Original Article

**Epigenetic impairments in development of parthenogenetic preimplantation mouse embryo**

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Running title: Epigenetics of mouse parthenogenetic embryo
**Abstract**

Parthenogenesis is an activation process of oocytes that occur without the participation of sperm. Evidence suggests that normal development of embryos requires proper expression of several imprinted genes inherited from both the paternal and maternal genomes. Compared to gene expression, histone modifications and chromatin remodeling are not well-documented. In this research, by using immunofluorescence staining for several developmental-associated histone modifications, we investigated whether epigenetic impairments in parthenogenetic embryos act as constraints for proper development. At early stages, fertilized embryos exhibited high methylation of histone H3 at lysine 9 (Me-H3-K9) and Heterochromatin Protein 1 (HP1) present in the maternal chromatin, while paternal chromatin showed weaker HP1 signals. We found that at the two-cell stage in fertilized embryos, HP1, initially detected around the nucleolus, colocalized with chromocenters at one pole of the blastomere, while parthenotes showed a diffused distribution pattern of HP1 throughout the entire nucleoplasm. At the four-cell stage, methylation of histone H3 at arginine 26 (Me-H3-R26) increased at nascent RNA repression sites in fertilized embryos, while parthenotes recorded weaker signals throughout the nucleoplasm, suggesting differences in pluripotency of the ICM cells between the two types of embryos. Moreover, at the blastocyst stage, we observed that the acetylation level of histone H4 at lysine 12 (Ac-H4-K12) was significantly decreased in parthenogenetic ICM compared to that in its fertilized counterpart. To summarize, differences in epigenetic modifications correlating with paternal chromatin’s capacity to regulate nascent RNA repression may contribute to aberrant development and lineage allocation in mouse parthenogenetic embryos.
Keywords: mouse parthenogenetic embryo, epigenetics, nascent RNA production, histone acetylation, histone methylation

Introduction

Parthenogenesis is a process in which zygotes are produced without sperm presence. Due to lack of paternal genes, parthenogenetic embryos cannot develop to full-term; however, these embryos show a great potential to generate histocompatible stem cells (parthenogenetic embryonic stem i.e. pES cells) for transplantation. Following fertilization, the paternal and maternal genomes undergo epigenetic changes and chromatin remodeling necessary to achieve transcriptional competence during early development [1]. In mice, the zygotic paternal genome is demethylated widely soon after fertilization, whereas the maternal genome remains highly methylated [2, 3]. This asymmetric pattern of DNA methylation in the two parental genomes at the one-cell stage persists at least until the two-cell stage of the embryo [4], and is believed to be responsible for functional differences between the parental genomes during development. Chromatin remodeling is a multi-stage process with the newly fertilized oocyte in transition between gametic chromatin and embryonic chromatin which subsequently becomes transcriptionally competent. The result is that both male and female pronuclei are remodeled in the same environment producing “opposing” epigenetic outcomes [5, 6].

As a part of the “histone code”, heterochromatin mediates many diverse functions in the cell nucleus, including centromere function, gene silencing, and nuclear organization [7]. The pericentromeric heterochromatin contains specific histone modifications, such as high level of methylation of histone H3 at lysine 9 (Me-H3-K9) and low level of acetylation of histone H3 at lysine 9 (Ac-H3-K9) or methylation of histone H3 at lysine 4 (Me-H3-K4) [8, 9]. Me-H3-K9 creates a high-affinity binding site for
Heterochromatin Protein 1 (HP1) [10], and HP1 proteins are thought to contribute to the transcriptional silencing of heterochromatin [8]. HP1 proteins might be recruited to pericentromeres by their binding to the newly deposited histone H3.3 that is methylated on lysine 9 prior to its deposition or becomes methylated after its incorporation into the chromatin [8, 11]. On the other hand, chromocenters represent major heterochromatic domains and appear to be important for regulating proper centromere function. Chromocenters also play an important role in gene regulation and association of genes with chromocenters have been found to correlate with silencing in somatic cells [12]. It has been previously demonstrated that in mammalian embryos, both parental genomes display a peculiar distribution of pericentric heterochromatin and centromeres, organized around nucleolar precursor bodies (NPB) [12]. Chromocenter genesis is finally achieved by the end of the two-cell stage in mice [13]. Remarkably, these dynamics of epigenetic marks and reorganization of nuclear architecture appear closely connected to the functional state of the genome. Moreover, previous researches have demonstrated asymmetric distribution of HP1 and trimethylation of histone H3 at lysine 9 (Me-H3-K9) at one- and two-cell stage in fertilized embryos [14], but there are only a limited number of reports on HP1 formation and localization at these stages in parthenogenetic embryos.

