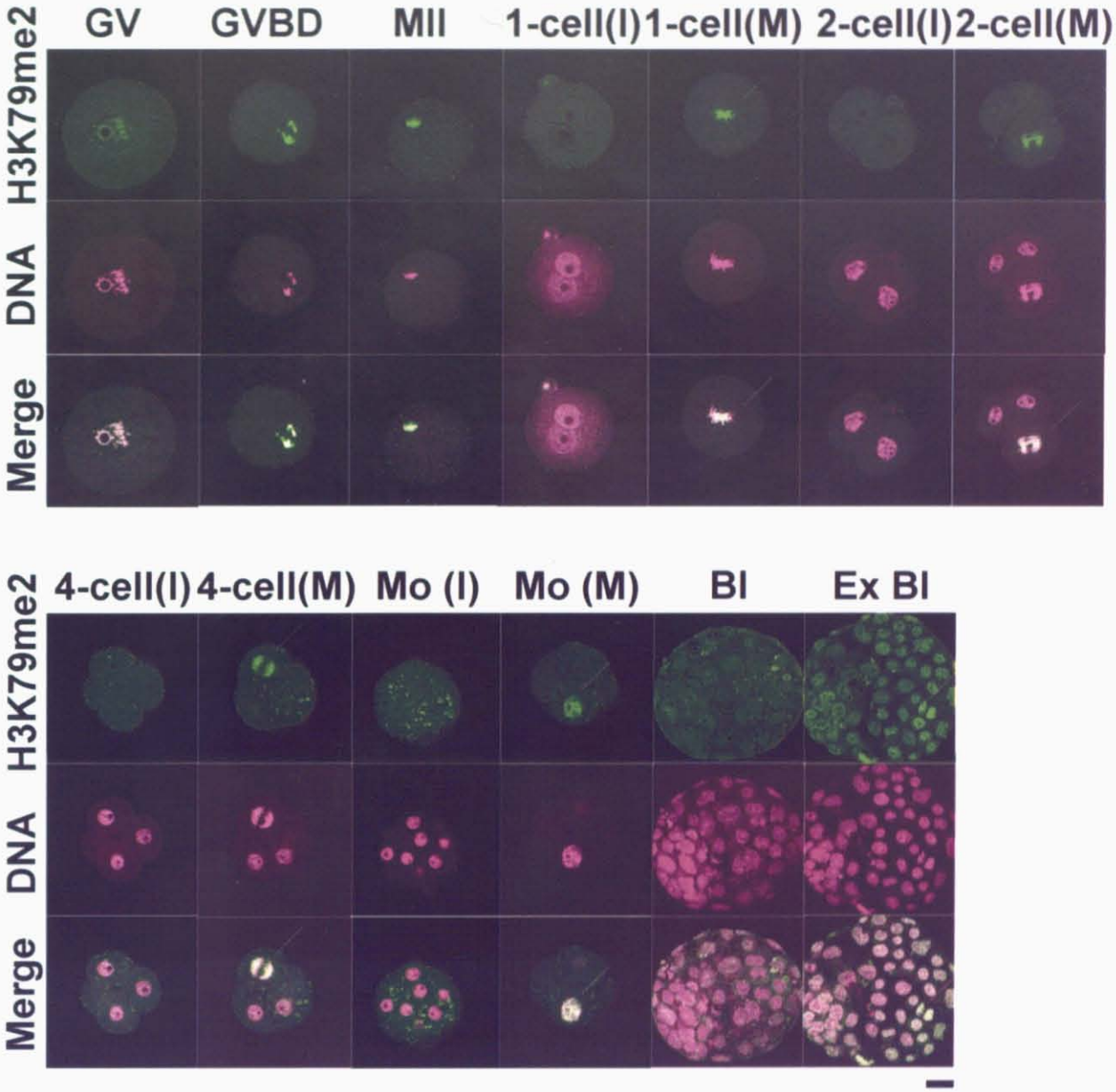


Fig. 3. Changes in H3K79me2 during meiotic maturation and preimplantation development. Oocytes and preimplantation embryos were immunostained with the anti-H3K79me2 antibody. The antibodies were localized with a FITC-conjugated secondary antibody (green). DNA was stained with propidium iodide (purple). In the merged images, the areas in which H3K79 methylation and DNA are co-localized look white. GV and GVBD indicate the oocytes at germinal vesicle stage and those which had undergone germinal vesicle breakdown 3 h after released from the ovary, respectively. MII indicates the unfertilized oocytes at MII stage. The embryos at interphase of 1-cell stage (1 cell (I)), M phase of 1-cell stage (1 cell (M)), interphase of 2-cell stage (2 cell (I)), M phase of 2-cell stage (2 cell (M)), interphase of 4-cell stage (4 cell (I)) and M phase of 4-cell stage (4 cell (M)) were collected 12, 14, 28, 37, 45 and 48 h after insemination, respectively. The morula stage embryos (Mo (I) and Mo (M)) were collected 60 h after insemination. The embryos at blastocyst and expanded blastocyst stages were collected at 96 and 118 h after insemination, respectively. The arrows indicate the blastomeres at M phase. The scale bar indicates 20 um.

