The Expression and Nuclear Deposition of Histone H3.1 in Murine Oocytes and Preimplantation Embryos

Machika KAWAMURA1), Tomohiko AKIYAMA1), Satoshi TSUKAMOTO2), Masataka G. SUZUKI1) and Fugaku AOKI1)

1) Department of Integrated Biosciences, Graduate School of Frontier Sciences, University of Tokyo, Chiba 277-8562, Japan
2) Laboratory of Animal and Genome Science Section, National Institute of Radiological Sciences, Chiba 263-8555, Japan

Abstract. Differentiated oocytes acquire totipotency through fertilization. During this transition, genome-wide chromatin remodeling occurs, which leads to change in gene expression. However, the mechanism that underlies this global change in chromatin structure has not been fully elucidated. Histone variants play a key role in defining chromatin structure and are implicated in inheritance of epigenetic information. In this study, we analyzed the nuclear localization and expression of H3.1 to elucidate the role of this histone variant in chromatin remodeling during oogenesis and preimplantation development. Analysis using Flag-tagged H3.1 transgenic mice revealed that Flag-H3.1 was not present in differentiated oocytes or early preimplantation embryos before the morula stage, although Flag-H3.1 mRNA was expressed at all stages examined. In addition, the expression levels of endogenous H3.1 genes were low at the stages where H3.1 was not present in chromatin. These results suggest that H3.1 is not incorporated into chromatin due to the inactivity of the histone chaperone and low mRNA expression level. The significance of the dynamics of H3.1 is evaluated in terms of chromatin remodeling that takes place during development.

Key words: H3.1, H3 variants, Oocyte, Preimplantation embryo, Reprogramming
H3.1 are discussed.

Materials and Methods

Transgenic mouse

The pCAGGS vector [17], which contains the CAG promoter and a 3’UTR region of rabbit β-globin, was used to generate CAG promoter-driven Flag-tagged H3.1 transgenic mice. For microinjection, the transgene was diluted to 4 ng/μl in Tris/EDTA buffer (pH 7.5) and microinjected into the pronucleus of C57 BL/6 1-cell embryos (Japan SLC, Shizuoka, Japan). One day after microinjection, normal 2-cell embryos were collected and transferred to day 0.5 pseudopregnant ICR (MCH) females (Japan SLC). Thirty-four mice were screened, and seven of them were positive for the transgene. The founder transgenic mouse was backcrossed twice with C57 BL/6 female mice (Japan SLC). The pups were subjected to genotyping of the transgene by PCR.

Oocyte collection

Growing oocytes were collected from nine-day-old mice. The ovaries were dissected in HEPES-buffered KSOM (potassium simplex optimized medium) [18]. The ovaries were further dissected after trypsin treatment to obtain additional growing oocytes. The growing oocytes were placed in α-minimum essential medium (Gibco BRL, Grand Island, NY, U.S.A.) supplemented with 5% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, U.S.A.) and 10 ng/μl epidermal growth factor (Invitrogen, Carlsbad, CA, U.S.A.) until use for immunocytochemistry or RT-PCR analysis. Full-grown oocytes were collected from three- to five-week-old mice 48 h after injection of 5 IU pregnant mare’s serum gonadotropin (PMSG; Aska Pharmaceutical, Tokyo, Japan).

In vitro fertilization and embryo culture

To obtain mature metaphase II (MII) oocytes, three- to five-week-old mice were superovulated by injection of 5 IU of human chorionic gonadotropin (hCG; Aska Pharmaceutical) 48 h after injection of PMSG. The oocytes were collected in human tubal fluid (HTF) medium [19] from the ampulla of the oviduct 16 h after injection of hCG. Sperm was collected from ICR mice (Japan SLC). In vitro fertilization was carried out in the HTF medium by fertilizing the MII stage oocytes with the sperm, which had been preincubated for 2 h, with a final concentration of 200,000–1,000,000 sperm/ml. The oocytes were washed in the KSOM medium [20] 6 h after insemination, and the fertilized oocytes with 2 pronuclei were selected. Embryos at various developmental stages were collected according to the following time schedule after fertilization: 1-cell embryos at 10 h, 2-cell embryos at 28–30 h, 4-cell embryos at 45 h, morulae at 72 h, and blastocysts at 96 h.