Previously, we have discussed the importance of paternal chromatin in regulating zygotic gene activation (ZGA) [15]. In mouse fertilized embryos, ZGA was found to be repressed at the late two-cell stage, illustrated by a reduction in nascent RNA synthesis. In mouse parthenogenetic embryos, on the other hand, this phenomenon was postponed until the late four-cell stage [15]. Moreover, as early as the four-cell stage, fertilized embryos started to show their differentiation fate and cell potency. A report in 2007 showed that the four-cell blastomeres, which possessed higher methylation of histone
H3 at arginine 26 (Me-H3-R26), directed their progeny to the ICM, and upregulation of this methylation led to a dramatic increase in Nanog and Sox2 expression in mouse fertilized embryos [16]. However, Me-H3-R26 level during this stage has not been investigated in the case of parthenogenetic embryos.

In addition, acetylation of histone H4 at lysine 12 (Ac-H4-K12) was also proved to be closely related to gene transcription. Specifically, data have shown that some portions of sperm chromatin bear H4 lysine acetylations that are passed into the zygote and may be involved in early embryo development [17]. Furthermore, Ac-H4-K12 was enriched in the area surrounding the transcription start site (TSS), and the genes in that region showed a significant bias towards developmental functions; some of these genes were expressed at high levels in early zygotes [18]. Nevertheless, Ac-H4-K12 expression levels during preimplantation development of mouse parthenogenetic embryos have not been elucidated yet.

In this study, we investigated HP1 formation and localization, and Me-H3-R26 and Ac-H4-K12 expression during preimplantation development of parthenogenetic embryos and compared them to fertilized embryos. We found that these histone signatures were impaired in parthenotes and that paternal chromatin played a vital role in controlling these expressions.

**Materials and Methods**

**Mice and reagents**

Female ICR mice were used as oocyte donors throughout the experiment; all mice were about 8-10 weeks old. Animals were maintained in accordance with the guidelines of
International University – Vietnam National University, Ho Chi Minh City. Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

**Parthenogenesis**

Female ICR mice were super-ovulated by intraperitoneal injection of 5 IU of equine chorionic gonadotropin (eCG), followed 48 h later by 5 IU of human chorionic gonadotropin (hCG). Oocytes were then mechanically denuded from cumulus cells in Hepes modified CZB (Hepes-mCZB) medium containing hyaluronidase (1 mg/mL), then incubated in activation medium consisting of Ca\(^{2+}\)-free mCZB medium supplemented with 10 mM SrCl\(_2\) and 5 µg/mL cytochalasin B (CB) for 6 h at 37°C in a humidified atmosphere with 5% CO\(_2\). After activation, oocytes containing two pronuclei (2PN) were selected and cultured in mCZB medium supplemented with 0.5% bovine serum albumin (BSA) during preimplantation development.

**Microinjection of spermatozoa**

For control studies, fertilized embryos were produced using intracytoplasmic sperm injection (ICSI) as described by Kimura and Yamanagichi [19] except that our experiment was performed at room temperature. Briefly, after washing, the sperm heads were separated from the tail by subjecting the head-tail junction to a few pulses using a piezo-activated micromanipulator system (Prime Tech, Aburaki, Tokyo, Japan). Only the sperm head was injected into each oocyte. After 10-20 min of recovery, ICSI embryos were cultured in mCZB medium supplemented with 0.5% BSA at 37°C in humidified air with 5% CO\(_2\) until the blastocyst stage.