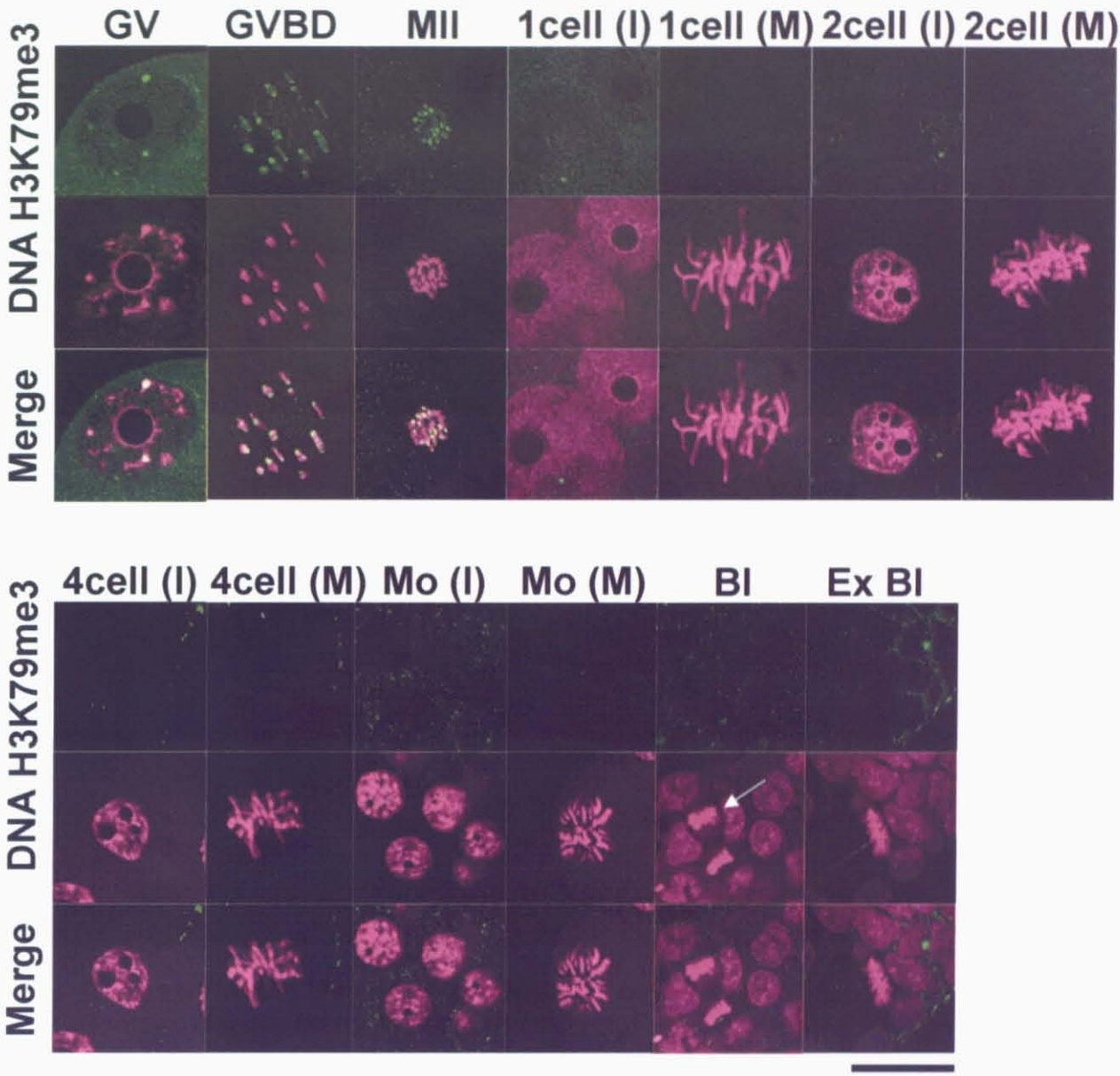


Fig. 4. Changes in H3K79me3 during meiotic maturation and preimplantation development. Oocytes and preimplantation embryos were immunostained with the anti-H3K79me3 antibody. The antibodies were localized with a FITC-conjugated secondary antibody (green). DNA was stained with propidium iodide (purple). In the merged images, the areas in which H3K79 methylation and DNA are co-localized look white. GV and GVBD indicate the oocytes at germinal vesicle stage and those which had undergone germinal vesicle breakdown 3 h after released from the ovary, respectively. MII indicates the unfertilized oocytes at MII stage. The embryos at interphase of 1-cell stage (1 cell (I)), M phase of 1-cell stage (1 cell (M)), interphase of 2-cell stage (2 cell (I)), M phase of 2-cell stage (2 cell (M)), interphase of 4-cell stage (4 cell (I)) and M phase of 4-cell stage (4 cell (M)) were collected 12, 14, 28, 37, 45 and 48 h after insemination, respectively. The morula stage embryos (Mo (I) and Mo (M)) were collected 60 h after insemination. The embryos at blastocyst and expanded blastocyst stages were collected at 96 and 118 h after insemination, respectively. The arrows indicate the blastomeres at M phase. The scale bar indicates 20 um.

