博士論文

Involvement of histone variants in the change of chromatin structure during early preimplantation development

（着床前初期発生におけるクロマチンリモデリングへの

ヒストンバリアントの関与）

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Involvement of histone variants in the change of chromatin structure during early preimplantation development

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ABBREVIATIONS

KD: Knock down

FRAP: Fluorescence recovery after photobleaching

ZGA: Zygotic gene activation

KO: Knock out

K: lysine

H3K4me3: histone H3 trimethylated at lysine 4

H3K9me2: histone H3 dimethylated at lysine 9

hpi: hours post insemination

eGFP: enhanced green fluorescent protein

siRNA: small interfering RNA

DAPI: 4’, 6’-diamidino-2-phenylindole

MII oocyte: Metaphase II oocyte

BSA: bovine serum albumin

IBMX: 3-isobutyl-1-methylxanthine

(an inhibitor of cAMP phosphodiesterase which inhibits spontaneous maturation)

BrdU: 5-bromo-2’-deoxyuridine

SUMMARY

In mice, chromatin structure is extremely loosened in one-cell stage embryos, and it becomes tighter at the two-cell stage. During this period, various epigenetic factors, *e.g.* histone variants and modifications, are changed. These alterations are collectively designated as chromatin remodeling. However, the mechanisms regulating these alterations have not been clarified. In this thesis, since the variants of linker histone H1 and core histone H3 are known to play important roles in the regulation of chromatin structure, I examined the involvement of these variants in chromatin remodeling during the one- and two-cell stages. Furthermore, I examined the roles of these variants in the gene expression and DNA replication in one- and two-cell stage embryos.

In chapter I, I examined the involvement of linker histone H1 variants in the chromatin remodeling. Immunocytochemistry showed that the nuclear deposition of H1FOO was at a high level at the one-cell stage and it drastically decreased at the two-cell stage, whereas the level of somatic linker histone variant H1D was low at the one-cell stage and increased at the two-cell stage. H1foo knockdown (KD) caused the chromatin structure to be tighter at the one-cell stage and delayed the DNA replication at the perinucleolar region. To examine the significance of the change in H1FOO during one- and two-cell stages, I overexpressed H1d and H1foo in one- and two-cell stage embryos, respectively. The overexpression of H1d caused the abnormally condensed chromatin structure at the one-cell stage and led to developmental arrest at the one- or two-cell stage. The overexpression of H1foo relieved the decrease of chromatin looseness at the two-cell stage. These results suggest that a high expression level of H1foo is involved in the formation of loosened chromatin structure in one-cell stage embryos and that the decrease in H1foo is associated with tightening chromatin

structure during the two-cell stage.

In chapter II, I examined the involvement of H3 variants, H3.1/3.2 in the change of chromatin structure during the one- and two-cell stages. It had been reported that the nuclear deposition of H3.1/3.2 was at a low level in one-cell stage embryos and increased during the two-cell stage. When H1foo was knocked down, the nuclear deposition of H3.1/H3.2 was increased in one-cell stage embryos in which DNA replication was delayed at the perinucleolar region. H3.1/3.2 KD at the two-cell stage caused the chromatin structure remained to be loosened state. Furthermore, H3.1/3.2 KD relieved some genes and a retrotransposon from the repression during the two-cell stage and had a detrimental effect on the development after the two-cell stage. These results suggested that the increase of H3.1/3.2 is involved in the formation of tightened chromatin structure and the repression of some genes and a retrotransposon during the two-cell stage.

Taken together, my research suggests that a high level of H1foo and a low level of H3.1/3.2 contribute the formation of loosened chromatin structure at the one-cell stage and that the decrease of H1foo and the increase of H3.1/3.2 are involved in the establishment of tight chromatin structure at the late two-cell stage to repress some genes and a retrotransposon which have been promiscuously transcribed at the one- and the early two-cell stage. In addition, I have shown that H1foo represses the nuclear deposition of H3.1/3.2, which would prevent from the delay in DNA replication at the perinucleolar region in the pronucleus of one-cell stage embryos. My study has elucidated the mechanism regulating chromatin remodeling during one- and two-cell stages and suggested that this remodeling plays roles in the regulation of gene expression and DNA replication.

GENERAL INTRODUCTION

Upon fertilization, an oocyte and a spermatozoon fuse to form a one-cell stage embryo. The maternal genome, derived from the oocyte, undergoes chromosomal segregation to extrude half of the chromosomes as a second polar body. In the paternal genome, derived from the spermatozoon, protamine which contributes to compact chromatin structure is removed and replaced with maternally supplied histones to establish nucleosomes (Nonchev and Tsanev, 1990). The paternal and maternal genomes separately form pronuclei in one-cell stage embryos. Syngamy occurs during the M phase and a nucleus containing both the maternal and paternal genomes forms after the cleavage into a two-cell stage embryo. During this process, dynamic alteration of chromatin structure and epigenome occur, which are referred to as chromatin remodeling (Fig. GI).

A line of evidence indicates that distinct changes in the chromatin structure occurs during one- and two-cell stages. Fluorescence recovery after photobleaching (FRAP) analysis demonstrated that the mobility of histones is extremely high in one-cell stage embryos and then decreases in two-cell stage embryos (Ooga et al., 2016). Since the mobility of histones and the degree of chromatin looseness are positively correlated (Martin et al., 2010, Chalut et al., 2012), it is likely that the chromatin structure is extremely loosened in one-cell stage embryos and then becomes more condensed in two-cell stage embryos. Electron spectroscopic imaging has shown that the chromatin is highly dispersed in one-cell stage embryos but becomes more condensed in two-cell stage embryos (Ahmed et al., 2010). A genome-wide

analysis of chromatin accessibility, using an assay for transposase-accessible chromatin in combination with high throughput sequencing (ATAC-Seq), showed that chromatin developed a widely open configuration within the transcribed region at the early two-cell stage, but this open chromatin was not observed at the late two-cell stage (Wu et al., 2016). Finally, although several chromocenters, where pericentromeric heterochromatin congregates, can be observed as densely stained foci by DNA staining with 4′,6-diamidino-2-phenylindole (DAPI) and DNA-fluorescent *in situ* hybridization analysis of major satellites in somatic cells, no focus of chromocenter has been observed in the pronuclei of one-cell stage embryos. Instead, pericentromeric heterochromatin with major satellites is localized in the peripheral region of a nucleolus in one-cell stage embryos (Probst et al., 2007, Probst et al., 2010). All of these results suggest that the chromatin structure is globally loosened at the one-cell stage and becomes relatively condensed at the two-cell stage.

Epigenetic modifications which are involved in the regulation of chromatin structure are also dynamically changed during one- and two-cell stages (Funaya and Aoki, 2017; Lim et al., 2016). Immunocytochemistry revealed that the level of H3K4me3 and H3K27ac, which are involved in the loosened chromatin, decrease during one- and two-cell stages (Dahl et al., 2016; Hayashi et al., 2011).

Chromatin structure is involved in the regulations of various cellular processes, *e.g.* transcription, DNA replication and DNA damage response *etc.* (Hauer and Gasser, 2017; Li et al., 2007; MacAlpine and Almouzni, 2013;

Venkatesh and Workman, 2015). Among them, the involvement in transcription has been most studied and numerous studies have demonstrated that chromatin structure plays a pivotal role in the activation and repression of gene expression in various types of cells (Li et al., 2007; Venkatesh and Workman, 2015). Indeed, it has been suggested that the change in chromatin structure is associated with the drastic alteration of gene expression during one- and two-cell stages.

In mouse oocytes, genes are actively transcribed during the growth phase, and transcription ceases when oocytes are fully grown (Moore et al., 1974). After fertilization, transcription restarts during the mid S-phase of the one-cell stage, but the transcriptional activity is low. A drastic activation of gene expression is observed during the mid-to-late two-cell stage. Therefore, the relatively low levels of gene activation from the one-cell stage to early two-cell stage and the high levels at the late two-cell stage are referred to as minor and major zygotic gene activation (ZGA), respectively (Aoki et al., 1997; Bouniol et al., 1995; Schultz RM, 1993; Fig. GI). Gene expression patterns are very different between these two phases (Abe et al., 2015; Flach et al., 1982). During minor ZGA, low levels of transcription occur in more than 90% of genes, and also from intergenic regions including retrotransposons. However, during major ZGA, the percentage of transcribed genes reduces to less than 80% and transcription from intergenic regions decreases, while the levels of transcription of particular genes greatly increase (Abe et al., 2015; Yamamoto et al., 2016, Yamamoto and Aoki, 2017).

The regulation of transcription is different between the one- and

two-cell stages. Reporter gene analyses revealed that transcription does not require an enhancer but only a core promoter at the one-cell stage, although a proximal promoter and enhancers are required for transcription at the two-cell stage as is observed in somatic cells (Abe et al., 2015, Hamamoto et al., 2014; Majumder et al., 1993; Wiekowski et al., 1993). This type of transcriptional regulation depending on only a core promoter, seems to induce promiscuous transcription at the one-cell stage as described above (Yamamoto and Aoki, 2017). Thus, the transition from promiscuous transcription all over the genome to more regulated transcription from particular genes occurs between the one- and two-cell stages.

In general, loosened chromatin is believed to facilitate the access of transcription factors to their target DNA sequences (Lee et al., 1993; Gaspar-Maia et al., 2011). This is supported by the finding that most upstream regions of active genes have a loosened chromatin configuration (Boyle et al., 2008). This hypothesis explains why enhancers are not required for transcription in one-cell stage embryos, since one of the functions of enhancers is to loosen the chromatin structure (Majumder et al., 1993, Smale and Kadonaga, 2003) and the chromatin of one-cell stage embryos is already loosened. At the two-cell stage, however, the chromatin structure is tightened so that enhancers are required for transcription. Reporter assay showed that butyrate, which inhibits histone deacetylase, relieved the dependency of enhancers for transcription in two-cell stage embryos (Wiekowski et al., 1993). Since acetylation of histones is associated with loosened chromatin structure, the increases in histone acetylation by

butyrate seems to have the chromatin structure remain to be loosened, leading to the enhancer-independent transcriptional regulation in two-cell stage embryos as is observed in one-cell stage embryos in which chromatin structure is originally loosened. Therefore, the change in the looseness of chromatin structure is likely to involve the alteration of gene expression during one- and one-cell stages, *i.e.* from minor to major ZGA.

It has been suggested that the change of gene expression from minor to major ZGA is associated with second round of DNA replication. Reporter assay showed that the embryos which had been treated with aphidicolin, an inhibitor of DNA replication, did not require the enhancers for transcription at the late two-cell stage (Majumder et al., 1997). In the transgenic embryos which had a reporter gene with the promoter of *Hsp70.1*, it was expressed at the one-cell and the early two-cell stage but repressed at the late two-cell stage. However, it was not repressed when the second round of DNA replication was inhibited (Christians et al., 1995). The DNA replication dependent repression of gene expression was also observed in some endogenous genes. Although transcription requiring complex (TRC), unidentified proteins which were detected at around 70 kDa on SDS PAGE, and *Eif1a* are transcribed at one- and early two-cell stages and then repressed at late two- and four-cell stage, they remained to be transcribed at the late two-cell stage when the second round of DNA replication had been inhibited by aphidicolin (Davis et al., 1996). However, it has not been clarified how DNA replication is involved in the change of gene expression during one- and two-cell stages.

In one-cell stage embryos, chromatin looseness is different even between male and female pronucleus. FRAP analysis revealed that histone mobility was high in one-cell stage embryos. Compared with the male pronucleus, histone mobility is lower in the female pronucleus but still higher than in two-cell stage embryos (Ooga et al., 2016; Table. GI). Epigenetic modifications are also different between male and female. The levels of H3K9me2/3 and H4K20me3 which are associated with tightened chromatin are lower of the male pronucleus than the female one (Santos et al., 2005; Kourmouli et al., 2004; Table. GI). It is thought that these differences are involved in the difference of gene expression between the male and female pronucleus. An *in vitro* transcription assay revealed that the male pronucleus has higher transcriptional activity than the female one (Aoki et al., 1997; Table. GI). In a reporter gene analysis, the male pronucleus showed transcriptional activity in the absence of an enhancer and the addition of an enhancer did not affect its activity (Majumder and DePamphilis, 1994; Wiekowski et al., 1993; Table. GI). On the other hand, the presence of an enhancer increased the transcriptional activity of the female pronucleus, but the degree of this increase was much smaller than that was seen in two-cell stage embryos (Wiekowski et al., 1993, Majumder et al., 1997; Table. GI). Therefore, the degree of dependence on enhancers in the female pronucleus seems to be intermediate between the male pronucleus and two-cell stage embryos. RNA sequencing analysis revealed that many genes are differentially expressed between parthenogenetic embryos and normal embryos (Park et al., 2013), suggesting that the gene

expression pattern is different between male and female pronuclei. In addition to transcription, it has been known that chromatin structure is involved in the DNA replication. In somatic cells, the timing of DNA replication at euchromatin is earlier than that of heterochromatin (O'Keefe et al., 1992). At the one-cell stage, DNA replication start in nucleoplasm of both male and female pronuclei, and then occur in peripheral region of nucleus and nucleolus (Aoki and Schultz, 1999). However, the timing of this DNA replication at nucleoplasm and those peripheral regions is different between male and female pronucleus (Aoki and Schultz, 1999; Table. GI). In the female pronucleus, DNA in the nucleoplasm and perinuclear regions is replicated earlier and that in perinucleolar regions is the last. Since pericentromeric heterochromatin is localized in the perinucleolar regions in the pronuclei, this order is the same as that of most somatic cells. However, in the male pronucleus, DNA replication of the nucleoplasm and perinucleolar regions is earlier and DNA replication of the perinuclear regions is the last. The difference in chromatin structure would be involved in this difference of the pattern of DNA replication between the parental pronuclei.

Taken together, it has been thought that the change of chromatin structure is involved in the alteration of gene expression and DNA replication during one- and two-cell stage. However, it is unknown the mechanism regulating the change of chromatin structure during this stage. Chromatin structure consists of nucleosomes, in which core histone octamers are wrapped with DNA, and linker histones which bind linker DNA between

nucleosomes. There are many core histone and linker histone variants transcribed from different genes whose expression patterns are varied among the tissues and cell types. These variants are known to have different functions from each other in the regulation of the chromatin structure in cultured cells (Biterge and Schneider, 2014). However, their involvement in the change of chromatin structure during one- and two-cell stages has not been clarified.

The purpose in this study is to elucidate the mechanism that regulates the change of chromatin structure during one- and two-cell stages. To clarify this mechanism, I investigated the involvement of the linker histone H1 and core histone H3 variants, in the change of chromatin structure during this period. Moreover, I examined the role of this change in the regulation of transcription and DNA replication.