**Incorporation of BrUTP into embryos and detection of new transcripts**

Each group of embryos (< 20) was washed twice in electrical permeabilization medium (EP: 0.25 M D-glucose, 100 µM CaCl\(_2\).2H\(_2\)O, 100 µM MgSO\(_4\), and 0.1% polyvinylpyrrolidone) and finally in one change of transcription buffer (EP + 10 mM
BrUTP; Sigma Aldrich). They were then transferred into a chamber between electrodes overlaid with a 20 µl droplet of transcription buffer. Two 80 µs electric pulses at 250 V/cm of direct current were triggered using an Electro Cell Fusion apparatus (Bex LF101L, Tokyo, Japan) with a 2 min interval between pulses. Two minutes after permeabilization, embryos were cultured in mCZB medium for 1 h further under the same conditions as above. The embryos were then fixed in 4% paraformaldehyde and new transcripts incorporating BrUTP were visualized by indirect immunofluorescence [15].

**Immunofluorescence microscopy**

Embryos were fixed in 4% Paraformaldehyde and treated as described previously [20]. The primary antibodies used were rabbit polyclonal anti-histone H4 at lysine 12 (Ac-H4-K12; Upstate Cell Signaling Solutions, Charlottesville, VA, USA), rabbit anti-trimethyl-histone H3 at lysine 9 (Me-H3-K9; Abcam, Cambridge, UK), mouse monoclonal anti-CREST (Santa Cruz Biotechnology, Inc., Europe), goat polyclonal anti-HP1β (Santa Cruz Biotechnology, Inc., Europe), rabbit polyclonal anti-dimethyl-histone H3 at arginine 26 (Me-H3-R26, Abcam, Cambridge, UK), mouse anti-histone H3 at lysine 9 (Ac-H3-K9; Upstate Cell Signaling Solutions, Charlottesville, VA, USA), and mouse monoclonal anti-BrdU (Roche Diagnostics, GmbH, Germany). The secondary antibodies were Alexa-Fluor-568-labeled goat anti-mouse IgG or Alexa-Fluor-568-labeled donkey anti-goat IgG or Alexa-Fluor-488-labeled chicken anti-rabbit IgG antibodies (Molecular Probes Inc., Eugene, OR, USA). DNA was counterstained with 2 µg/ml 4,6-diamidino-2-phenylindole (DAPI; Molecular Probes).

**Quantitation of fluorescence intensity in nuclei**

Embryos were observed using a fluorescence microscope (Olympus Optical Co., Tokyo, Japan). Fluorescence intensities of nuclei were measured by manually outlining all
nuclei in the display as described previously [21]. Briefly, nuclei were selected randomly. The total intensity in each nucleus was measured from five different regions and the background value for the cytoplasm was subtracted. This calculated intensity was multiplied by the nuclear volume to estimate the total amount of fluorescence for the nucleus. One-cell embryos were measured at 10, 12, and 14 h after sperm injection or activation and intensity was calculated based on a mean of two pronuclei. For two-cell, late two-cell, four-cell, late four-cell and eight-cell, and morula/blastocyst stages, embryos were measured at 24, 32, 48, 54, 62, and 84 h after sperm injection or activation, respectively [15].

Statistical analysis

Student’s \( t \)-test was used to estimate the significance of any differences between experimental groups in immunofluorescence studies. Each experiment was repeated at least three times and about 60 immunostained oocytes were examined in each group. The data were subjected to arcsine transformation for each replication to normalize them. The transformed values were analyzed by one-way ANOVA using Microsoft® Excel (Microsoft® Software by Microsoft Corporation) and significance level was considered at \( p < 0.05 \).