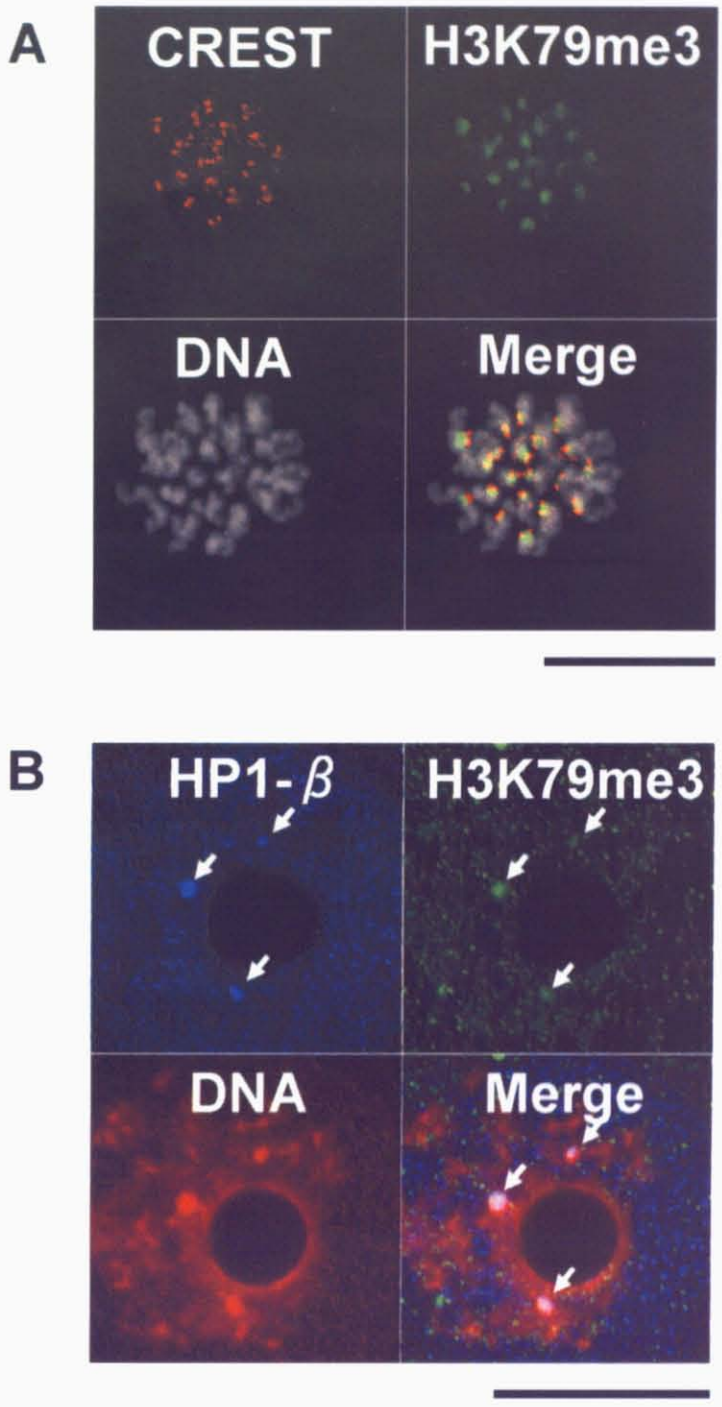


Fig. 5. Localization of H3K79me3 on the pericentromeric region in the nucleus of mouse oocytes. (A) Mouse MII stage oocytes were triple-stained with the antibodies against H3K79me3 and centromere proteins (CREST), and propidium iodide (DNA). The antibodies against H3K79me3 and CREST were probed with FITC-conjugated (green) and rhodamin-conjugated (red) secondary antibodies, respectively. DNA is colored with gray in the images. (B) GV stage oocyets were triple-stained with the antibodies against H3K79me3 and HP1 β , which were probed with FITC-conjugated (green) and Cy5-conjugated (blue) secondary antibodies, respectively, and propidium iodide (DNA; red). The scale bars indicate 20 um.