Immunocytochemistry

The oocytes and embryos were fixed in 3.7% paraformaldehyde (Wako Pure Chemical Industries, Osaka, Japan) with 0.2% Triton X-100 (Sigma-Aldrich) for 40 min at room temperature and washed with PBS containing 1% BSA (1% BSA/PBS) three times. The cells were then incubated with anti-Flag mouse IgG primary antibody (Sigma-Aldrich) diluted 1:100 with 1% BSA/PBS containing 0.2% Tween 20 (MP Biomedicals, Solon, OH, U.S.A.) overnight at 4 C. After being washed in 1% BSA/PBS three times, they were incubated with the Alexa Fluor 488-conjugated mouse IgG secondary antibody diluted 1:100 with 1% BSA/PBS containing 0.2% Tween 20 for 1 h at room temperature. The cells were then mounted on VECTASHIELD (Vector Laboratories, Burlingame, CA, U.S.A.), an anti-bleaching reagent, and were observed using a confocal laser scanning microscope (Carl Zeiss 510, Oberkochen, Germany).

Reverse transcription PCR (RT-PCR)

For RT-PCR analyses to detect the expression of Flag-H3.1 transgene, 10 oocytes/embryos were placed in 400 μl ISOGEN (Nippon Gene, Tokyo, Japan). RNA was extracted by following the manufacturer’s protocol (Nippon Gene), and reverse transcription was carried out with an Oligo dT Primer using a PrimeScript RT-PCR Kit (TaKaRa, Shiga, Japan). PCR was carried out using Ex Taq DNA Polymerase (TaKaRa) and 0.4 oocytes/embryos cDNA in a tube as a template. Flag-H3.1 was detected with the following primers: 5’ATGAGCGCAGATAAGGGAGGAGG3’ (forward) and 5’CTCGGTGCACTTCTGGTAGC3’ (reverse). PCR conditions were as follows: 95 C for 30 sec (denaturation), 57 C for 30 sec (annealing) and then 72 C for 30 sec (elongation). Fifty pg of Rabbit α-globin mRNA (Sigma-Aldrich) was added into the samples before RNA extraction as an external control and was detected with the following primers: 5’GTGGGACAGGAGCGTTGAAT3’ (forward) and 5’GCAGCCACCGTGGCGGATTG3’ (reverse). The PCR conditions were: 95 C for 20 sec (denaturation), 58 C for 30 sec (annealing), and 72 C for 30 sec (elongation).

For real-time PCR analyses to examine the expression levels of endogenous histone H3 variants, 40 oocytes/embryos from three-week-old ddY mice (Japan SLC) were placed in 400 μl ISOGEN (Nippon Gene), and RNA was extracted. The contaminated DNA was digested with DNase I (Promega, Madison, WI, U.S.A.) for 40 min at 37 C, and then RNA was purified with phenol/chloroform or ISOGEN. Primers were based on the common sequences of several genes encoding H3.1 as well as to the individual sequences of H3.1 genes (Table 1). The primers of common sequences in each of H3.2 and H3.3 were also prepared. Two-step PCR reactions were carried out with the following conditions using a Thermal Cycler Dice Real Time System (TaKaRa): 95 C for 5 sec (denaturation) and 60 C for 30 sec (annealing/elongation). After PCR, the samples were subjected to agarose gel electrophoresis to confirm that only a band with the expected size was observed.