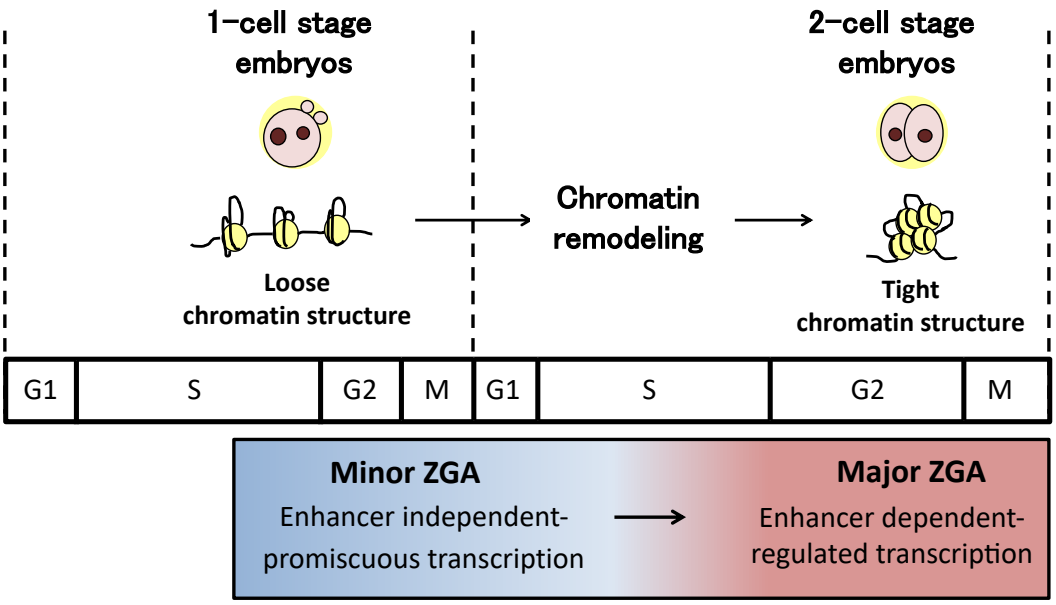

Figure. GI. Chromatin remodeling during the one- and two-cell stage. The chromatin structure is extremely loose at the one-cell stage and then becomes tighter during the two-cell stage in a DNA replication dependent manner. This alteration of chromatin structure is a distinct part of chromatin remodeling which occurs during fertilization and the early preimplantation development. Between the early and the late two-cell stage, gene expression change from minor to major ZGA, i.e. from the enhancer independent-promiscuous to the enhancer dependent-regulated transcription.

	Male	Female
Chromatin looseness	high	low
Epigenetic factors		
whole nucleus		
H3K9me2,3	low	high
nucleolar periphery		
H3K9me3	low	high
H4K20me3	low	high
Transcription		
activity	high	low
dependence on enhancers	independent	partly dependent
DNA replication	fast	slow

Table. GI. Summary of the differences in chromatin structure, epigenetic factors, transcription and DNA replication between the male and female pronucleus.

CHAPTER 1

The involvement of linker histone H1 variants in the change of chromatin structure during early preimplantation development

INTRODUCTION

Linker histones are involved in higher-order chromatin structure (Pan and Fan, 2016, Saeki et al., 2005). In mice, there are 11 linker histone variants encoded by individual genes that are classified into somatic and germ cell types. The somatic cell type (somatic linker histone) contains *H1a*, *H1b*, *H1c*, *H1d*, *H1e*, *H1f0*, and *H1fx*, which are ubiquitously expressed in somatic cells. All of these variants except *H1f0* and *H1fx* are located on chromosome 13 as a cluster (Wang et al., 1997), whereas *H1f0* and *H1fx* are located on chromosomes 15 and 6, respectively. In germ cell types, *H1t*, *H1t2*, and *Hils1* are specifically expressed in sperm cells (Lennox et al., 1984, Martianov et al., 2005, Yan et al., 2003), whereas *H1foo* is specifically transcribed in oocytes (Tanaka et al., 2001). The experiment using knock-in mice with each tagged H1 variant showed that the nuclear deposition of some linker histone variants (H1A, B, C, D, E, FX) changed dynamically during preimplantation development (Izzo et al., 2017).

The pattern of localization in the genome is also different among somatic linker histone variants. DNA adenine methyltransferase identification analysis which map the localization of specific proteins on genome showed that although H1B, C, D, and E are depleted at transcription start site (TSS) of active genes, this degree of depletion at TSS of active genes was weak in H1A (Izzo et al., 2013). Furthermore, the distribution of H1B, C, D, E is correlated with that of heterochromatin protein 1 (HP1) which is associated with inactive transcription, while the distribution of H1A showed a weak correlation with H3K4me3, which is associated with active transcription (Izzo et al., 2013). These data suggest that each linker histone variant have different functions.

Although linker histones generally condense the chromatin structure, the degree of condensation differs among the variants. H1A and H1C have less ability to condense

chromatin structure than other somatic linker histone variants (H1B, D, E) (Clausell et al., 2009, Khadake and Rao., 1995). Chromatin assembly was examined in the extract of *Drosophila* embryos lacking any linker histones, and it was found that adding H1B, H1E, or H1F0 to the extract condensed the chromatin structure, whereas H1A and H1C had no effect on chromatin condensation (Clausell et al., 2009). It has also been shown that the degree of condensation by H1FOO is lower than that by H1C, since ectopic expression of H1FOO reduced the heterochromatin area in mouse fibroblast cells, while H1C overexpression had no effect on the heterochromatin (Kunitomi et al., 2016). As the looseness of chromatin structure is affected by the associated linker histone variants, it is possible that the replacing linker histone variants may induce the change in the chromatin looseness during one- and two-cell stage embryos.

In this chapter, I examined the involvement of linker histone variants in the change of chromatin structure during one- and two-cell stage embryos.

RESULTS

H1foo is highly expressed and involved in the loosened chromatin structure at the one-cell stage embryos

To identify the candidate linker histone variant involved in the change of the chromatin structure during the one- and two-cell stages, I analyzed the expression patterns of all identified variants during preimplantation development using RNA sequencing data (Abe et al., 2015). Among them, *H1a* and *H1foo* showed markedly high expression levels at the one-cell stage (Fig. 1-1). However, the level of *H1a* remained high at the two-cell stage. Although it decreased at the four-cell stage, the *H1a* expression level remained high until the blastocyst stage. By contrast, the expression level of *H1foo* drastically decreased at the two-cell stage and continued to decrease until the blastocyst stage at which time the RPKM value was almost 0. I also examined the changes in the expression levels of linker histone variants during preimplantation development by real-time PCR and obtained the results which were consistent with RNA-seq data (Fig. 1-2): *H1foo* was the only H1 variant which was highly expressed at the one-cell stage but not at the other preimplantation stages. Immunocytochemistry using the antibody against H1FOO showed that H1FOO was localized in pronuclei only at the one-cell stage during preimplantation development (Fig. 1-3A). On the other hand, the level of the nuclear deposition of H1D, a somatic linker histone variant, was low at the one-cell stage and increased drastically at the two-cell stage (Fig. 1-3B). It has been shown that H1FOO has less ability to condense chromatin than other linker histone variants (Kunitomi et al., 2016). Therefore, I recognized H1foo as a potential candidate for the linker histone involved in the formation of the loosened chromatin structure at the one-cell stage.

Effect of H1foo KD on the loosened chromatin structure in one-cell stage embryos

To examine whether H1foo is involved in the loosened chromatin structure of one-cell stage embryos, FRAP analysis was performed in H1foo knockdown (KD) embryos. First, I confirmed that small interfering RNA (siRNA) effectively decreased the H1FOO level so that no H1FOO signal was detected in the pronuclei of one-cell-stage embryos (Fig. 1-4A). The FRAP results showed that the mobile fraction in the male pronucleus was significantly lower in H1foo KD embryos than control ones (Fig. 1-4B), which suggests that H1FOO is necessary for the formation of loosened chromatin structure. In female pronuclei, the mobile fraction was slightly lower in H1foo KD embryos than in control ones but the difference was not significant.

Effects of H1foo KD on DNA replication and cleavage of one-cell stage embryos

I analyzed the effects of H1foo KD on early preimplantation development. The timing of cleavage into two-cell stage embryos was delayed in H1foo KD embryos (Fig. 1-5A), although these embryos developed to the blastocyst stage at a similar rate as controls (Fig. 1-5B). It is possible that this delay in cleavage was caused by a delay in the completion of DNA synthesis at the one-cell stage, because H1foo KD induced the change in chromatin structure (Fig. 1-4B) and chromatin structure is involved in the timing of DNA replication (O'Keefe et al., 1992). Therefore, I investigated the timing of DNA replication in H1foo KD embryos using 5-bromo-2'-deoxyuridine (BrdU) assay. It was shown in somatic cells that DNA replication occurs earlier in euchromatin and later in heterochromatin (O'Keefe et al., 1992). In the embryos, DNA replication occurred in both male and female nucleoplasm, where euchromatin is localized, at 8 hours post insemination (hpi) (Fig. 1-6A), and then DNA replication in the male pronucleus was

completed, whereas it still continued at the perinucleolar region of the female pronucleus, where pericentromeric heterochromatin is localized, at 10 hpi (Fig. 1-6A). Finally, DNA replication is completed in the female pronuclei at 12 hpi. Since the completion of DNA replication is later in the female pronucleus than the male one, the timing of completion of DNA replication in the whole genome is determined by that in the heterochromatin region of the female pronucleus. However, in H1foo KD embryos, BrdU assay showed that the timing of DNA replication in male pronucleus was delayed in nucleoplasm and perinucleolar region at 10, 12 hpi, while the timing of DNA replication in female pronucleus was delay only in perinucleolar region, at 12 hpi. The delay was more evident in the male pronucleus than in the female pronucleus (Fig. 1-6A): the percentage of embryos that had not completed DNA replication at perinucleolar region, i.e., the embryos incorporating BrdU, was higher in H1foo KD embryos than in controls at 10 hpi in the male pronucleus and at 12 hpi in both male and female pronuclei (Figure. 1-6B).

Effects of overexpressing linker histone variants on chromatin structure and the development in one- and two-cell stage embryos

The results of immunocytochemistry showed that H1FOO was localized in the pronuclei at the one-cell stage and this nuclear deposition decreased dramatically at the two-cell stage (Fig. 1-3A). On the contrary, a somatic linker histone variant, H1D, was at a low level at the one-cell stage and increased at the two-cell stage (Fig. 1-3B). To evaluate the significance of a high and low level of H1FOO at the one- and two cell stages, respectively, in the regulation of chromatin structure, I overexpressed H1d and H1foo in one- and two-cell stage embryos, respectively. Since H1FOO and H1D has a

weak and strong ability, respectively, to condense chromatin, the high and low level of H1FOO and H1D may be involved in the loosened chromatin structure in one-cell stage embryos. To address this hypothesis, I overexpressed H1d by microinjecting complementary RNA (cRNA) encoding H1d in one-cell stage embryos. In this experiment, H1a was used as a control, because H1a is expressed all through the preimplantation development and has a weak ability to condense chromatin when compared to H1D. The results showed that the embryos overexpressing H1d had abnormally condensed pronuclei, although those of H1a did not show any abnormal feature (Fig. 1-7). Furthermore, H1d overexpressing embryos were arrested at one- or two-cell stage (Fig. 1-8). These results suggest that the low expression level of somatic linker histone variants at the one-cell stage is involved in the loosened chromatin structure and important for the preimplantation development.

Next, I examined the effect of overexpression of H1foo in the chromatin structure of two-cell stage embryos to examine whether or not the reduction of H1foo is associated with the decrease in the looseness of chromatin structure at the two-cell stage. The microinjection of cRNA encoding H1foo and H1c, which is ubiquitously expressed in somatic cells and was used as a control for H1foo, caused the ectopic deposition of H1FOO and H1C, respectively, in the nuclei of the two-cell stage embryos (Fig. 1-9A). FRAP analyses using enhanced green fluorescent protein (eGFP)-H2B were conducted to examine the looseness of the chromatin structure in these embryos. In agreement with a previous report (Ooga et al., 2016), the looseness decreased during the one- and two-cell stages in the control embryos without injecting cRNA of linker histone (Fig. 1-9B). However, the degree of this decrease was reduced by the overexpression of H1foo (Fig. 1-9B), whereas it was not changed by the overexpression of H1c. Therefore,

it seems that the reduction in H1foo is involved in the decrease in chromatin looseness during in the two-cell stage. The embryos overexpressing H1foo at the two-cell stage normally developed until the blastocyst stage (Fig. 1-10).

DISCUSSION

In this chapter, I have shown that H1foo is highly expressed and involved in the loosened chromatin structure at the one-cell stage. Furthermore, I showed that H1foo is necessary for the fair progression of DNA replication. On the other hand, somatic linker histone variants are expressed at a low level in one-cell stage embryos and this low expression is involved in the loosened chromatin structure and required for the preimplantation development.

RNA-seq data showed the expression pattern of all linker histone variants during preimplantation development. *H1foo* is highly expressed and somatic linker histone variants, especially *H1b*, *c*, *d*, *e,* are expressed at a low level in one-cell stage embryos (Fig. 1-1). *H1foo* expression is regulated by NOBOX, an oocyte-specific transcription factor, and *H1foo* is transcribed during oocyte growth (Rajkovic et al., 2004). Although *H1foo* is not transcribed after fertilization (Abe et al., 2015), H1foo mRNA remains abundant in one-cell stage embryos. On the other hand, somatic linker histone variants are expressed in a DNA replication-dependent manner and DNA replication does not occur during oocyte growth and maturation (Pan et al., 2016). Therefore, somatic linker histone variants are expressed at a low level in one-cell stage embryos. The differences of the mechanism regulating transcription among H1foo and somatic linker histone variants may contribute to the difference in the expression level among these variants in one-cell stage embryos.

Although several reports have shown that chromatin structure is loosened at the one-cell stage and becomes tighter at the two-cell stage (Ahmed et al., 2010; Majumder et al., 1993; Ooga et al., 2016), the mechanism regulating this change in chromatin structure remains to be elucidated. My results suggest that H1foo is involved in the

formation of the loose chromatin structure at the one-cell stage and that the reduction in its nuclear deposition causes the decrease in chromatin looseness at the two-cell stage (Fig. 1-4, 1-9). The C-terminal domain of H1FOO seems to be responsible for forming the loosed chromatin structure. Linker histones have several domains and their C-terminal domains contain positively charged amino acids which are involved in the formation of condensed chromatin structure (Clausell et al., 2009; Lu et al., 2004). When the C-terminal domain of linker histone variant H1E, which has a strong ability to condense chromatin, was substituted with the C-terminal domain of linker histone H1C with a relatively weak ability to condense chromatin, the ability to condense the chromatin was reduced (Clausell et al., 2009). As H1FOO has more negatively charged amino acids (aspartic and glutamic acids) in its C-terminal tail domain than the other variants, it is thought to have a weaker ability to condense the chromatin structure than others (Bednar et al., 2016). Therefore, the abundant deposition of H1FOO with a weak ability to condense the chromatin is a possible reason why chromatin structure is loosened in the pronuclei of one-cell stage embryos. It is likely that in H1foo KD embryos, other variants with a stronger ability to condense the chromatin than H1foo would be incorporated into the chromatin, so that the chromatin structure became tighter. At the two-cell stage, nuclear deposition of H1FOO decreases, while somatic linker histone variants are increasingly incorporated into chromatin (Izzo et al., 2017; Tanaka et al., 2001; Fig. 1-3B), which suggests that the change in the nuclear deposition of H1 variants regulates chromatin structure during this period. When I overexpressed H1foo in two-cell stage embryos, the overexpression induced the loosened chromatin structure, not so much as that of one-cell stage embryos (Fig. 1-9). It is thought that this difference between H1foo overexpression two-cell stage embryos and one-cell stage embryos is

involved in the H3.1/3.2 (see Chapter 2).