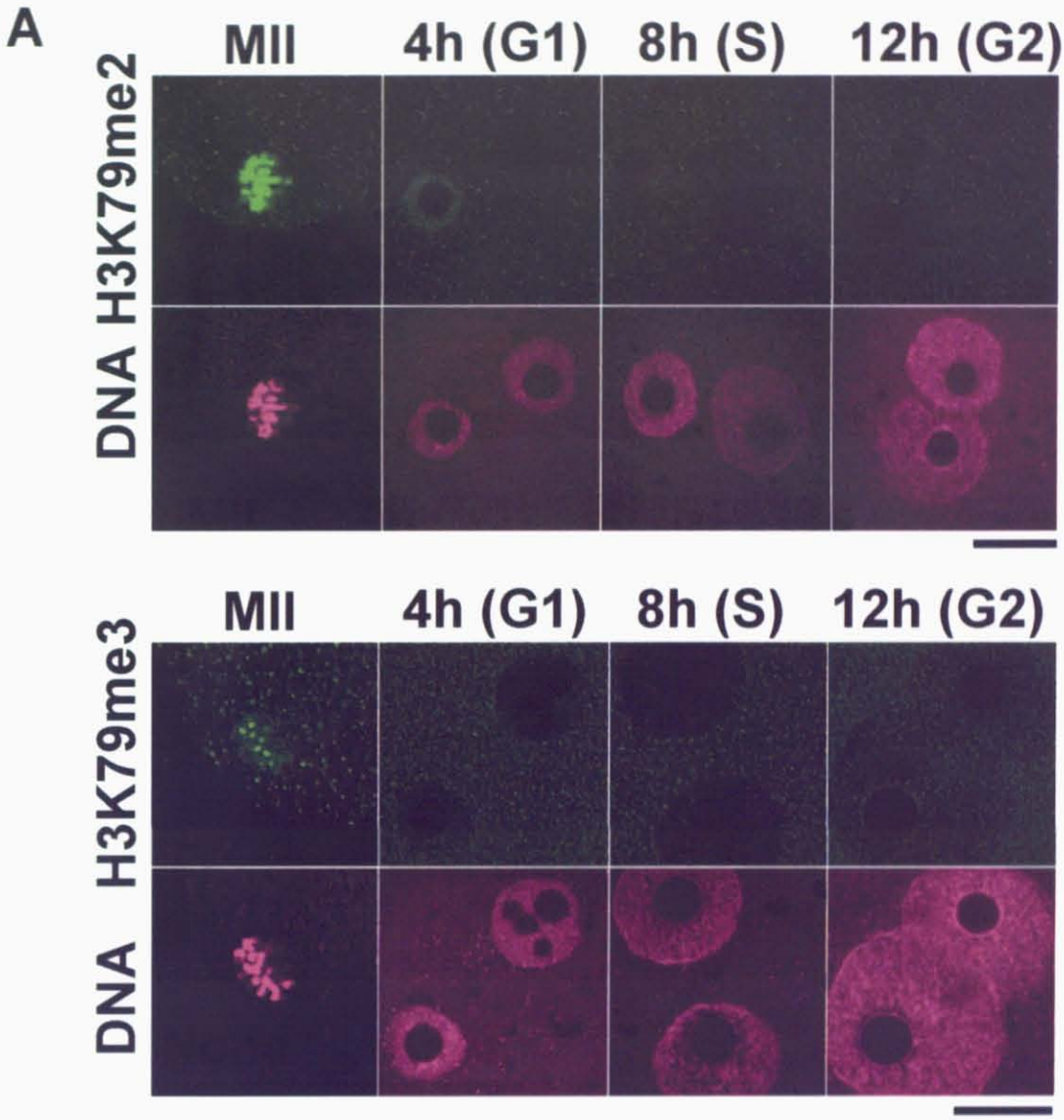


Fig. 6. Cell cycle-independent demethylation of H3K79 after fertilization.
The unfertilized oocytes (MII) and 1-cell stage embryos which had been collected 4, 8 and 12 h after insemination were immunostained with the antibodies against H3K79me2, H3K79me3 antibodies respectively (A). The embryos which had been incubated with (Aphi.) and without (None) aphidicolin were collected 12 h after insemination and immunostained for H3K79me2 (B). The antibodies were localized with a FITC-conjugated secondary antibody (green). DNA was stained with propidium iodide (purple). The scale bars indicate 20 um.