Results

Flag-H3.1 is absent from chromatin before fertilization

We have previously shown that when Flag-tagged H3.1 mRNA was microinjected, Flag-H3.1 protein was not incorporated into the nuclei before fertilization or during early preimplantation development, although the protein was efficiently translated [10]. These results showed that the de novo incorporation of endogenous H3.1 into chromatin does not occur during these periods, but it is still unknown whether or not H3.1 is present in the chromatin because preexisting H3.1 that had been incorporated into chromatin before fertilization may remain there after fertilization and during early preimplantation.
H3.1 IN OOCYTES AND EMBRYOS

Therefore, we generated Flag-tagged H3.1 transgenic mice that ubiquitously express Flag-H3.1 in all tissues to analyze whether Flag-H3.1 is present during oogenesis and preimplantation development. In these mice, Flag-H3.1 was absent in the nuclei of both growing and full-grown oocytes (Fig. 1A). Similar to the results of mRNA microinjection [10], Flag-H3.1 was not detected until the morula stage after fertilization, although Flag-H3.1 mRNA was expressed in all examined stages from the growing stage of oocytes to the blastocyst stage (Fig 1B). These results strongly suggest that H3.1 is not incorporated into chromatin due to the absence and/

Table 1. Primers used for detection of endogenous H3.1 genes

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Ref Seq accession no.</th>
<th>Sequences</th>
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<tbody>
<tr>
<td>A. Common PCR primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H3.1</td>
<td>Forward 5'-TGCAGGAGGCCCTGTGA-3'</td>
<td>Reverse 5'-TGGATGTCCTTGCCATG-3'</td>
</tr>
<tr>
<td>H3.2</td>
<td>Forward 5'-TGCAGGAGGCCGAGGA-3'</td>
<td>Reverse 5'-TGGATGTCCTTGCCATG-3'</td>
</tr>
<tr>
<td>H3.3</td>
<td>Forward 5'-ATCCACCCGTGTAACAG-3'</td>
<td>Reverse 5'-CTTCACCCCTCAAGTGAAGG-3'</td>
</tr>
<tr>
<td>B. Individual PCR primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hist1h3a</td>
<td>NM_013550.4</td>
<td>Forward 5'-GTGCTCACCCAGCTTTC-3'</td>
</tr>
<tr>
<td>Hist1h3g</td>
<td>NM_145073.2</td>
<td>Forward 5'-CACCTTTCCCTACGGTTACT-3'</td>
</tr>
<tr>
<td>Hist1h3h</td>
<td>NM_178206.2</td>
<td>Forward 5'-CTTGGTGGCAGCTTACC-3'</td>
</tr>
<tr>
<td>Hist1h3i</td>
<td>NM_178207.2</td>
<td>Forward 5'-CTTATTCACCTTTCTAGTGAG-3'</td>
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Fig. 1. Nuclear deposition of Flag-H3.1 in the oocytes and preimplantation embryos of Flag-tagged H3.1 transgenic mice. (A) Flag-H3.1 was detected by immunocytochemistry with ant-Flag antibody in growing (GO) and full-grown oocytes (FGO) and preimplantation embryos. Scale bar= 10 μm. (B) Expression of Flag-H3.1 mRNA was examined by RT-PCR in growing (GO) and full-grown oocytes (FGO), 1-cell embryos (1-C) and blastocysts (Bl) obtained from transgenic mice and FGO from wild-type mice (Control). Rabbit α-globin mRNA was added into each sample to control the RNA extraction and reverse transcription during the process of RT-PCR assay.

development. Therefore, we generated Flag-tagged H3.1 transgenic mice that ubiquitously express Flag-H3.1 in all tissues to analyze whether Flag-H3.1 is present during oogenesis and preimplantation development. In these mice, Flag-H3.1 was absent in the nuclei of both growing and full-grown oocytes (Fig. 1A). Similar to the results of mRNA microinjection [10], Flag-H3.1 was not detected until the morula stage after fertilization, although Flag-H3.1 mRNA was expressed in all examined stages from the growing stage of oocytes to the blastocyst stage (Fig 1B). These results strongly suggest that H3.1 is not incorporated into chromatin due to the absence and/
or inactivity of a histone chaperone before fertilization and early preimplantation development.