In the H1foo KD embryos, the timing of the completion of DNA replication was delayed both in the nucleoplasm and at the perinucleolar region in the male pronucleus, but only at the perinucleolar region in the female one (Fig. 1-6). FRAP analysis revealed that H1foo KD induced the tightened chromatin in the male pronucleus, but not the female one (Fig. 1-4). Since it has been known that the chromatin structure is associated with the timing of DNA replication (O'Keefe et al., 1992), H1foo KD caused the tightened chromatin structure, leading to the delay in DNA replication in the male pronucleus. In this context, the overexpression of H1d induced the abnormally condensed chromatin (Fig. 1-7). It is likely that abnormally condensed chromatin by this overexpression induced the timing of the delay in DNA replication and lead to developmental arrest at one- or two-cell stage (Fig. 1-8).

The role of the abundant deposition of H1FOO in the pronuclei of one-cell stage embryos in the reproduction is unclear. Although H1foo KD caused the delay in the cleavage into two-cell stage, it did not affect the rate of development to the blastocyst stage (Fig. 1-5). The Mouse Genome Informatics database showed that H1foo-Knockout (KO) female mice are fertile, but that their litter sizes have not been analyzed (http:// www.informatics.jax.org/reference/J:101326). Therefore, the effects of H1foo KO on the reproduction of female mice has not been clarified in detail. Although H1foo KD did not have significant effect on the percentage of embryos which had developed into the blastocyst stage (Fig. 1-5B), the possibility could not be excluded that the timing of the development to the blastocyst stage would be delayed in H1foo KD embryos. Indeed, although H1foo KD delayed the cleavage from one- to two-cell stage embryos, it did not have significant effect on the percentages of the embryos

which finally developed to the two-cell stage at 24 hpi (Fig. 1-5A). Therefore, the delay in the timing of the cleavage from one-cell to two-cell stage embryos by H1foo KD would be maintained or amplified at the timing of the development to blastocysts, which might cause the reduction of the offspring number. Furthermore, some experiments showed that linker histone variants compensated for each other and that knocking out a single H1 variant did not cause lethality (Fan et al., 2001; Rabini et al., 2000). Some linker histone variants (H1a, H1f0, and H1fx) are highly expressed in one-cell stage embryos (Fig. 1-1). H1A has a less ability to condense the chromatin structure than other somatic linker variants (Clausell et al., 2009; Khadake and Rao, 1995). Therefore, it is possible that H1a compensates for the function of H1foo in the regulation of chromatin structure, so that H1foo KD had a little effect on preimplantation development to the blastocyst stage.

Figure. 1-1.

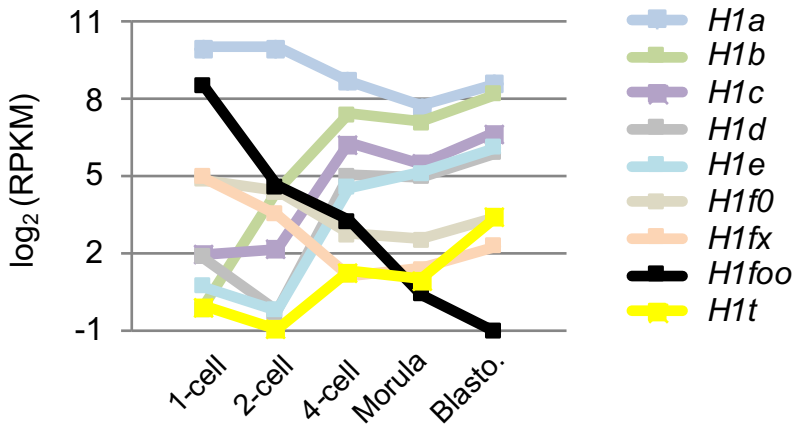

Figure. 1-1. Expression of linker histone variants during preimplantation embryos.

Changes in the expression of linker histone variants during preimplantation development. The expression patterns of all identified linker histone variants were analyzed using RNA- sequencing data (Abe et al., 2015). *H1t2* and *Hils* are not shown in the figure because their RPKM values were 0 throughout preimplantation development.

Figure. 1-2.

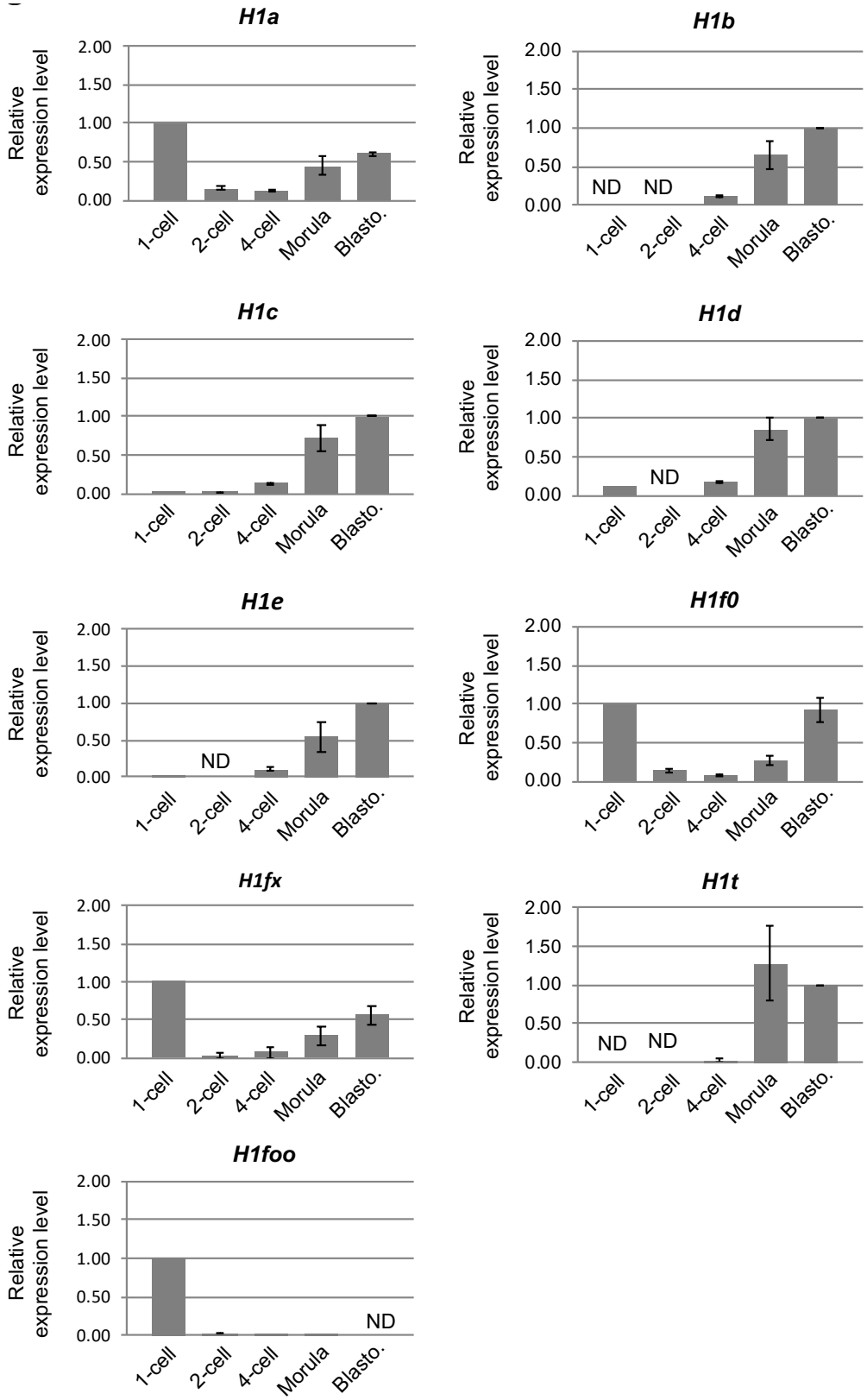
H1a
H1b
H1c
H1d
H1e
H1f0
H1fx
H1t
H1foo
Relative expression level
2.00
1.50
1.00
0.50
0.00
1-cell
2-cell
4-cell
Morula
Blasto.
ND

Figure. 1-2. Analysis for the expression of linker histone variants in the preimplantation embryos by using reverse transcription real-time PCR. Twenty embryos at the one-, two-, and four-cell, morula, and blastocyst stages were collected at 13, 32, 48, 72, and 96 hours post insemination (hpi), respectively. The value of one-cell (*H1a, H1f0, H1fx, H1foo*) or blastocyst (*H1b, H1c, H1d, H1e, H1t*) stages was set to 1 and relative values were calculated. The gene expression of *H1t2* and *Hils1* were not examined because their RPKM values were 0 throughout preimplantation development. Three independent experiments were conducted for each variant. Error bars represent S.E.M. ND = Not Detected

Figure. 1-3.

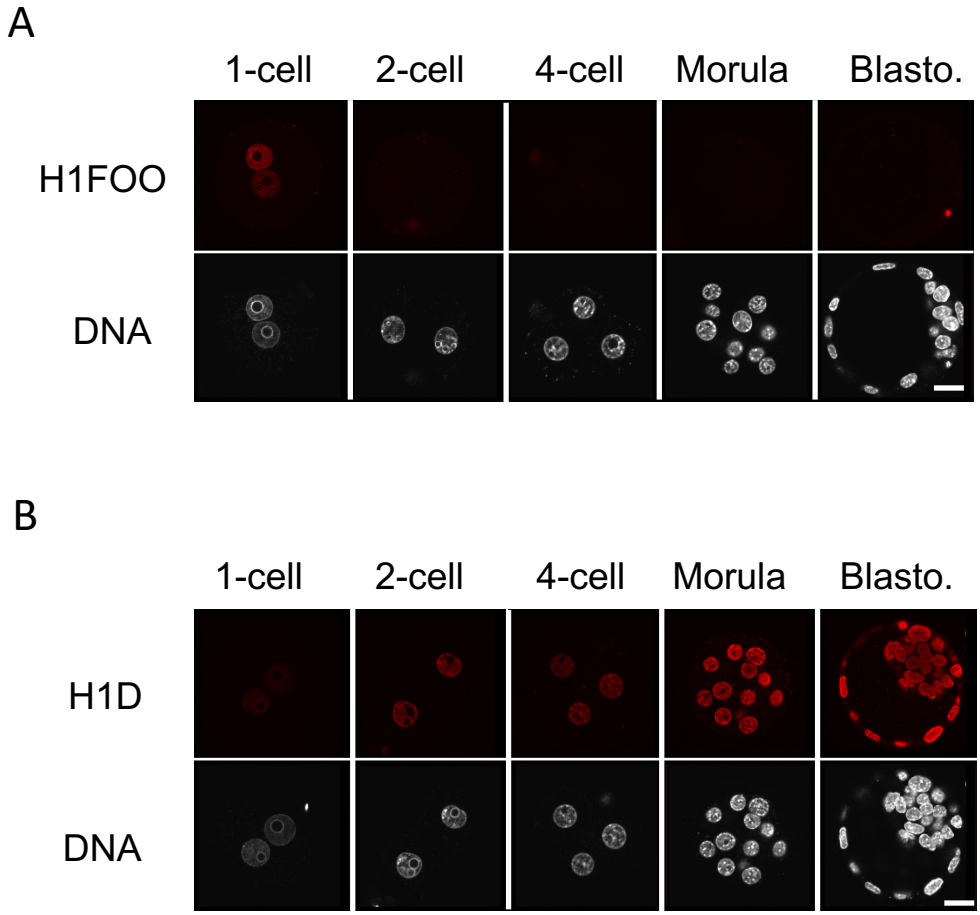

Figure. 1-3. Localization of linker histone variants in the preimplantation embryos.

Nuclear localization of H1FOO (A) and H1D (B) in the preimplantation embryos. Embryos at the one-, two-, and four-cell, morula, and blastocyst stages were collected at 11, 28, 42, 72, and 96 hpi, respectively, and were immunostained with the antibodies against H1FOO (A) and H1D (B). DNA was stained with DAPI. Three independent experiments were performed and similar results were obtained for each experiment. Representative images are shown. Scale bar = 20 μm.

Figure. 1-4.

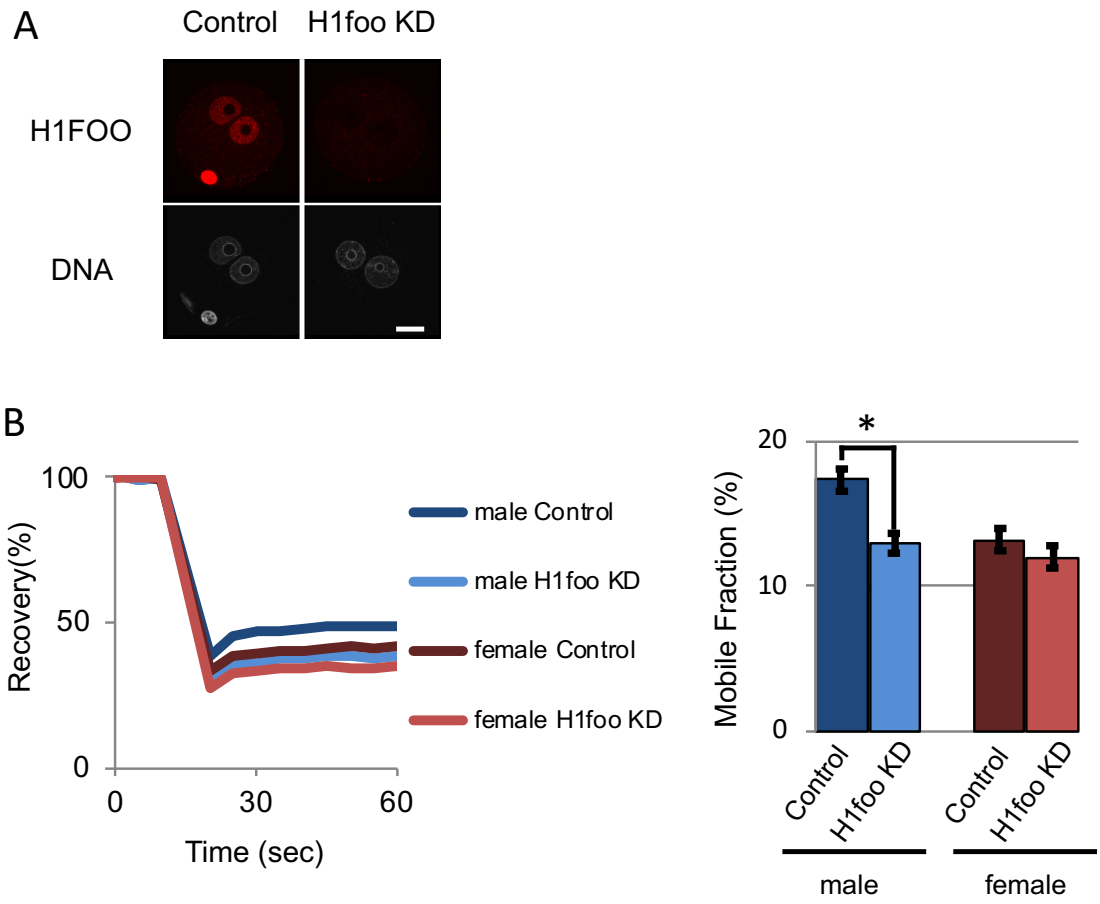

Figure. 1-4. Effects of H1foo-knockdown (KD) on looseness of the chromatin structure in one-cell stage embryos.