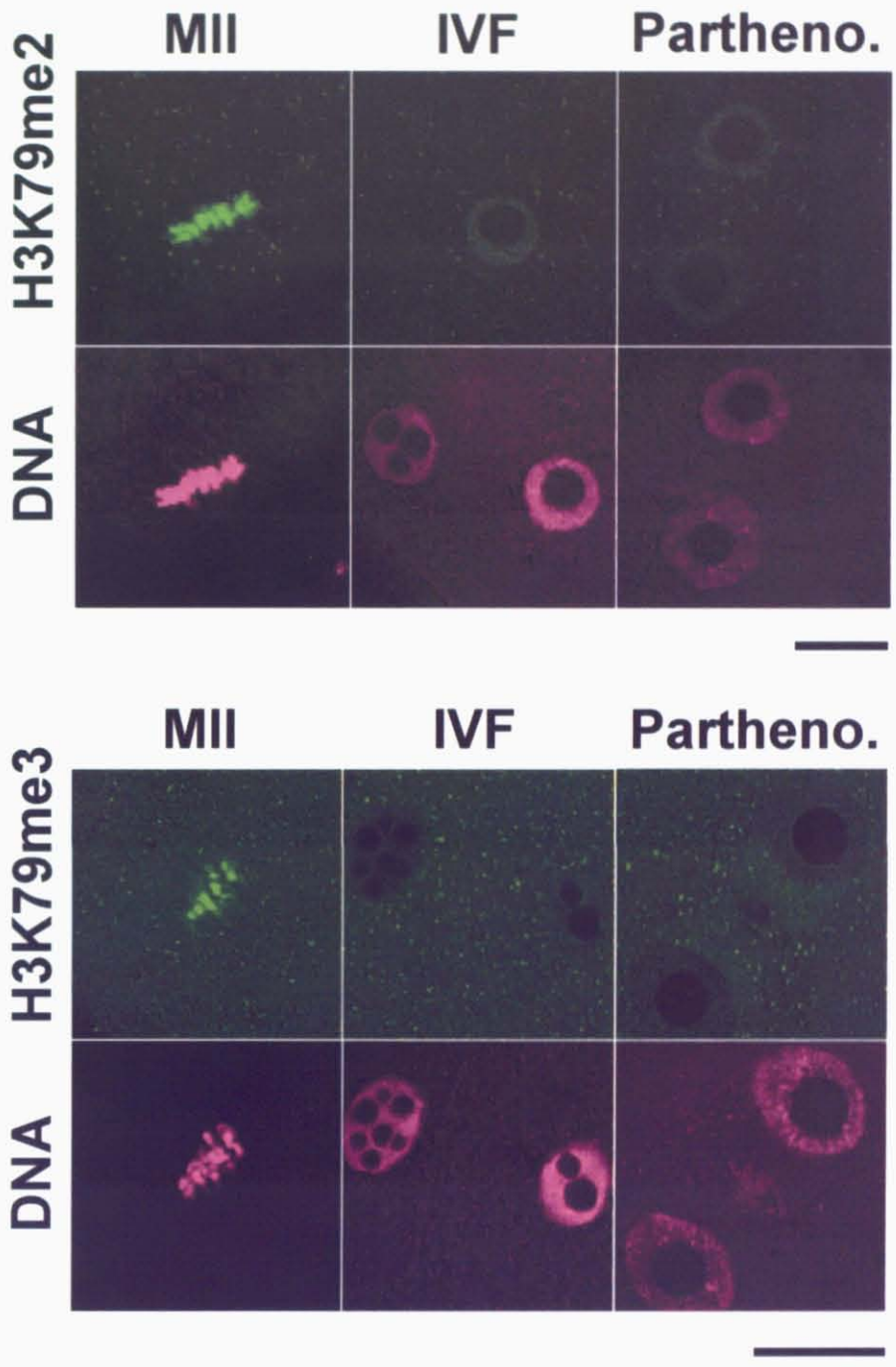


Fig. 7. H3K79 demthylation in parthenogenetically activated oocytes. MII stage oocytes, the embryos which had been collected 5 h after imsemination, and parthenogenetically activated oocytes which had been collected 4 h after the stimulation with Sr2+, were immunostained with the antibody against H3K79me2 and H3K79me3. DNA was stained with propidium iodide. The scale bars indicate 20 um.