Results

Me-H3-K9 and HP1 are asymmetrically distributed in maternal and paternal genomes of fertilized embryos

One of the reasons that arrests parthenogenetic embryo development at E9.5 is the lack of paternal genes. Our previous results have shown that male-derived factors play a key role in the regulation of transcription repression in the two-cell to four-cell transition through differences between male and female chromatin remodeling, DNA synthesis, and nascent RNA production [15]. In order to clarify the role of paternal chromatin in
this phenomenon, we first examined acetylation of histone H3 at lysine 9 (Ac-H3-K9), methylation of histone H3 at lysine 9 (Me-H3-K9) and Heterochromatin Protein 1 (HP1) allocation in early fertilized embryos. Soon after fertilization, maternal chromatin showed high level of HP1 expression while this signal was not expressed in paternal chromatin (Fig. 1Ak). After 3 h of fertilization, a weak signal of HP1 started to appear in paternal chromatin while HP1 signal was still maintained at a high level in the maternal chromatin (Fig. 1Al). Although both pronuclei expressed Ac-H3-K9, they showed an asymmetric methylation of H3-K9 between maternal and paternal chromatin in the 1-cell stage (Fig. 1Bc). It is suggested that in mouse early embryos, pericentromeric heterochromatin (PCH) contains Suv39h/HP1 in a parental-specific manner. The targeting mechanism present in the paternal PCH is absent in the maternal PCH due to the presence of the Suv39h/HP1 pathway; therefore, H3K9me3-independent accumulation of HP1 occurs in the paternal pronuclei [11]. These results indicate the role of paternal chromatin in regulating epigenetic asymmetries between two pronuclei of mouse embryos in the first cell cycle.

Centromere relocation at the chromocenter of nucleoplasm was delayed in parthenogenetic embryos compared to fertilized embryos.

In the next step, we focused more on the formation of chromocenters during the first two cell cycles. These structures are involved in centromere function and regulation of gene transcription [22, 23]. HP1 is associated with heterochromatin and is usually enriched in chromocenters [22]. Localization of kinetochores and pericentric chromatin were detected using anti-CREST antibody.

At the 1-cell stage, HP1 was present in the nucleoplasm and presented a perinucleolar ring around the NPB, colocalizing with DNA in the female pronuclei while in the male pronuclei, HP1 signal was dimmer and homogeneously distributed in the nucleoplasm.
until 4 h after fertilization (Fig. 1An). There was accumulation of HP1 around the NPB in male pronuclei concomitant with the pericentric heterochromatin forming a ring around the NPB after 6 h of fertilization (Fig. 2Ab). In fertilized embryo, at the early two-cell stage, pericentric DNA became localized at one pole of nucleus, and centromeres accumulated around the nucleolus (Fig. 2Ba, 2Bb). In the late 2-cell stage, kinetochores were polarized and compacted at the chromocenters at one pole of the nucleus (Fig. 2Be, 2Bd). In parthenogenetic embryos, at the early 2-cell stage, centromeres were grouped around the nucleolus (Fig. 2Be, 2Bf); however, at the late 2-cell stage, the positions of kinetochores and chromocenters were still diffused throughout the nucleoplasm (Fig. 2Bg, 2Bh), and the kinetochores were colocalized with the compacted chromocenters at the 4-cell stage (data not shown). This indicates that relocation of the centromeres at the chromocenter of nucleoplasm was delayed in parthenogenetic embryos.

Methylation of histone H3 at Arginine 26 express a diffused distribution pattern in four-cell stage parthenogenetic embryos

Recent findings have demonstrated that cells in mouse embryos can differ in their developmental fate and potency at the four-cell stage [24]. Since epigenetics is suggested to be involved in maintaining pluripotency, we inspected the expression of methylation of histone H3 at arginine 26 (Me-H3-R26) at the two- and four-cell stage in parthenogenetic embryos. This methylation is one of the most common posttranslational modifications; it promotes DNA accessibility and biases cells to their ICM fate [16]. At the two-cell stage, nascent RNA distribution was found to be uniform throughout the nuclei corresponding with high levels of Me-H3-R26 expression in both fertilized and parthenogenetic embryos (Fig. 3Ab, 3Af, 3Ac, 3Ag). We have reported previously that the two-cell to four-cell transition resulted in a repression of nascent RNA production in
some regions of the nuclei in fertilized but not in parthenogenetic embryos [15]. In this study, a closer look at the early four-cell blastomere from both types of embryos revealed high levels of Me-H3-R26 compacted in which nascent RNA was repressed in fertilized embryos (Fig. 3Bb, 3Bc). In contrast, the methylation level of H3-R26 seemed to be weaker and diffused throughout the nucleoplasm concomitant with uniform distribution of nascent RNA in parthenogenetic embryos (Fig. 3Bf, 3Bg). We found that H3-R26 was methylated higher in fertilized than in parthenogenetic embryos at the four-cell stage. This may lead to the higher levels of methylation of H3-R26 observed in the ICM of fertilized blastocysts as compared to parthenogenetic blastocysts (Fig. 3C).