(A) Immunofluorescence images of H1foo KD embryos stained with anti-H1FOO antibody. Fully grown oocytes were injected with siRNA against H1foo (H1foo KD) or control siRNA (Control), matured, and fertilized *in vitro*. They were collected at 11 hpi and subjected to immunocytochemistry. Scale bar = 20 µm. (B) Analyses of the looseness of the chromatin structure in H1foo KD embryos. H1foo siRNA (H1foo KD) or control siRNA (Control) were co-injected with cRNA encoding eGFP-tagged H2B into fully grown oocytes. They were matured and fertilized *in vitro* and then subjected to FRAP analyses at 10–12 hpi. Recovery curve and mobile fraction of male and female pronuclei in H1foo KD and control embryos are shown on the left and right, respectively. Four independent experiments were conducted for each group to analyze 29 or more embryos. Error bars indicate S.E. An asterisk indicates a significant difference ($P < 0.05$; by Student's t-test).

Figure. 1-5.

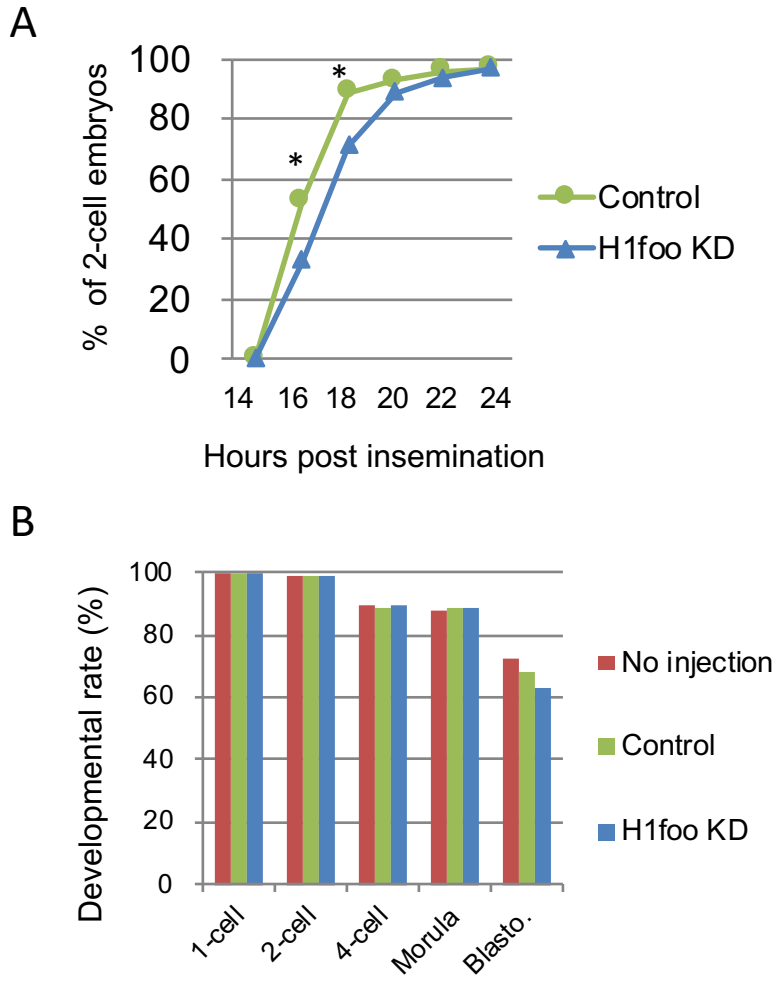

Figure. 1-5. Effect of H1foo KD on preimplantation development.
Fully grown oocytes were injected with siRNA against H1foo (H1foo KD) or control siRNA (Control), matured, and fertilized *in vitro*. (A) The percentage of embryos that had cleaved into two-cell stage embryos. At each time point post-insemination, the percentage of embryos that had cleaved into the two-cell stage embryos was examined. Asterisks indicate significant difference from Control groups ($P < 0.05$; by $\chi 2$ test or Fisher's exact test). Four independent experiments were conducted for each group to analyze a total of 54 or more embryos. (B) H1foo KD, Control, No injection embryos were observed at 12, 28, 45, 72, 96 hpi to evaluate the development to the one-, two- and four-cell, morula, and blastocyst stages, respectively. Six independent experiments were conducted for each group to analyze a total of 142 or more embryos.

Figure. 1-6.

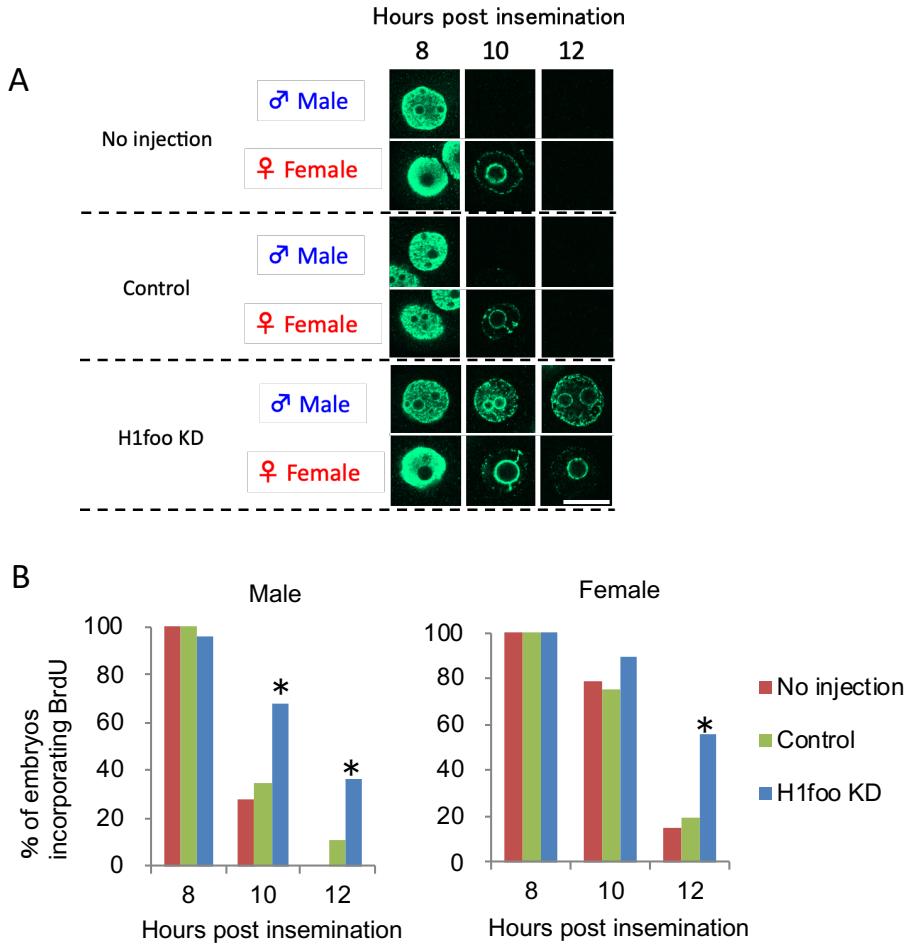

Figure. 1-6. Effects of H1foo KD on DNA replication in one-cell stage embryos. Fully grown oocytes were injected with siRNA against H1foo (H1foo KD) or control siRNA (Control), matured, and fertilized *in vitro*. (A) At each time point post insemination (8, 10, 12 hpi), the occurrence of DNA replication in the nuclei was examined by detecting 5-bromo-2'-deoxyuridine (BrdU) incorporation. Noninjected embryos (No injection) served as a control. Scale bar = 20 µm. (B) The percentage of embryos in which BrdU incorporation was detected at the perinucleolar region in the male and female pronuclei. At each time point post insemination, the occurrence of DNA replication in the peripheral regions of male and female pronuclei was examined by detecting BrdU incorporation. Asterisks indicate significant difference from both of No injection and Control groups ($P < 0.05$; by $\chi 2$ test or Fisher's exact test). Four independent experiments were conducted for each group to analyze a total of 27 or more embryos.

Figure. 1-7.

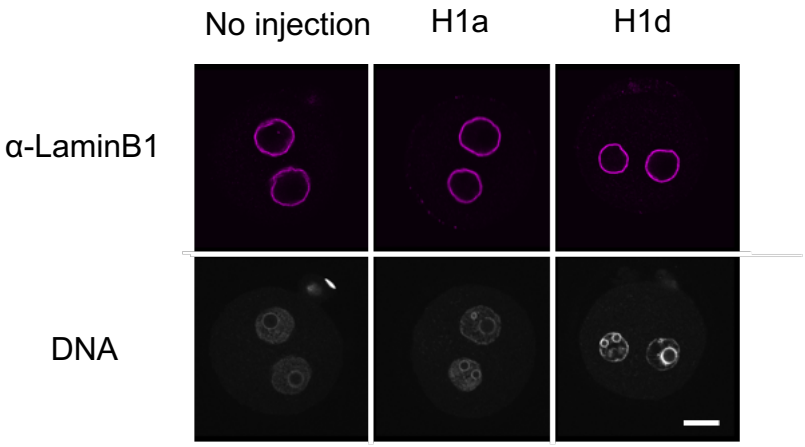

Figure. 1-7. Effect of the overexpression of linker histone variants on chromatin structure in one-cell stage embryos.
Mll stage oocytes were injected with cRNA encoding linker histone variant H1a or H1d, or none (No injection), and fertilized *in vitro*. The embryos were collected at 11 hpi and stained with DAPI to evaluate the condensation of chromatin. These embryos were double stained with anti-LaminB1 (α-LaminB1) antibody to clarify the outline of nuclei. Scale bar = 20 μm. At least three independent experiments were performed in each experimental group, and more than 17 embryos were observed in total.

Figure. 1-8.

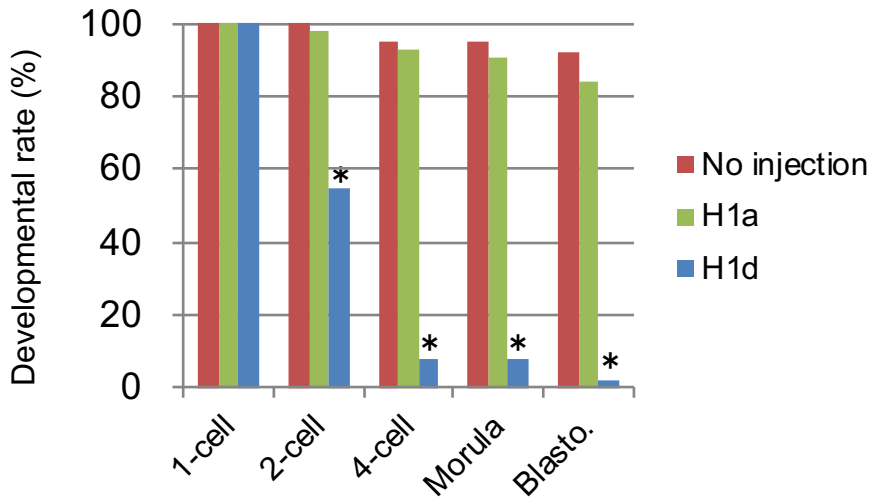

Figure. 1-8. Effect of the overexpression of linker histone variants on the preimplantation development.

Mll stage oocytes were injected with cRNA of linker histone variants H1a or H1d, or none (No injection), and fertilized *in vitro*. These embryos were observed at 12, 24, 45, 72, 96 hpi to evaluate the development to the one-, two- and four-cell, morula, and blastocyst stages, respectively. Asterisks indicate significant difference from both of No injection and H1a groups ($P < 0.05$; by $\chi 2$ test or Fisher's exact test). Three independent experiments were conducted for each group to analyze a total of 56 or more embryos.

Figure. 1-9.

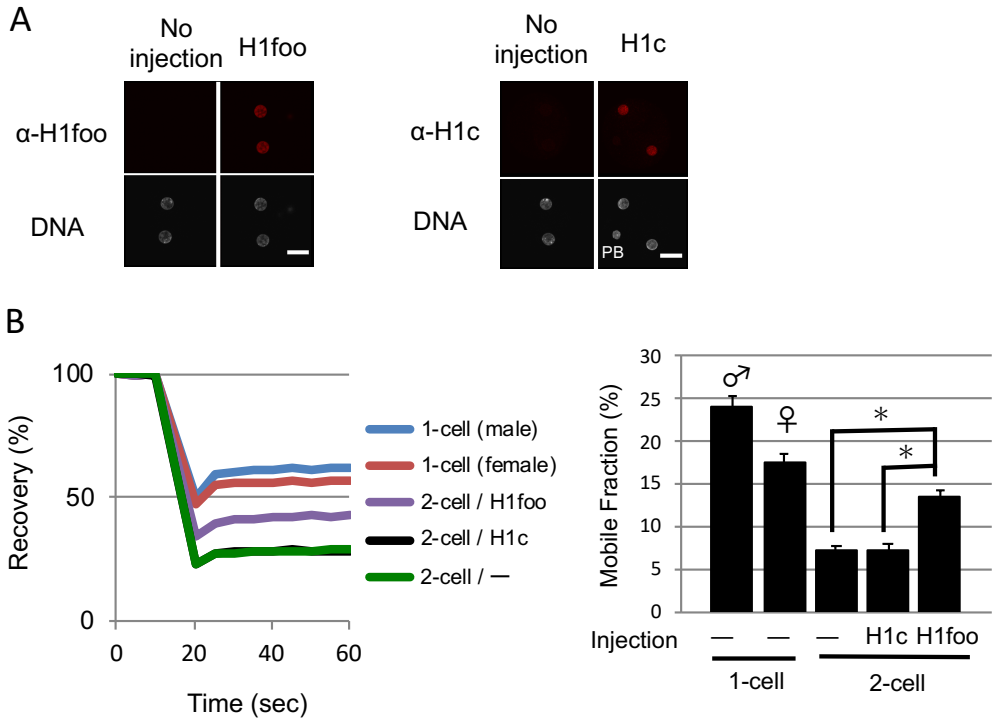

Figure. 1-9. Effects of the overexpression of H1foo on chromatin structure in two-cell stage embryos.

Embryos were injected with cRNA encoding H1foo or H1c at the one-cell stage. (A) Immunofluorescence images of two-cell stage embryos overexpressing H1foo and H1c at 32 hpi. No injected two-cell embryos (No injection) were examined as controls. The embryos were stained with the antibodies against H1FOO (α-H1foo) or H1C (α-H1c). Scale bar = 20 µm. PB = Polar body (B) Analyses of chromatin structure by FRAP in H1foo-overexpressing embryos. cRNA encoding eGFP-H2B was co-injected with cRNA of H1foo, H1c, or none (-) into one-cell stage embryos at 4–7 hpi. FRAP analyses were performed in one-cell embryos at 10–12 hpi and in two-cell embryos at 28–30 hpi. The diagrams of the recovery curve and mobile fraction are shown on the left and right respectively. ♂ and ♀ represent male and female pronuclei, respectively. The experiments were carried out four times in two-cell embryos and twice in one-cell embryos, and similar results were obtained for each experiment. More than 26 embryos were analyzed in each group. Error bars indicate S.E. An asterisk indicates a significant difference ($P < 0.05$; by Student's t-test).