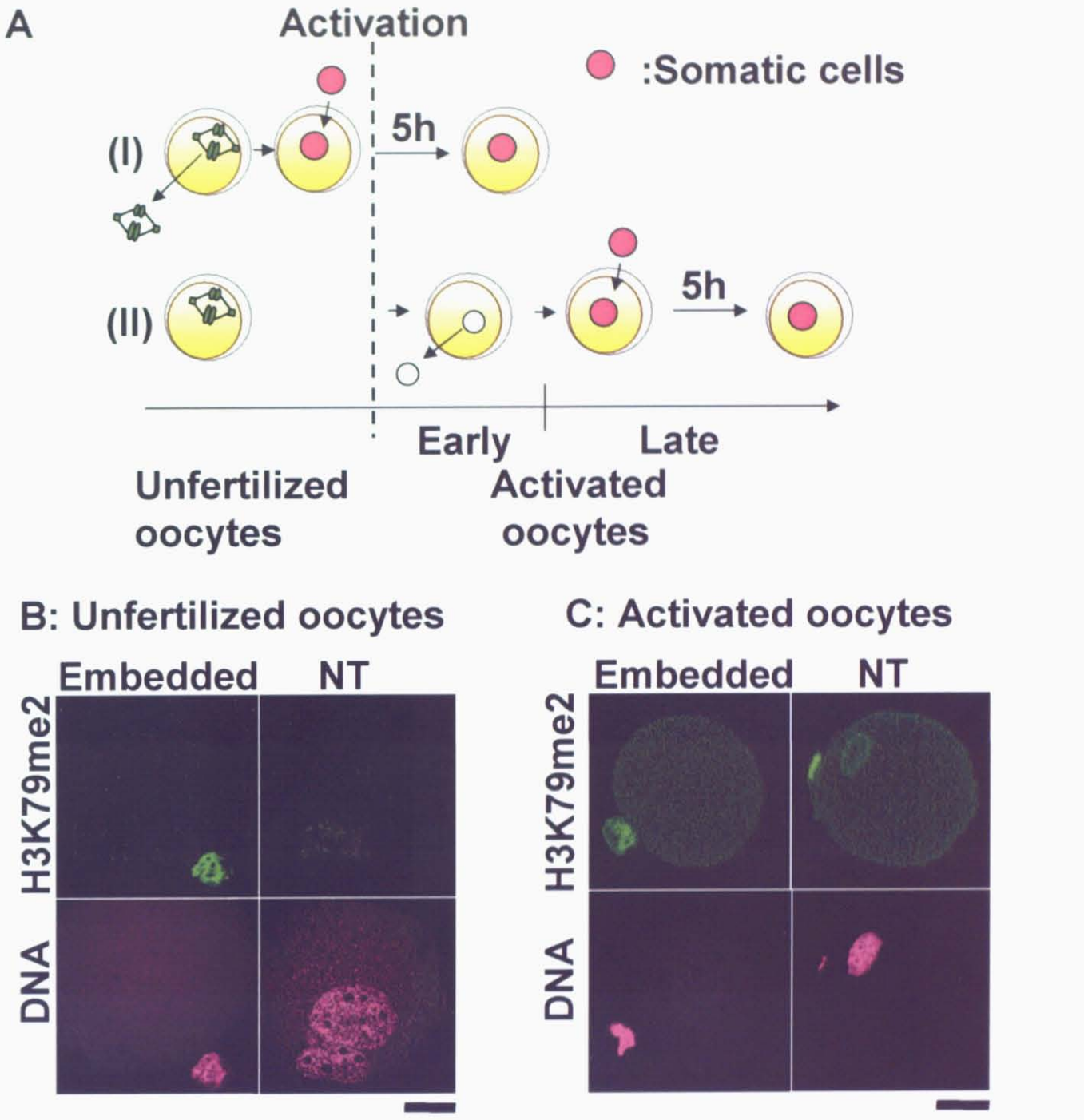
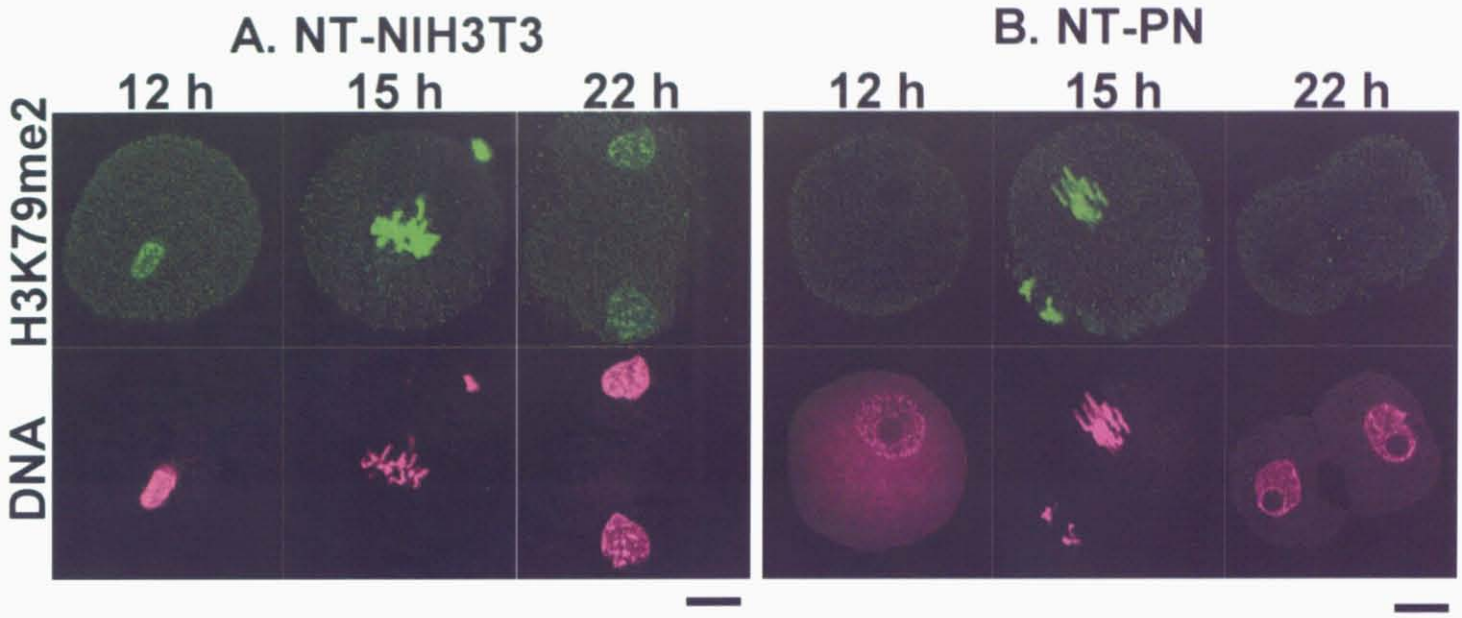