Acetylation level of histone H4 at lysine 12 was lower in ICM of parthenogenetic than that in fertilized blastocysts

In the next step, we investigated another paternal chromatin contribution by examining acetylation of histone H4 at lysine 12 (Ac-H4-K12) from the one-cell to blastocyst stage in parthenogenetic embryos. This common histone acetylation has been known to be involved in zygotic gene transcription and exhibited a close relationship with the expression of pluripotent genes, such as Oct4 and Nanog [25].

Our previous results demonstrated that in the case of fertilized embryos, at the one-cell stage, the acetylation level of H4-K12 was higher in expanded paternal pronucleus, and weaker in maternal pronucleus [15]. In this study, we found that H4-K12 was highly acetylated in both fertilized and parthenogenetic embryos from the two-cell to morula stage. However, at the blastocyst stage, the acetylation level of H4-K12 was lower in ICM of parthenogenetic blastocysts as opposed to that in fertilized embryos (Fig. 4). Such unusual levels of acetylation may induce a downregulation in expression of important pluripotent genes, contributing to developmental defects in parthenotes.
Discussion

The presence of both parental chromatin enables the establishment of asymmetric epigenetics in the zygote, which is essential for proper nuclear reprogramming and restoration of cell totipotency during preimplantation development [26]. The absence of one parental genome can lead to aberrant expression of imprinted, developmental and pluripotent genes, triggering changes in cell potency [4, 14]. In this study, we have investigated some important histone modifications in fertilized and parthenogenetic embryos, then demonstrated the role of paternal chromatin in controlling these epigenetic expressions. Soon after fertilization, transcriptional activity was immediately associated with paternal chromatin, and this induced transcription was also higher in the male pronucleus [27]. Various epigenetic modifications are known to be involved in the reprogramming of the parental genome [28, 29]. The first observation of nucleosomal histones, including acetylation and methylation, is important for regulating gene transcription and silencing [30, 31]. Modifications can occur at several amino acid residues, in which lysine 9 of histone H3 is a notable example, which can be acetylated or methylated [32]. We demonstrated that soon after ICSI, the paternal pronucleus expanded quickly and showed a high level of acetylation of H4-K12, whereas the maternal pronucleus was smaller, with weak staining for acetylation, in addition, at the two-cell stage, male chromatin and female chromatin contributed separately to show different patterns of histone methylation [15]. This combination of the male and female chromatin might induce a rapid decrease in nascent RNA production during the two-cell to four-cell transition in fertilized embryos.