Figure. 1-10.

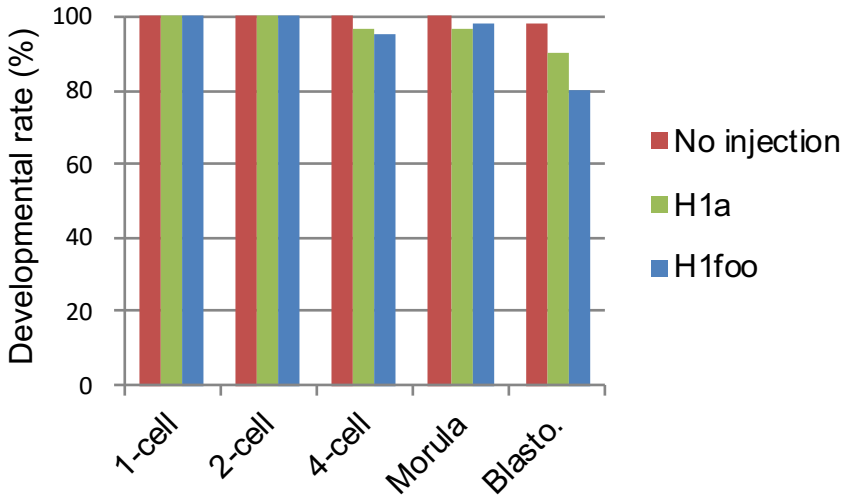

Figure. 1-10. Effects of H1foo overexpression on preimplantation development. Embryos were injected with cRNA of linker histone variants H1foo, H1a, or none (No injection) at the one-cell stage. These embryos were observed at 12, 24, 45, 72, 96 hpi to evaluate the development to the one-, two- and four-cell, morula, and blastocyst stages, respectively. Three independent experiments were conducted for each group to analyze a total of 60 or more embryos.

CHAPTER 2

The involvement of core histone H3 variants in the change of chromatin structure during early preimplantation development

INTRODUCTION

Various studies showed that core histone H3 variants play important roles in the regulation of chromatin structure. There are three major non-centromeric histone H3 variants in mammals, H3.1, H3.2 and H3.3. It is known that H3.3 is expressed throughout the cell cycle and incorporated into the chromatin in a DNA replication-independent manner by the histone chaperone called HIRA. H3.3 is preferentially modified with H3K9ac, H3K14ac and H3K79me2 which abundantly deposited in the upstream regions of active genes (Goldberg et al., 2010; Jin et al., 2009; Yukawa et al., 2014) and are associated with active transcription (Hake et al., 2006; Lawrence et al., 2016), suggesting that H3.3 is involved in the formation of loosened chromatin structure. On the other hand, H3.1 and H3.2 are expressed and incorporated into chromatin in a DNA replication-dependent manner by the histone chaperone called CAF-1. H3.1 and H3.2 have the modifications associated with the transcriptional repression, *e.g.* H3K9me2 in H3.1 and H3K27me2/3 in H3.2 (Hake et al., 2006; Hake and Allis, 2006), suggesting that H3.1/3.2 are involved in the formation of tightened chromatin structure.

The immunocytochemistry using the antibody against H3.3 showed that it is abundantly deposited in the nuclei throughout the preimplantation development. However, the antibody which detects H3.1 and H3.2 (cannot discriminate them) revealed that the level of H3.1/3.2 are very low in the nuclei of the one-cell stage embryos and that they are greatly increased at the late two-cell stage. At the one-cell stage, H3.1/3.2 showed different localization patterns at the perinucleolar region between the male and female pronuclei. The levels of H3.1/3.2 are higher in the female pronucleus than the male one (Kawamura, 2016). At the late two-cell stage, H3.1/3.2 are

abundant at chromocenters, where chromatin is condensed (Kawamura, 2016).

Several studies have suggested that H3 variants are involved in the regulation of chromatin structure in preimplantation embryos. The KD of H3.3 using siRNA caused the failure in the formation of de novo nucleosome in the male pronucleus of one-cell stage embryos (Inoue et al., 2014). The KD of H3.3 using morpholino at the two-cell stage reduced the levels of H4K16ac and H3K36me2 which are associated with loosened chromatin and active transcription and induced highly condensed chromatin and developmental arrest at the morula stage (Lin et al., 2013). On the other hand, H3.1/3.2 overexpression led to the accumulation of H3.1/3.2 at the perinucleolar region of the male pronuclei, delayed DNA replication at this region and caused developmental arrest (Kawamura, 2016). These results suggested that H3 variants are involved in the regulation of chromatin structure in the preimplantation embryos. However, the role of H3 variants in the drastic changes of chromatin structure during the one- and two-cell stages has not been clarified. In this chapter, to understand the mechanism underlying these phenomena, I conducted H3.1/3.2 KD specifically at the two-cell stage and analyzed the consequences on the chromatin structure.

RESULTS

H1foo is involved in the nuclear deposition of H3.1/3.2

It was shown that the level of H3.1/3.2 was very low in the pronuclei at the one-cell stage, increased during the two-cell stage and then remained high during the preimplantation development (Kawamura, 2016). I have confirmed these results in one- and two-cell stage embryos (Fig. 2-1). It was also shown that although the level of H3.1/3.2 was low in one-cell stage embryos, it was relatively low in the perinucleolar region in the male pronucleus but not the female one and that the increase in the H3.1/3.2 level by their overexpression caused a delay in DNA replication in the perinucleolar regions of the male pronucleus (Kamamura, 2016). Since this pattern of the delay in DNA replication is reminiscent of the effect of H1foo KD (Fig. 1-6A, B), I hypothesized that H1foo KD would increase the level of H3.1/3.2 in the perinucleolar region, leading to the delay in DNA replication. To address this issue, I examined the effects of H1foo KD on nuclear deposition of H3 variants by immunocytochemistry. Nuclear deposition of H3.1/H3.2 but not H3.3 increased by H1foo KD in male and female pronuclei of one-cell stage embryos (Fig. 2-2A, B). Furthermore, in the perinucleolar region, the effects of H1foo KD was evident only in the male pronucleus. H3.1/3.2 was detected in half of the H1foo KD embryos, whereas it was detected only in 10% of control embryos (Fig. 2-2C, D). However, H3.1/3.2 in female pronucleus was detected in 60% of both the H1foo KD and control embryos. Taken together, these results suggest that H1foo repress the deposition of H3.1/3.2 in the perinucleolar region of the male pronucleus at the one-cell stage.

As described above, the level of H3.1/3.2 increases at the two-cell stage (Kawamura, 2016; Fig. 2-1), while nuclear localization of H1FOO decreased

dramatically at the two-cell stage (Fig. 1-3A). To examine whether this reduction of H1FOO causes the increase of H3.1/3.2, I overexpressed H1foo and examined the nuclear deposition of H3.1/3.2 in two-cell stage embryos. Unexpectedly, the level of nuclear deposition of H3.1/3.2 in H1foo overexpressing two-cell stage embryos was not different from the embryos without overexpression or those overexpressing H1c (Fig. 2-3), suggesting that H1foo suppresses the nuclear deposition of H3.1/3.2 at the one-cell stage but not two-cell stage.

Effects of H3.1/3.2 KD on the chromatin structure in two-cell stage embryos.

The level of H3.1/3.2 increased between the early and late two-cell stages (Kawamura, 2016; Fig. 2-1) and the chromatin structure was tightened during this period (Ooga et al., 2016). Therefore, I examined whether the increase of H3.1/3.2 is involved in the tightened chromatin structure in the late two-cell stage embryos. I knocked down H3.1/3.2 by microinjecting siRNA with the common sequence between H3.1 and H3.2 into fully grown oocytes. These oocytes were allowed to mature and fertilize *in vitro*. At the one-cell stage, the level of H3.1/3.2 in both male and female pronuclei of H3.1/3.2 KD embryos was not different from that of control embryos (Fig. 2-4A, B). However, at the two-cell stage, the level of H3.1/3.2 was much lower in H3.1/3.2 KD embryos than the control ones, while the level of H3.3 was increased in H3.1/3.2 KD embryos (Fig. 2-4C, D). FRAP analyses revealed that the chromatin looseness was maintained in H3.1/3.2 KD embryos during the one- and two-cell stages (Fig. 2-5). These results suggest that the increase of H3.1/3.2 is involved in the formation of tightened chromatin in the late two-cell stage embryos.

Effects of H3.1/3.2 KD on the modifications of histone H3 in two-cell stage embryos

H3 variants are preferentially modified with specific histone modifications (Hake et al., 2006). Among these modifications, it has been suggested that H3K9me2 and H3K4me3 play important roles in the formation of tight and loosened chromatin structure. H3.1/3.2 are more likely to acquire H3K9me2 and H3.3 is preferentially modified with H3K4me3. Therefore, I examined these modifications in H3.1/3.2 KD two-cell stage embryos. Immunocytochemistry showed that the level of H3K9me2 decreased in H3.1/3.2 KD embryos (Fig. 2-6A, B). On the other hands, the level of H3K4me3 increased slightly but not significantly (Fig. 2-6C, D). H3.1/3.2 KD thus affected the level of H3K9me2 in two-cell stage embryos. Since H3K9me2 is associated with heterochromatin, it may not be efficiently formed in H3.1/3.2 KD embryos.

Effects of H3.1/3.2 KD on the timing of DNA replication in two-cell stage embryos

Since H3.1/3.2 KD induced the change in chromatin structure in the late two-cell stage (Fig. 2-5) and it is known that chromatin structure is involved in the timing of DNA replication (O'Keefe et al., 1992), I examined the timing of DNA replication in H3.1/3.2 KD embryos by using a BrdU assay. No change was observed in the timing of DNA replication by H3.1/3.2 KD at the two-cell stage (Fig. 2-7).

Effects of H3.1/3.2 KD on the change in gene expression pattern during the early and late two-cell stages.

To investigate the involvement of the nuclear deposition of H3.1/3.2 in the changes of gene expression pattern from minor to major ZGA, I examined the effect of H3.1/3.2 KD in the expression of some genes which are transcribed during minor ZGA (at the

one- and early two-cell stages) but repressed during major ZGA (at the late two-cell stage). *Eif1a, Zfp352,* and *Zscan4d* are transcribed at the one-cell stage and their expression level increased at the early two-cell stage, and then decreased at the late two-cell stage (Abe et al., 2015; Fig. 2-8A). However, the degree of this decrease between the early and late two-cell stages was reduced by H3.1/3.2 KD (Fig. 2-8A). In addition, it has been shown that *LINE-1* is also actively transcribed at the one- and early two-cell stages and then suppressed at the late two-cell stage (Jachowicz et al., 2017; Fig. 2-8A). This suppression did not occur sufficiently in H3.1/3.2 KD embryos. On the other hands, *Wsb1, Timd2* and *Sord*, whose expression level was very low at the one- and early two-cell stages and then abruptly increased at the late two-cell stage, remained to be actively transcribed at the late two-cell stage in H3.1/3.2 KD embryos (Fig. 2-8B). These results suggest that the nuclear deposition of H3.1/3.2 is involved in the transition from minor ZGA to major one by repressing the genes which had been transcribed during minor ZGA.

Effects of H3.1/3.2 KD on preimplantation development

I observed the development of H3.1/3.2 KD embryos to examine the effects of H3.1/3.2 KD on preimplantation development. The results showed that H3.1/3.2 KD had a detrimental effect on preimplantation development after the two-cell stage (Fig. 2-9).

Effects of H3.1/3.2 KD on DNA damage and chromosomal segregation

One of the causes in developmental arrest by H3.1/3.2 KD may be a failure in the repression of *LINE-1* expression at the late two-cell stage, since it was reported that the de-repression of *LINE-1* induced DNA damage at the morula stage (Hatanaka et al.,

2015). Therefore, I examined the DNA damage in H3.1/3.2 KD embryos at the late two-cell stage. Immunocytochemistry showed that the number of foci of γH2AX, a DNA damage marker, was higher in H3.1/3.2 KD embryos than the control ones (Fig. 2-10A, B). Furthermore, micronuclei were observed in more than half of H3.1/3.2 KD embryos at the four-cell stage, whereas no micronucleus was observed in most of control embryos (Fig. 2-10C, D). These results suggest that DNA damage occurred in H3.1/3.2 KD embryos, which led to the abnormal segregation during the cleavage to cause the developmental arrest after the two-cell stage.

DISCUSSION

It was shown that the level of nuclear deposition of H3.1/3.2 was low at the one-cell stage and increased between the early and late two-cell stages (Kawamura, 2016). However, the significance and mechanism of this change has not been elucidated. In this chapter, I have shown that H1foo repressed the nuclear deposition of H3.1/3.2 in one-cell embryos and that the increase of H3.1/3.2 had the chromatin structure tightened and repressed the expression of some genes and a retrotransposon during the two-cell stage.

The nuclear deposition of H3.1/3.2 was at a low level at the one-cell stage and increased at the two-cell stage (Fig. 2-1). A previous study examined the nuclear incorporation of each Flag-tagged H3 variant in one-cell stage embryos and found that the incorporation of H3.1 and 3.2 was less active than H3.3 (Kawamura, 2016). Since the nuclear deposition of H3.1/3.2 was increased by H1foo KD at the one-cell stage (Fig. 2-2), H1foo appears to repress the incorporation of H3.1/3.2. Although the mechanism underlying this repression is unclear, it has been shown that linker histone and core histone H3 reciprocally affect their incorporation into chromatin. A previous study showed that H3.3 KD increased the nuclear deposition of somatic linker histone variants, H1A, B, C, D, and E in mouse embryos (Lin et al., 2013). In these cells, H3.1 and/or H3.2 would be incorporated into the chromatin instead of H3.3, which leads to the incorporation of those somatic linker histone variants (H1A, B, C, D, and E) into the chromatin. By contrast, knockout (KO) of three somatic linker histone variants H1c, d, and e decreases the level of H3K27me3 in embryonic stem cells (Fan et al., 2005). Although in that study, nuclear deposition of H3 variants was not analyzed in the triple KO ES cells, deposition of H3.2 was low because H3K27me3 is enriched in H3.2 (Hake

et al., 2006), which suggests that linker histone variants affect the incorporation of H3 variants into chromatin. Therefore, we hypothesized that linker histones and core histones H3 interact with each other and that there are some preferable combinations between these variants. As described above, H3.1 and/or H3.2 seem to prefer somatic linker histone variants. It is also likely that H1FOO preferentially interacts with H3.3 rather than H3.1/3.2. H3.3 and H1FOO are deposited at high levels in the pronuclei of one-cell stage embryos, thereby suppressing the incorporation of H3.1/3.2 and other linker histone variants into chromatin. At the two-cell stage, the nuclear deposition of somatic linker histone variants increased, which would contribute to the increased incorporation of their preferable H3 variants, H3.1 and H3.2.