Fig. 8. Demethylation of H3K79 in the somatic nucleus transplanted into enucleated unfertilized oocytes and embryos. (A); A scheme of experimental procedures. (I): Unfertilized oocytes were enucleated. Somatic nucleus were transplanted into the enucleated oocytes and following cultured for 5 h after activation and collected for immunocytochemistry. (II): Activated oocytes were enucleated. Somatic nuclear were transplanted into this enucleated activated oocytes 7 h after activation. After 5 h culturing the reconstructed oocytes were collected for immunocytochemistry. (B),(C); An interphase nucleus of NIH 3T3 cell was embedded in the perivitelline space of enucleated oocytes (embedded). Five hours after the electrofusion, the transferred oocytes before activation (NT; prepared discribed as (I) in fig. 9A) and transferred oocytes after activation (NT; prepared discribed as (II) in fig. 9A) were collected for immnostaining with anti-H3K79me2 antibody which was probed with a FITC-conjugated secondary antibody (green). DNA was stained with propidium iodide (purple). The scale bars indicate 20 um.



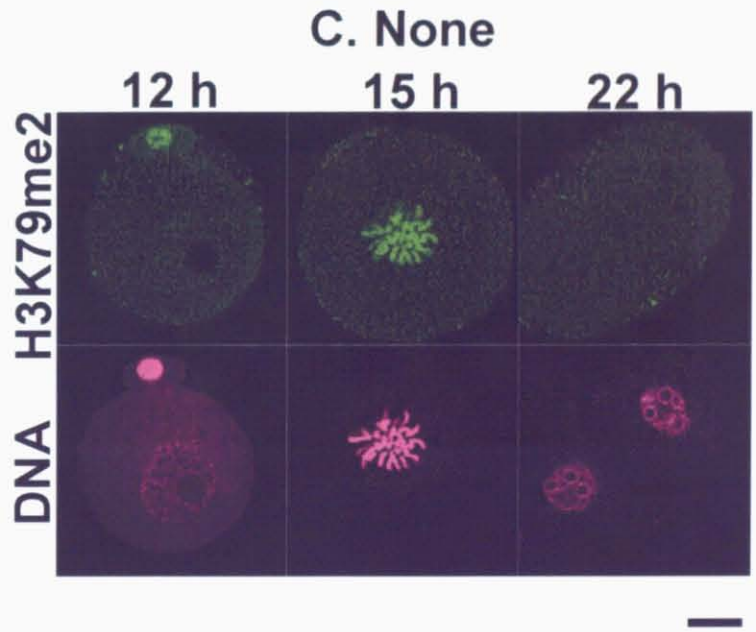


Fig. 9. H3K79me2 occurs in M phase specifically during preimplantation development. Results of nuclear transfer experiments in to late 1cell stage enucleated activated oocytes using nuclei of NIH 3T3 (A) and Pronucleus (B) as nuclei donor was indicated. The reconstructed oocytes were collected after 12h, 15h, 22h from activation for immunocytochemistry using antibody against H3K79me2(green). They corresponding to interphase on 1cell stage, M phase on 1cell stage, interphase on 2cell stage. Normally parthenogenetic activated oocytes were collected similarly and immunostained (C). DNA was stained with propidium iodide (purple). Scale bars indicated 20um