HP1, a heterochromatin that interacts with Me-H3-K9, is a component of silent chromatin at telomeres and centrosomes. The time-lapse observation revealed that the chromatin association of HP1beta is regulated in a cell cycle-dependent manner [33]. As
a reflection of chromatin organization, HP1 signals started to appear in paternal pronucleus at 4 h after fertilization. It was located around the nucleolus of pronuclei, and was higher in maternal chromatin, reconfirming exclusive expression of methylation in female pronucleus. Moreover, heterochromatin is also implicated in other basic cellular functions, including repression of inadequate transcription [34], cohesion of kinetochores [35], and stability of chromosomes [36]. Previous results have suggested the existence of a stage-specific nuclear organization that was reminiscent of mouse antral oocytes [37]. Specifically, prior to ovulation, the kinetochores and pericentric heterochromatin of mouse pre-ovulatory oocytes cluster around the nucleolus and gene transcription is shut down [38]. Transcription can first be detected at the end of the first cell cycle [39]. Thus, the nuclear organization in one-cell embryos can provide a necessary constraint for further development. At the two-cell stage, Rabl-like configuration is typical for the nuclear organization, in which polarized arrangement of kinetochores resembles a half-moon [14]. This was in accordance with the distribution previously described in two-cell stage mouse embryos by Mayer’s group [40] and Martin’s group [12]. The polarity in a Rabl-like configuration as well as the large numbers of chromocenters may imply some constraints on nuclear restructure implying regulations of gene expression. In addition, the presence of this configuration may correlate in time with the major wave of genome activation; however, whether there is a causal connection between the two is still unknown. It has been demonstrated that in fertilized embryos, nascent RNA production was repressed at the late two-cell stage, but not in the case of parthenogenetic embryos, in which this phenomenon was maintained until late four-cell stage [15]. At the late two-cell stage, fertilized embryos exhibited chromocenters associated with HP1 and polarized in one pole of the blastomere. On the other hand, not all chromocenters in parthenogenetic embryos were
linked with HP1, and high number of chromocenters were still diffused throughout the nucleoplasm, confirming a delay in nascent RNA repression in parthenogenetic blastocysts. Lack of the paternal genome might contribute to this abnormality, and this may be one of the reasons affecting parthenotes’ later development. At this stage, zygotic genes should be silenced so that the embryonic genes can be expressed to sustain proper development of the embryos [41], and it appears that paternal genome might be involved in activation of the embryonic genome.

The evidence provided in this study has demonstrated that non-uniform epigenetics between blastomeres imposes heritable instructions for lineage-specific differentiation [26, 28]. Among common histone modifications, methylation of histone H3 at arginine 26 in blastomeres, at the four-cell stage, was proved to significantly display lineage allocation and cell fate determination [16, 24]. In fertilized embryos, since nascent RNA expression had already been repressed at the late two-cell stage; as a result, at the early four-cell stage, upregulation of Me-H3-R26 expression indicated the onset of embryonic genes. High signals of Me-H3-R26 were concentrated at the location where nascent RNA was repressed. On the other hand, parthenogenetic four-cell blastomere showed weaker and uniform expression of Me-H3-R26 throughout the nucleoplasm. The reason for this phenomenon may be because nascent RNA repression does not occur at this stage in the parthenogenetic blastocyst. Due to the balance in the level of gene expression, differentiated genes could not be expressed in the parthenogenetic blastocyst. Moreover, in 2015, Chen and Yu’s group [42] proposed decreased Nanog and Sox2 expression in parthenotes in the blastocyst stage, given that these gene expressions were upregulated by CARM1-mediated Me-H3-R26 [16, 43]. As a result, low levels of Me-H3-R26 at the four-cell stage might lead to lower number of cells directing their progeny to the ICM, and decreased expression of Me-H3-R26 in
parthenogenetic ICM cells. Consequently, aberrant pluripotency in the ICM cells might
directly affect pES establishment rate and differentiation capacity.

In addition, previous studies have suggested that changes in chromatin structure
underlie, at least in part, zygotic gene activation in the mouse [44]. Specifically, histone
H4 acetylation is incorporated with chromatin remodeling during differentiation and
development. It has been suggested that this acetylated region preferentially initiates
transcription in the mouse embryos at the one- and two-cell stage. At later stages,
chromatin formation bearing these acetylated histone H4 may be required for countering
a transcriptionally repressive state that follows the first mitosis [45-47]; hence,
sustaining continued gene expression for normal development of the embryos [44].

Recently, Ac-H4-K12 has been found to be important in paternal influence on early
gene expression in the zygote [17]. We observed homogenous expression of Ac-H4-
K12 in all the blastomeres of both parthenogenetic and fertilized embryos from the two-
cell to the morula stage. At the blastocyst stage, ICM cells of parthenogenetic embryos
expressed significantly lower levels of Ac-H4-K12 than that of fertilized ones,
indicating impairments of gene expression in the parthenogenetic ICM cells. Because
Ac-H4-K12 was required for the recruitment of the transcription factor Oct4 to its
binding site in the Nanog promoter [25]; therefore, the reduction of Ac-H4-K12 may be
one of the reasons contributing to reduced Nanog and Oct4 expression in
parthenogenetic ICM cells. This might have an impact on pES cell maintenance and
differentiation, and we will study these aspects in future studies. In conclusion,
differences in epigenetic modifications correlating to paternal chromatin’s capacity to
regulate nascent RNA repression may contribute to the aberrant development and
lineage allocation in mouse parthenogenetic embryos.
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Conflicts of interest

The authors declare no conflicts of interest in the research.