The low level of the nuclear deposition of H3.1/3.2 in one-cell stage embryos, especially at the perinucleolar region of the male pronucleus, may be important for the formation of a unique chromatin structure at this region and the accurate proceeding of DNA replication. Although pericentromeric regions are localized at the perinucleolar regions in both of male and female pronuclei in one-cell stage embryos (Probst et al., 2007), H3.1/3.2 was localized in the perinucleolar region only in the female pronuclei but not male ones (Kawamura, 2016). Since H3.1/3.2 is involved in the formation of heterochromatin (Hake and Allis, 2006), it is possible that heterochromatin is not formed at the pericentromeric regions in the male pronucleus. This unique chromatin is likely be involved in the timing of DNA replication, because DNA replication occurs later in heterochromatin regions than euchromatin regions, and the timing of the completion of DNA replication is determined by that in heterochromatin regions (O'Keefe et al., 1992). Consistent with this hypothesis that the timing of the completion of DNA replication at the perinucleolar region is much earlier in the male pronucleus

than the female one (Aoki and Schultz, 1999; Fig. 1-6A). A previous report showed that the overexpression of H3.1 or H3.2 induced its accumulation at the perinucleolar region of male pronucleus and the delay in timing of DNA replication at this region (Kawamura, 2016). In this chapter, H1foo KD also induced the similar phenotype (Fig. 1-6, 2-2). Therefore, the delay in the completion of DNA replication in H1foo KD embryos would be mediated by the accumulation of H3.1/3.2 and heterochromatin formation at the perinucleolar regions of male pronucleus.

Pioneering studies on the regulation of ZGA showed a quarter-century ago that the change in chromatin structure is involved in a drastic alteration in the regulation of gene expression from minor to major ZGA. The chromatin structure is permissive for transcription without an enhancer at the one- and early two-cell stage when minor ZGA occurs, whereas it become repressive at the mid- to late two-cell stage during which major ZGA proceeds. Under the repressive state, a functional enhancer is required for efficient transcription to relieve the repression (Majumder et al., 1993; Nothias et al., 1995; Schultz and Worrad, 1995; Wiekowski et al., 1993). It was also shown that the second round of DNA replication was associated with this alteration in the regulation of gene expression (Christians et al., 1995; Majumder et al., 1997). However, the mechanism underlying this alteration has not been elucidated. In this chapter, I have shown that H3.1/3/2 KD caused the chromatin structure to remain loosened (Fig. 2-5). It has been reported that H3.1/H3.2 are incorporated in the transcriptionally inactive genome regions and enriched in H3K9me2/3 which are associated with the repression of gene expression and heterochromatin formation (Lawrence et al., 2017; Hake and Allis, 2006; Yukawa et al., 2014). Therefore, I suggest that the increase in H3.1/3.2 causes the chromatin structure tightened at the late two-cell stage. This hypothesis also

well explains the mechanism by which DNA replication is associated with the change in the regulation of gene expression during two-cell stage: the incorporation of H3.1/3.2 into chromatin is dependent on DNA replication.

Recent studies have shown that a low level of transcription promiscuously occurs from most of genes and even intergenic regions during minor ZGA and that this promiscuous transcription ceased and high levels of transcription from specific genes occur during major ZGA (Abe et al., 2015; Yamamoto and Aoki, 2017). This alteration in the pattern of gene expression would be caused by the change in the chromatin structure. As described above, the chromatin structure is loosened at the one-cell and the early two-cell stage, which would allow the transcription from the various genome regions without enhancers. However, at the late two-cell stage, the chromatin structure becomes tight, which is repressive for transcription. To relieve this repression, enhances are required. It seems that this enhancer-dependent transcription is more stable and efficient than the enhancer-independent one (Majumder et al., 1997; Smale and Kadonaga, 2003). During major ZGA, some genes which are required for development would be transcribed efficiently and appropriately under the regulation of enhancers, while the other genes which are not required for development, e.g. oocyte specific genes, could be repressed because of the absence of an adequate enhancer. Although the change in the chromatin structure is thus important in the progression of gene expression program and the regulation of development, its underlying mechanism has not been clarified. In this chapter, I have shown that the chromatin structure remained loose and some genes and a retrotransposon were relieved from the repression at the late two-cell stage when H3.1/3.2 had been knocked down (Fig. 2-5, 8). These results suggest that the increase of H3.1/3.2 induce the formation of tight chromatin structure,

leading to repressing the expression of some genes and retrotransposon which do not have functional enhancers at the late two-cell stage (Fig. 2-11). This hypothesis elucidates in part the mechanism regulating the transition from minor to major ZGA.

Figure. 2-1.

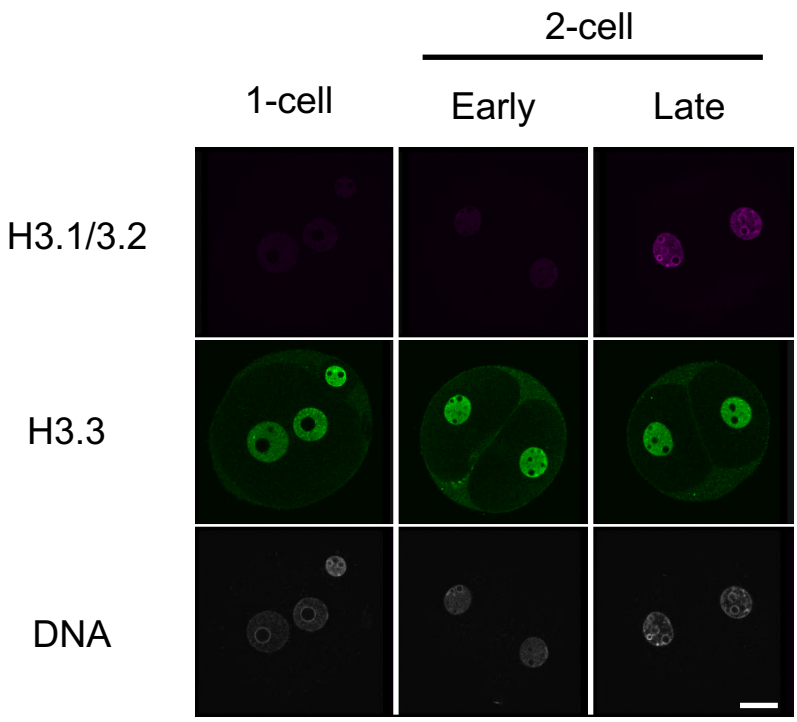

Figure. 2-1. Localization of H3 variants during one- and two-cell stage embryos. Nuclear localization of H3.1/3.2 and H3.3 during one- and two-cell stages. Embryos at the one-, early, late two-cell stages were collected at 11, 15, 28 hpi, respectively, and immunostained with anti-H3.1/3.2 and H3.3 antibodies. DNA was detected by DAPI staining. Two independent experiments were performed and representative images are shown. Scale bar = 20 μm.

Figure. 2-2.

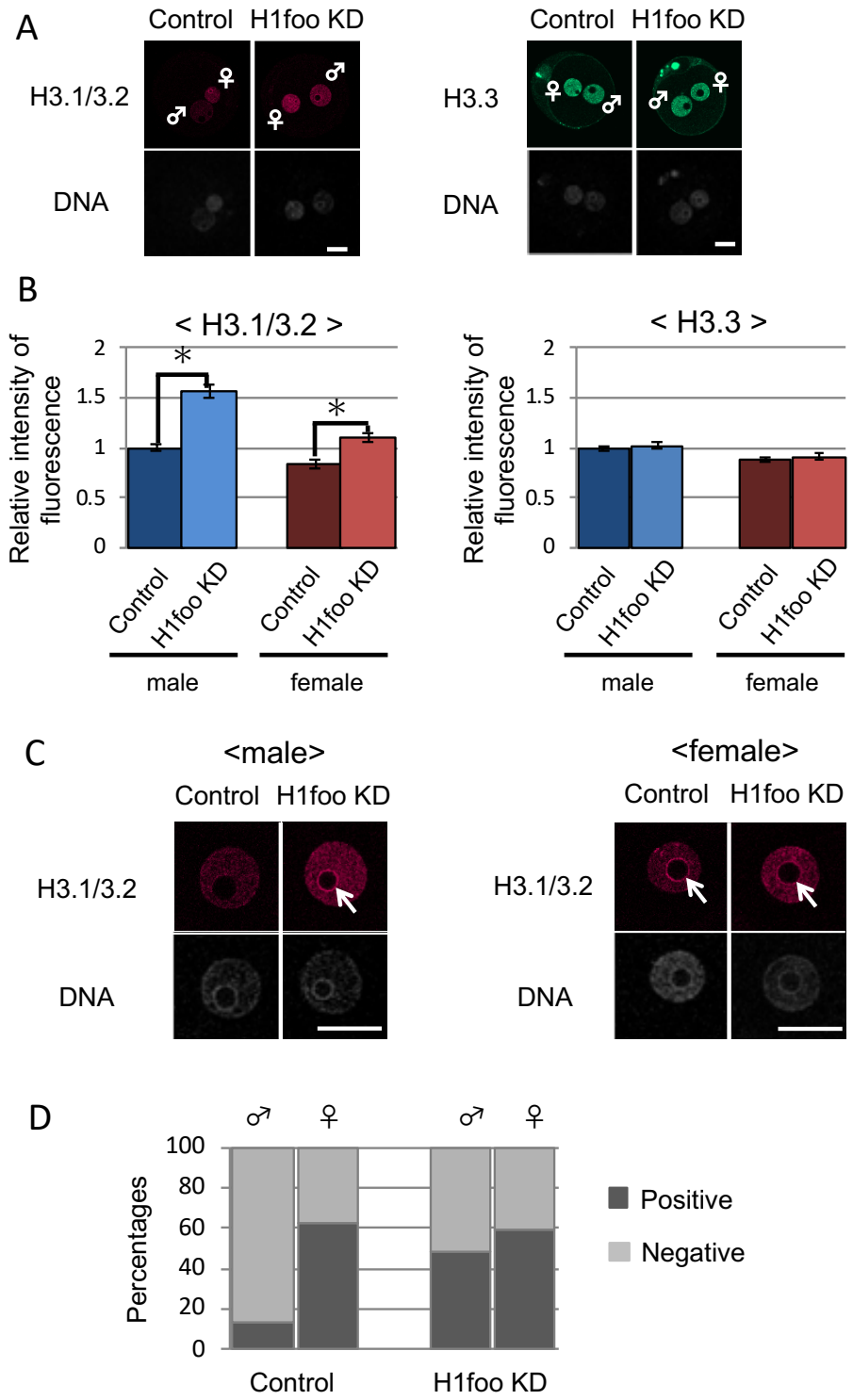
A
Control
H1foo KD
H3.1/3.2
H3.3
DNA
B
< H3.1/3.2 >
< H3.3 >
Relative intensity of fluorescence
2
1.5
1
0.5
0
*
Control
H1foo KD
male
female
C
<male>
<female>
D
Percentages
100
80
60
40
20
0
Positive
Negative

Figure. 2-2. The nuclear deposition of H3.1/3.2 variants is regulated by H1foo in one-cell stage embryos

Fully grown oocytes were injected with siRNA against H1foo (H1foo KD) or control siRNA (Control), matured, and fertilized *in vitro*. (A) Immunofluorescence images of H1foo KD and control one–cell stage embryos stained with anti- H3.1/3.2 and H3.3 antibodies at 11 hpi. Scale bar = 20 µm. (B) Quantification of the fluorescence signals in the experiments of (A). Signal intensities of H3.1/3.2 and H3.3 were corrected with those of DAPI. The value of the male pronucleus in the control embryo was 1 and relative values were calculated. (C) Enlarged immunofluorescence images of the male and female pronuclei of H1foo KD and control one-cell stage embryos stained with anti-H3.1/3.2 antibody. Scale bar = 20 µm. (D) Percentages of the male and female pronuclei in which peripronucleolar localization of H3.1/3.2 were observed (positive) or not (negative). At least four independent experiments were performed in each experimental group, and more than 14 embryos were analyzed in total. Error bars indicate S.E. Asterisks indicate a significant difference ($P < 0.05$; by Student's t-test). White arrows indicate the localization of H3.1/H3.2 at the perinucleolar region.

Figure. 2-3.

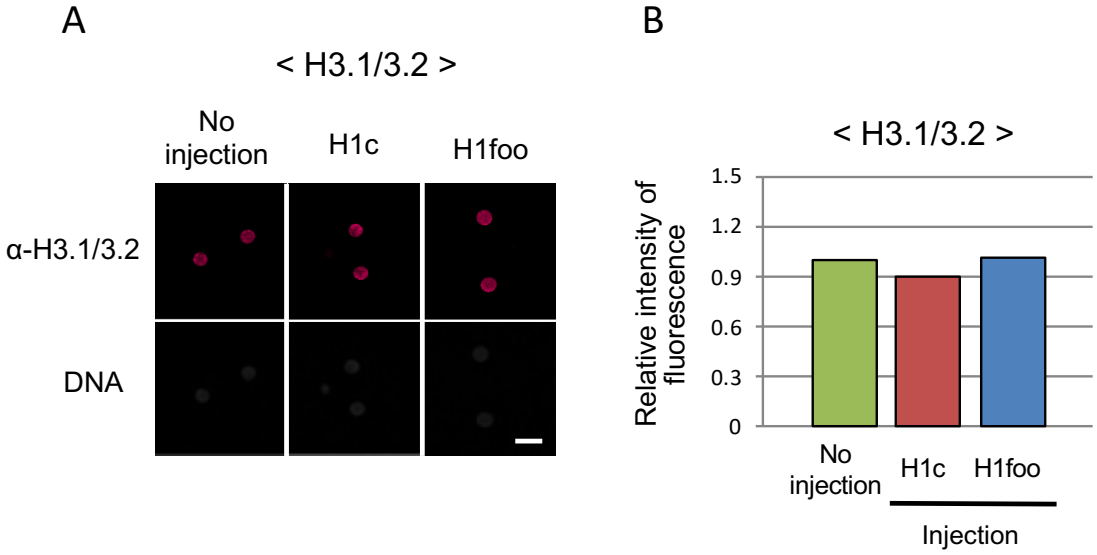

Figure. 2-3. Effects of the overexpressing H1foo on nuclear deposition of H3.1/3.2 in two-cell stage embryos.

Embryos were injected with cRNA encoding H1foo or H1c at the one-cell stage. (A) Immunofluorescence images of two-cell stage embryos overexpressing H1foo and H1c, stained with anti-H3.1/3.2 antibody (α-H3.1/3.2), at 32 hpi. Scale bar = 20 μm. (B) Quantification of the fluorescence signals in the experiments in (A). Signal intensities of H3.1/3.2 was corrected with those of DAPI. The value of the No injection embryos was set to 1 and relative values were calculated. At least two independent experiments were performed in each experimental group, and more than 31 embryos were analyzed in total.

Figure. 2-4.