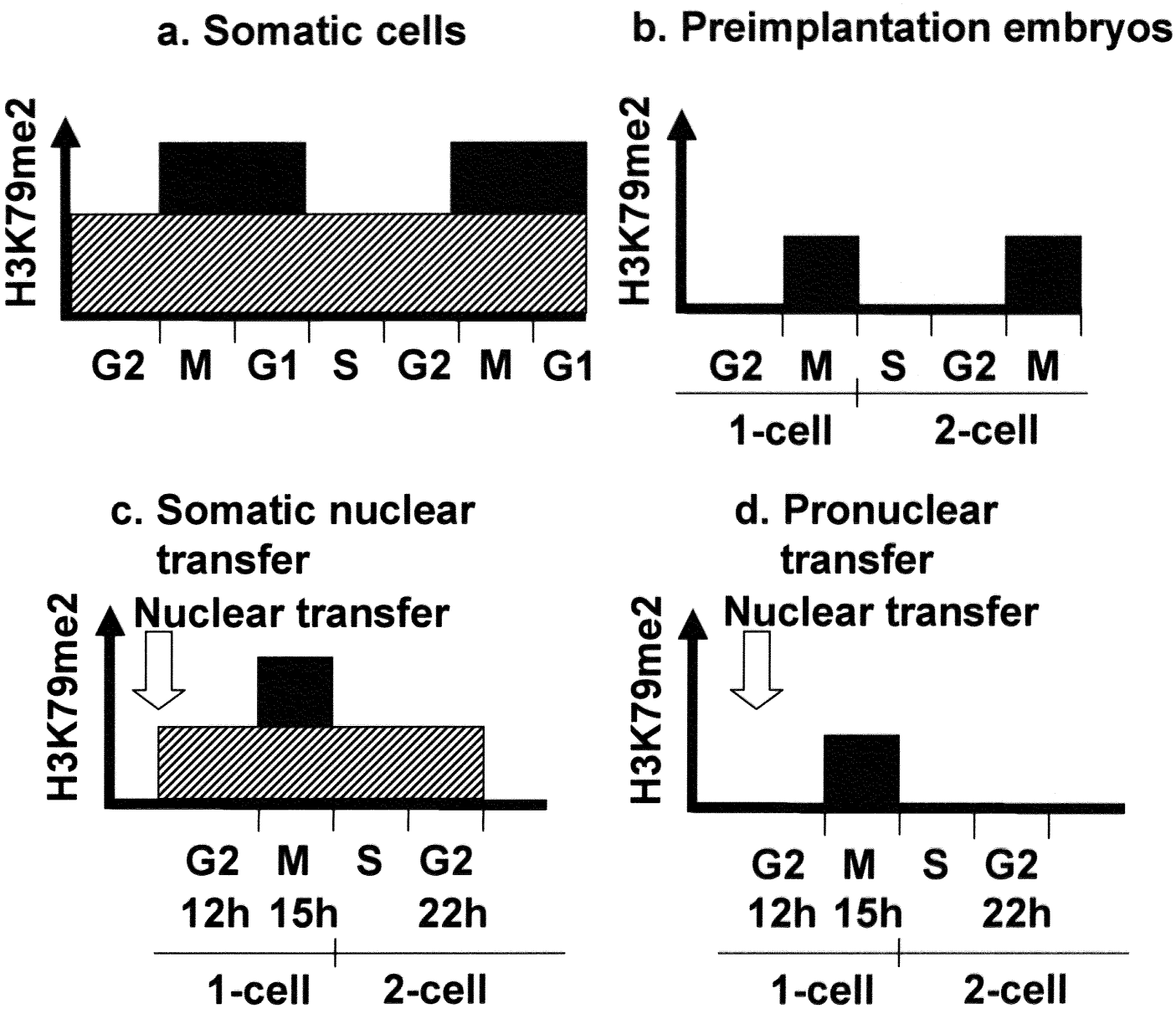


Fig.10. Hypothetical view of the regulation of H3K79me2 during early preimplantation development. (A) In somatic cells, H3K79me2 functioning as active gene marker is maintained all though the cell cycle (diagonally hatched box) and the additional H3K79me2 occur at M phase to play a role in some M pahse-specific events (solid box). The additional H3K79me2 which occurred at M phase persists at G1 phase and decreases during S phase. (B) In the embryos, all H3K79me2 is eliminated after soon fertilization. After that, little or no H3K79me2 as active gene marker occurs at interphase during preimplantation development before blastocyst stage. However, H3K79me2 occurs at M phase, which would play a role in some M pahse-specific events (solid box). Since G1 phase is very short during the early preimplantation development, the H3K79me2 which occurred at M phase is lost during S phase soon after cleavage. (C) Somatic nuclei which had had H3K79me2 as active gene marker (diagonally hatched box) were transplanted into the late 1-cell stage embryos. H3K79me2 level increased at M phase (solid box). It decreased after cleavage but still remain at the original level that had been observed before transplantation. (D) Male pronuclei which had had no H3K79me2 as active gene marker were transplanted into the late 1-cell stage embryos. H3K79me2 level increased at M phase (solid box). It was completely lost after cleavage, since there was no pre-existing H3K79me2 as active gene marker.