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**Figure Legends**

**Fig. 1.** Histone H3 modifications and Heterochromatin Protein 1 formation in one-cell embryo after fertilization (A) Expression of methylation of histone H3 at lysine 9 (Me-H3-K9) (f-j) and HP1 (k-o) in paternal and maternal chromatin 5 h after fertilization. Maternal chromatin shows high expression level of Me-H3-K9, while paternal chromatin showed hypomethylation. Soon after fertilization, maternal chromatin showed high level of HP1 expression while this signal was not expressed in paternal chromatin. (B) Acetylation of histone H3 at lysine 9 (Ac-H3-K9) and Me-H3-K9 in 1-cell embryo at 6 h after fertilization. Although both pronuclei show Ac-H3-K9 (b); they showed an asymmetric methylation of H3-K9 between maternal and paternal chromatin in the 1-cell stage (c). The DNA was counterstained with DAPI. Bar = 10 µm.

**Fig. 2.** Distribution of Heterochromatin Protein 1 (HP1) and kinetochores in one-cell and two-cell embryos. (A) There was accumulation of HP1 around the NPB in
male pronuclei concomitant with the pericentric heterochromatin ring around the NPB at 1-cell stage, 6 h after fertilization. (B) At the early two-cell stage, centromeres aggregated around the nucleolus in both types of embryos (a, b, e, f). At the late two-cell embryos, kinetochores were polarized and compacted at the chromocenters at one pole of the nucleus in fertilized embryos (c, d; Arrows). However, the position of kinetochores and chromocenters were diffused throughout the nucleoplasm in parthenotes (g, h). The DNA was counterstained with DAPI. Bar = 10 µm.

Fig. 3. Expression of methylation of histone H3 at arginine 26 (Me-H3-R26) and nascent RNA at two- and four-cell stages in fertilized and parthenogenetic embryos. Embryos were subjected to electrical permeabilization in the presence of BrUTP, cultured for 1 h and collected for the detection of BrUTP-labeled RNA transcripts and Me-H3-R26 by immunostaining. (A) At the two-cell stage, nascent RNA distribution was uniform throughout the nuclei, corresponding with high levels of Me-H3-R26 expression in both fertilized and parthenogenetic embryos (B) High levels of Me-H3-R26 were compacted in which nascent RNA was repressed in fertilized embryos (b and c). In contrast, methylation level of H3-R26 seemed to be weaker and diffused throughout the nucleoplasm concomitant with uniform distribution of nascent RNA in parthenogenetic embryos (C) Intensity of Me-H3-R26 expression during preimplantation development of fertilized and parthenogenetic embryos. Expression levels were significantly different (asterisks) at the 4-cell stage and in ICM cells of fertilized and parthenogenetic embryos. The DNA was counterstained with DAPI. Bar = 10 µm.

Fig. 4. Expression of histone H4 acetylation at lysine 12 (Ac-H4-K12) in fertilized and parthenogenetic embryos during preimplantation development. (A) Acetylation level of H4-K12 was maintained at a high level in both fertilized and
parthenogenetic embryos in the two-cell, four-cell, eight-cell, and morula stage. (B) At
the blastocyst stage, acetylation level of H4-K12 was lower in ICM of parthenogenetic
compared to that in fertilized embryos. The circles in blastocysts indicate their ICM.
Bar = 10 µm.
Figure 1

A

DNA

Me-H3-K9

HP1

B

1 h 2 h 3 h 4 h 5 h

DNA Me-H3-K9 HP1

♂ ♀ ♂ ♀ ♂ ♀ ♂ ♀ ♂ ♀ ♂ ♀ ♂ ♀ ♂ ♀ PB ♂ ♀ ♂ ♀ ♂ ♀ ♂ ♀ ♂ ♀

Figure 2
Figure 3
Figure 4