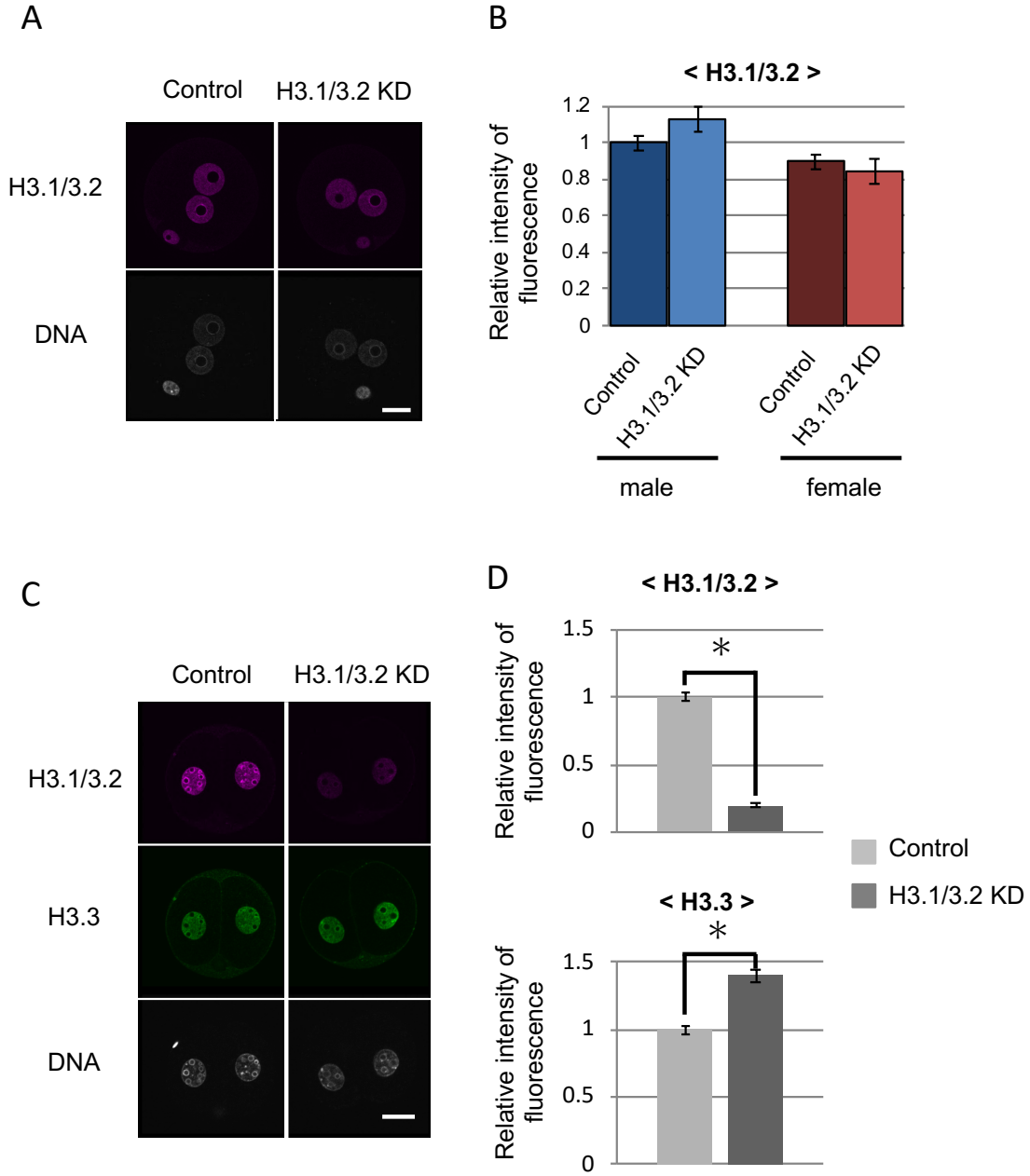
A
Control
H3.1/3.2 KD
H3.1/3.2
DNA
B
< H3.1/3.2 >
Relative intensity of fluorescence
1.2
1
0.8
0.6
0.4
0.2
0
Control
H3.1/3.2 KD
Control
H3.1/3.2 KD
male
female
C
Control
H3.1/3.2 KD
H3.1/3.2
H3.3
DNA
D
< H3.1/3.2 >
Relative intensity of fluorescence
1.5
1
0.5
0
*
< H3.3 >
Relative intensity of fluorescence
1.5
1
0.5
0
*
Control
H3.1/3.2 KD

Figure. 2-4. KD of H3.1/3.2 by siRNAs in embryos.
Fully grown oocytes were injected with siRNA against H3.1/3.2 (H3.1/3.2 KD) or control siRNA (Control), matured, and fertilized *in vitro*. (A) Immunofluorescence images of H3.1/3.2 KD and Control one-cell stage embryos stained with anti-H3.1/3.2 antibody at 11 hpi. Scale bar = 20 µm. (B) Quantification of the fluorescence signals in the experiments in (A). Signal intensities of H3.1/3.2 was corrected with those of DAPI. The value of the male pronucleus in the Control embryos was set to 1 and relative values were calculated. (C) Immunofluorescence images of H3.1/3.2 KD and Control two-cell stage embryos stained with anti-H3.1/3.2 and H3.3 antibodies at 30 hpi. Scale bar = 20 µm. (D) Quantification of the fluorescence signals in the experiments in (C). Signal intensities of H3.1/3.2 and H3.3 were corrected with those of DAPI. The value of the Control embryos was set to 1 and relative values were calculated. At least four independent experiments were performed in each experimental group, and more than 23 embryos were analyzed in total. Error bars indicate S.E. Asterisks indicate significant differences ($P < 0.05$; by Student's t-test).

Figure. 2-5.

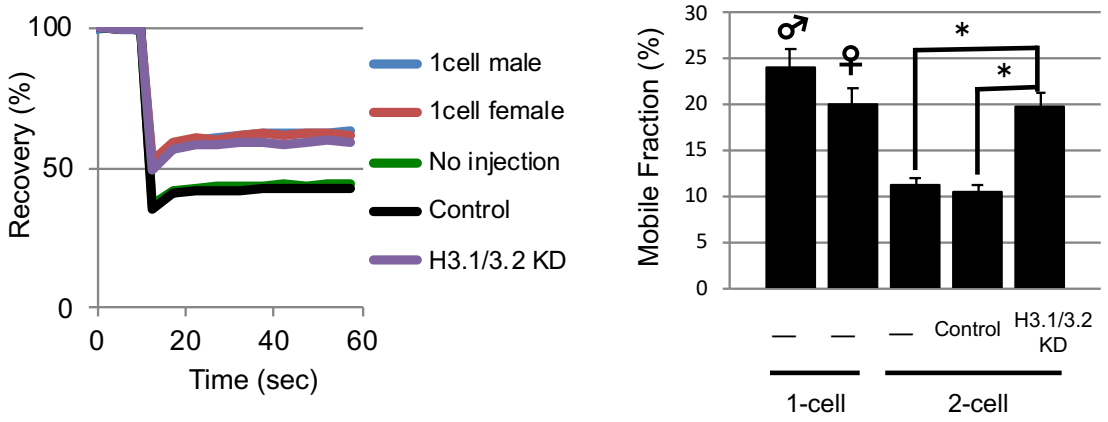

Figure. 2-5. Effects of H3.1/3.2 KD on the chromatin structure in two-cell stage embryos.

Analyses of the looseness of the chromatin structure in H3.1/3.2 KD embryos. cRNA encoding eGFP-H2B was co-injected with siRNA against H3.1/3.2 (H3.1/3.2 KD), control siRNA (Control) or none (-) into fully grown oocytes. They were matured and fertilized *in vitro* and then subjected to FRAP analyses in one-cell embryos at at 10–12 hpi and in two-cell embryos at 30–32 hpi. The diagrams of the recovery curve and mobile fraction are shown on the left and right respectively. ♂ and ♀ represent male and female pronuclei respectively. Four independent experiments were conducted for each group to analyze 23 or more embryos. Error bars indicate S.E. Asterisk indicates a significant difference ($P < 0.05$; by Student's t-test).

Figure. 2-6.

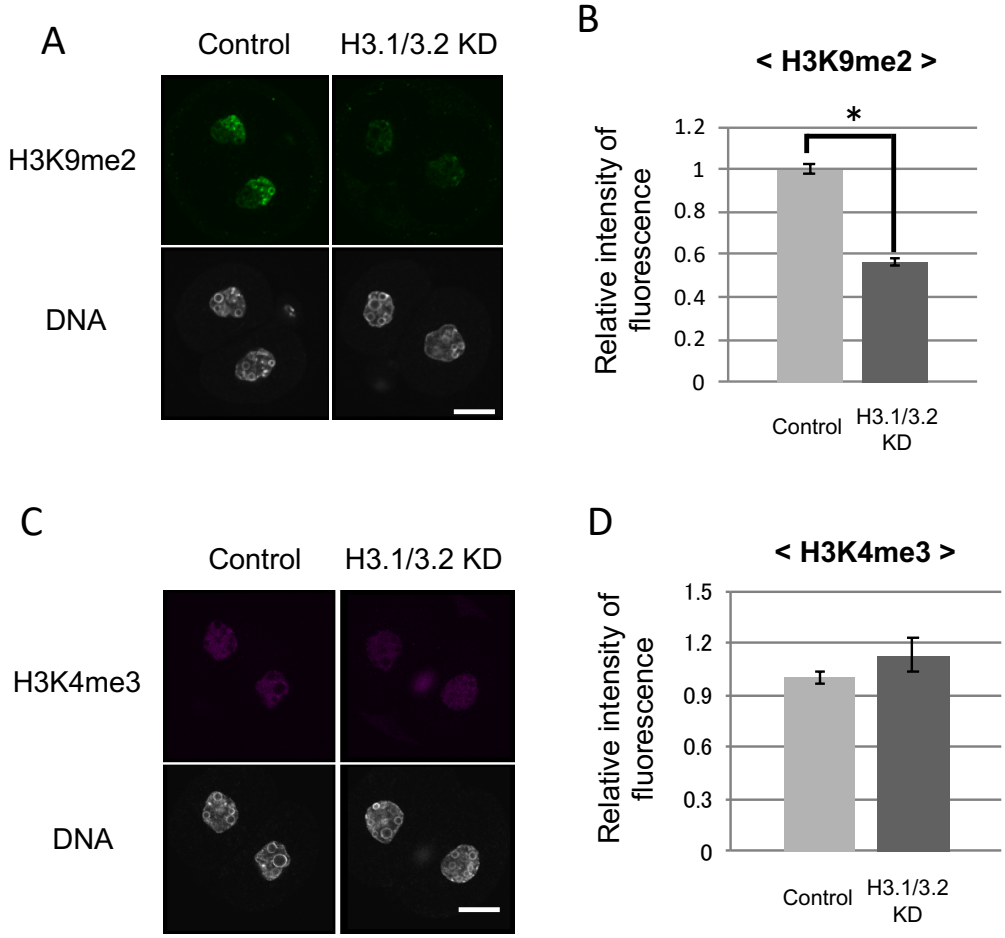

Figure. 2-6. Effect of H3.1/3.2 KD on histone modifications in two-cell embryos. Fully grown oocytes were injected with siRNA against H3.1/3.2 (H3.1/3.2 KD) or control siRNA (Control), matured, and fertilized *in vitro*. (A) Immunofluorescence images of H3.1/3.2 KD and Control two-cell stage embryos stained with anti-H3K9me2 antibody at 30 hpi. Scale bar = 20 µm. (B) Quantification of the fluorescence signals in the experiments in (A). Signal intensities of H3K9me2 was corrected with those of DAPI. The value of the Control embryos was 1 and relative values were calculated. (C) Immunofluorescence images of H3.1/3.2 and Control embryos stained with anti-H3K4me3 antibody at 30 hpi. Scale bar = 20 µm. (D) Quantification of the fluorescence signals in the experiments in (C) Signal intensities of H3K4me3 was corrected with those of DAPI. The value of the Control embryos was 1 and relative values were calculated. At least three independent experiments were performed in each experimental group, and more than 19 embryos were analyzed in total. Error bars indicate S.E. Asterisks indicate a significant difference ($P < 0.05$; by Student's t-test).

Figure. 2-7.

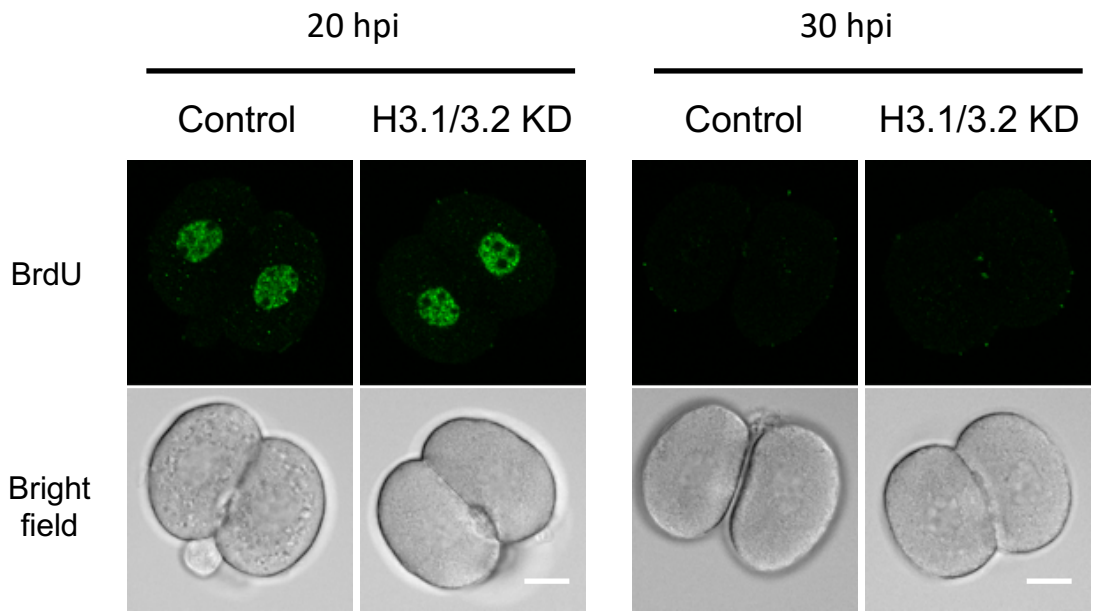

Figure. 2-7. Effects of H3.1/3.2 KD on the timing of DNA replication in two-cell stage embryos.

Fully grown oocytes were injected with siRNA against H3.1/3.2 (H3.1/3.2 KD) or control siRNA (Control), matured, and fertilized *in vitro*. At each time point post insemination (20, 30 hpi), the occurrence of DNA replication was examined by detecting BrdU incorporation. Three independent experiments were conducted for each group to analyze a total of 21 or more embryos and similar results were obtained in each experiment. Scale bar = 20 μm.

Figure. 2-8.