**The expression level of endogenous H3.1 is low at the stages where Flag-H3.1 is not present in chromatin**

Flag-H3.1 was absent from the nucleus before the morula stage (Fig. 1A). In order to investigate the expression of endogenous H3 variants in oocytes and preimplantation embryos, we performed real-time PCR and examined overall expression of H3.1, H3.2 and H3.3. Each of the H3 variants is encoded by several genes. First, we investigated the overall expression of H3.1, H3.2, and H3.3 by designing a common primer that recognizes all the genes for each variant (Fig. 2). The result showed that H3.1 transcript was expressed at low levels in oocytes and early preimplantation embryos, whereas its nuclear deposition was not observed in the transgenic mice expressing the Flag-H3.1. The expression increased from the morula stage, which is consistent with the increase in the nuclear deposition of Flag-H3.1. In contrast, H3.2 and H3.3 was expressed at high levels in full-grown oocytes and 1-cell stage embryos.

Next, we examined the expression of individual H3.1 genes. H3.1 is coded by 4 genes, Hist1h3a (NM_013550.4), Hist1h3g (NM_145073.2), Hist1h3h (NM_178206.2), and Hist1h3i (NM_178207.2), included in the same histone cluster on chromosome 13 [21, 22]. However, its individual expression in mammalian preimplantation embryos has not been reported. Here, we investigated individual gene expression using primers that specifically recognize each gene. Similar expression patterns were observed for all 4 genes, with the expression level being low in full-grown oocytes to 4-cell stage embryos and increasing from the morula stage (Fig. 3), suggesting that all H3.1 genes are regulated by a common mechanism. Taken together, the nuclear incorporation of H3.1 may be limited due to its low expression level along with the absence of the histone chaperone activity.

**Discussion**

In this study, we found that Flag-H3.1 is absent from chromatin in early preimplantation embryos (Fig. 1A), which is consistent with our previous finding that Flag-H3.1 was not incorporated into the nucleus in these embryos [10]. Furthermore, our results showed that Flag-H3.1 was also absent in the chromatin of differentiated oocytes, both growing and full-grown oocytes. To our knowledge, this is the first study to analyze the dynamics of Flag-H3.1 during oogenesis. We have also shown that the expression level of H3.1 was low at the stages in which the presence of Flag-H3.1 was not detected in chromatin (Fig. 2). Consequently, these results suggest that the presence of H3.1 in chromatin is regulated by its expression level along with the activity of a histone chaperone(s).

Our results may be regarded as both consistent and inconsistent with previous reports for several reasons. First, we previously analyzed the dynamics of Flag-H3.1 by microinjection of Flag H3.1 mRNA into embryos and found that Flag-H3.1 was incorporated from the 4-cell stage [10]. However, the analysis using transgenic mice revealed that Flag-H3.1 is present in chromatin from the morula stage (Fig. 1A). Since nuclear incorporation of H3.1 would have just started at the 4-cell stage, a significant amount of H3.1 would not yet be accumulated in the nucleus. Therefore, Flag-H3.1 was not detected at this stage in the transgenic embryos. Next, according to a previous study, the endogenous expression of H3.1 was not detected in preimplantation mouse embryos [23]. Although there is no description about which genes were examined in that study, we could detect all of the genes encoding H3.1 (Fig. 3). It is likely that the expression was not detected because a small amount of embryo cDNA was used for analysis in the previous study, i.e., 0.05 embryos per tube in the PCR assay [23], whereas we used 4 embryos per tube.

After fertilization, Flag-H3.1 was detected from the 4-cell stage, and thus may be essential for heterochromatin formation. It is known that heterochromatin formation changes dynamically during preimplantation development [24]. Before the 4-cell stage, heterochromatin is detected only in the peripheral region of the nucleolus, and thus there are few chromocenters [24, 25]. Heterochromatin formation progressively takes place from the 4-cell to the blastocyst stage. This pattern of heterochromatin formation is similar to that of Flag-H3.1 nuclear deposition, suggesting that H3.1 is involved in heterochromatin formation. Indeed, it has been suggested that H3.1 is associated with constitutive heterochromatin [6]. Moreover, in the embryos expressing mutated CAF-1, a histone chaperone for H3.1, heterochromatin formation was delayed during preimplantation development [24], supporting our hypothesis that H3.1 is responsible for the formation of heterochromatin in the late preimplantation stage.

We have previously shown by microinjecting Flag-H3.1 mRNA into embryos that the Flag-H3.1 protein was not incorporated into the nucleus after fertilization when genome reprogramming occurs...
to transform the differentiated oocytes into totipotent embryos, and we suggested that the absence of H3.1 is essential for the genome reprogramming to establish totipotency [10]. However, as described in the Results section, those experiments could not exclude the possibility that the H3.1 that had been incorporated into the chromatin during oogenesis still remained there after fertilization. In the present study, we could ensure the actual deposition of the histone variant into chromatin by analyzing the transgenic mice ubiquitously expressing Flag-H3.1 and obtained results that strongly support that H3.1 is absent from the chromatin after fertilization. We also previously examined the dynamics of histone variant replacement to investigate their involvement in genome remodeling in the somatic nucleus transplanted into the enucleated oocyte [26]. Soon after the transplanted oocytes were activated, all three H3 variants derived from the somatic cell were removed from the chromatin, and then oocyte-derived H3 variants were incorporated. At this time, H3.1 was incorporated as well as other H3 variants, although H3.1 was not usually incorporated in the 1-cell stage embryos. This incorporation may be due to the presence of a somatic cell-derived histone chaperone or a factor involved in the nuclear import of H3.1. Moreover, this anomalous incorporation of H3.1 would be responsible for the failure in genome reprogramming resulting in the low success rate of somatic nuclear cloning.

The lack of H3.1 in differentiated oocytes may facilitate the acquisition of totipotency after fertilization. During preimplantation development, embryos gradually lose their totipotency and finally start to differentiate at the morula/blastocyst stage [1, 27]. The incorporation of H3.1 occurs coincidentally with this process, suggesting that H3.1 is associated with differentiation. Similarly, the cell lines derived from differentiated tissues are rich in H3.1, whereas embryo-derived cell lines have high levels of H3.3 [7]. Therefore, the lack of H3.1 would be a preparatory state for oocytes to acquire totipotency soon after fertilization.

We found that the expression levels differ among H3 variants in oocytes and preimplantation embryos. Notably, H3.1 is expressed at low level in oocytes and increases from the morula stage, whereas H3.2 and H3.3 are highly expressed in oocytes (Fig. 2). Although genes that code for H3.3 are separately located in the genome, those that code for H3.1 and H3.2 are located as histone clusters. In the case of all four genes that code for H3.3, all are localized in the major histone cluster HIST1 on Chromosome 13 [21, 22]. More than half of the genes encoding H3.2 are also localized in the same cluster on Chromosome 13. Since the expressions of these H3.1 and H3.2 genes thus seem to be regulated in the identical mechanism, the difference in the expression patterns between H3.1 and H3.2 may be due to a difference in stability of mRNA. The stability of histone mRNA is regulated by the stem-loop binding protein (SLBP) [28, 29]. In addition, factors associated with histone mRNA processing such as the 100 kDa zinc finger protein (ZFP100) may influence the stability of mRNA [29, 30].
Taken together, mammalian-specific H3.1 is absent from the chromatin in oocytes and early preimplantation embryos. This absence of H3.1 may be due to the inactivity of histone chaperone and the low level of gene expression. The absence of H3.1 may make it possible for the chromatin structure to easily alter for the chromatin remodeling to establish totipotency after fertilization. H3.1 is present in chromatin in late preimplantation embryos to establish the heterochromatin structure and promote differentiation.

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References