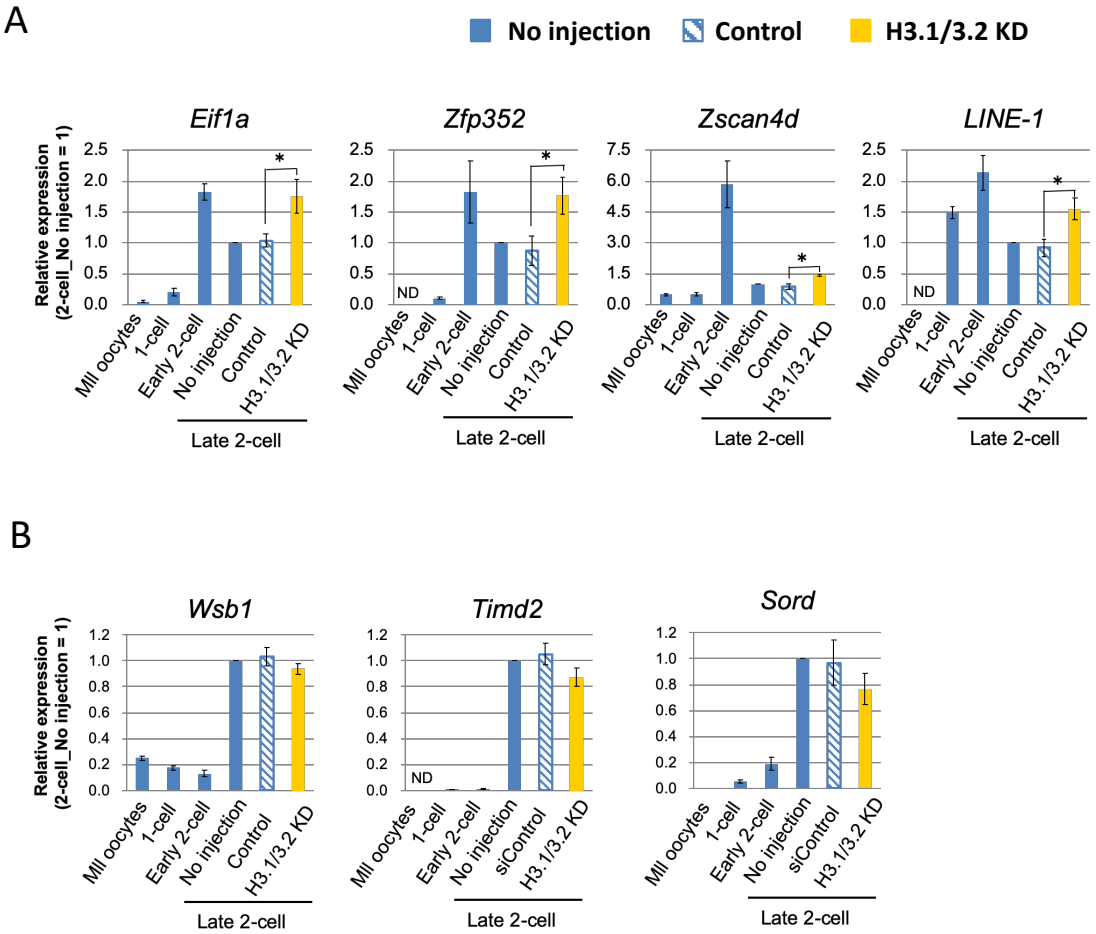

Figure. 2-8. Effects of H3.1/3.2 KD on the change of gene expression pattern during early and late two-cell stages.

(A, B) Fully grown oocytes were injected with siRNA against H3.1/3.2 (H3.1/3.2 KD), control siRNA (Control) or none (No injection), matured, and fertilized *in vitro*. Twenty-five Mll oocytes and embryos at the one- and two-cell stages were collected at 12, 18, 32 hpi, respectively. The expression level of each gene was examined by using reverse transcription and real-time PCR in oocytes and embryos. The value of two-cell stage embryos with no injection was set to 1 and relative values were calculated. Five independent experiments were conducted for each variant and similar results were obtained for each experiment. Error bars represent S.E. Asterisks indicate a significant difference ($P < 0.05$; by Student's t-test).
ND = Not Detected

Figure. 2-9.

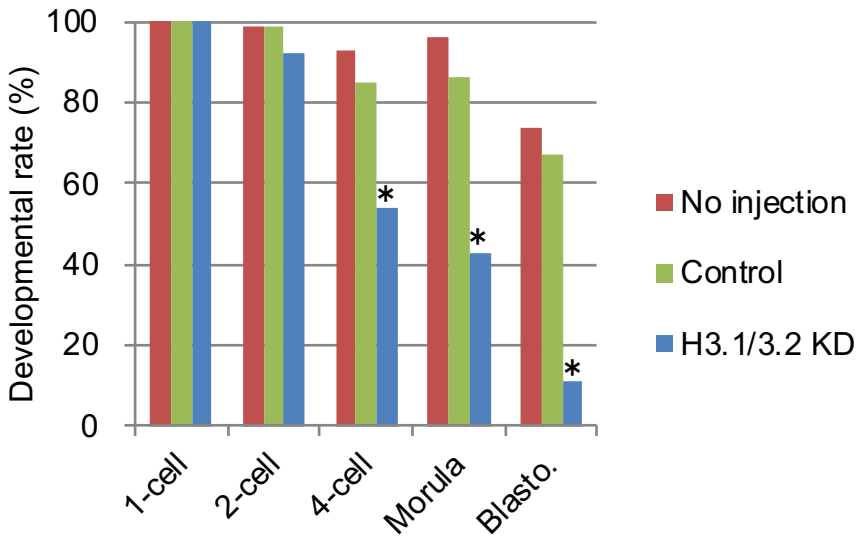

Figure. 2-9. Effect of H3.1/3.2 KD on preimplantation development.
Fully grown oocytes were injected with siRNA against H3.1/3.2 (H3.1/3.2 KD), control siRNA (Control) or none (No injection), matured, and fertilized *in vitro*. These embryos were observed at 12, 24, 45, 72, 96 hpi to evaluate the development to the one-, two- and four-cell, morula, and blastocyst stages, respectively. Asterisks indicate significant differences from both of No injection and Control groups ($P < 0.05$; by $\chi 2$ test or Fisher's exact test). Four independent experiments were conducted for each group to analyze a total of 74 or more embryos.

Figure. 2-10.

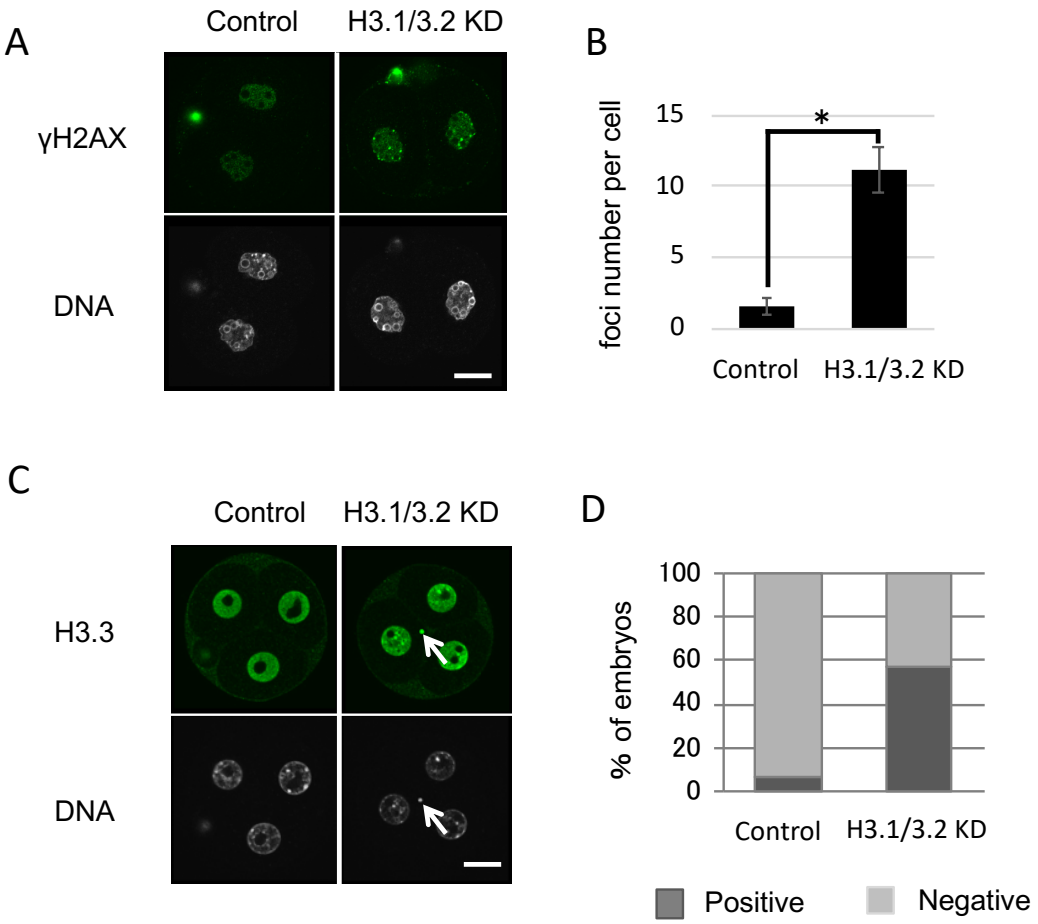

Figure. 2-10. Effects of H3.1/3.2 KD on DNA damage of two-cell stage embryos. Fully grown oocytes were injected with siRNA against H3.1/3.2 (H3.1/3.2 KD) or control siRNA (Control), matured, and fertilized *in vitro*. (A) Immunofluorescence images of H3.1/3.2 KD and Control two-cell stage embryos stained with anti-γH2AX antibody at 30 hpi. Scale bar = 20 µm. (B) The number of γH2AX foci in the nucleus in the immunofluorescence images in (A). The foci whose size occupied more than 0.15% of the area of whole nucleus were recognized as γH2AX foci. An asterisk indicates a significant difference ($P < 0.05$; by Student's t-test). Error bars indicate S.E. (C) Immunofluorescence images of H3.1/3.2 KD and Control four-cell stage embryos stained with anti-H3.3 antibody at 42 hpi. Scale bar = 20 µm. An arrowhead indicates a micronuclus. (D) Percentages of the embryos with micronuclei (positive) or not (negative). At least three independent experiments were performed in each experimental group, and more than 20 embryos were analyzed in total.

Figure. 2-11.

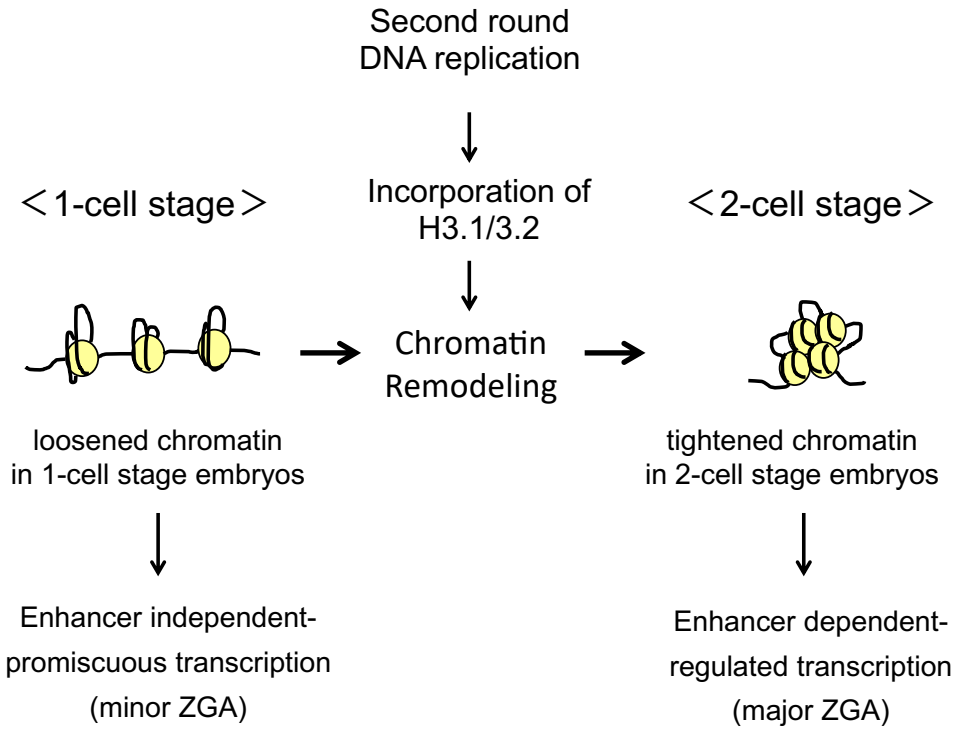

Figure. 2-11. The involvement of H3.1/3.2 in the change of the chromatin structure and gene expression during one- and two-cell stages.
During the one- and two-cell stages, the nuclear localization of H3.1/3.2 increases in a DNA replication dependent manner. The increase of H3.1/3.2 causes the chromatin structure to be tighter at the late two-cell stage and this alteration of chromatin structure is involved in the transition from minor to major ZGA.

CONCLUSION & PERSPECTIVE

In this thesis, I have shown that H1FOO is abundantly deposited in the pronuclei of one-cell stage embryos and that it abruptly decreases at the two-cell stage. Since H1FOO has weaker ability to condense the chromatin than other H1 variants, it would be involved in the formation of loosed chromatin structure in one-cell stage embryos. Furthermore, H1foo KD caused the increase in the nuclear deposition of H3.1/3.2 at the one-cell stage, which suggests that H1foo suppresses the deposition of H3.1/3.2 to a low level. Since H3.1/3.2 is known to involve the tight chromatin structure, the abundant nuclear deposition of H1FOO contributes the establishment of loosened chromatin structure via the reduction of H3.1/3.2 in addition to its own function (Fig. C&P). In addition, the repressing the nuclear deposition of H3.1/3.2 by H1foo is required to avoid the delay in the completion of DNA replication. At the two-cell stage, the nuclear deposition of H1FOO decreases, whereas somatic linker histone variants and H3.1/3.2 increases. The alterations of these histone variants would contribute to the change in chromatin structure during one- and two-cell stages (Fig. C&P). Furthermore, H3.1/3.2 KD decreased the degree of the reduction of chromatin looseness and reduced the suppression of some genes and a retrotransposon at the late two-cell stage. These results suggest that the chromatin remodeling regulated by the changes in H1foo and H3.1/3.2 is involved in the alteration of gene expression pattern during one- and two-cell stages.

My results have shown that H1foo and H3.1/3.2 are involved in the change of chromatin structure during one- and two-cell stages. However, the effect of these variants on the chromatin structure of particular regions on the genome could not be analyzed, since I examined the chromatin looseness by FRAP which analyzes the chromatin in whole nucleus but not the specific regions in genome.

Immunocytochemistry also provides only limited information about the localization of the variants in genome. Recently, STAR-Chromatin immunoprecipitation sequencing (Zhang et al., 2016) and Cleavage Under Targets and Release Using Nuclease (CUT&RUN)-sequencing (Meers et al., 2019; Sken and Henikoff, 2017), which can analyze the distribution of target proteins in genome using a small number of cells (less than 100), have been developed. Using these methods, the distribution of histone variants in genome could be analyzed in one- and two-cell stage embryos. Since ATAC-seq provides the information of "opened regions" which are likely to be loosened regions in genome and the results of this analysis for the preimplantation embryos has been published (Wu et al., 2016), the roles of histone variants in the looseness of particular regions would be clarified by comparing the data of ATAC-seq and the deposition of the variants in the particular regions during the chromatin remodeling.

It is important to comprehensively examine the effects of H3.1/3.2 KD on the change of gene expression during one- and two-cell stages. Although I examined the expression of three genes which are transcribed during minor ZGA and repressed during major ZGA. During minor and major ZGA, gene expression pattern dynamically changed, it is likely that H3.1/3.2 KD also affect the genes whose expression patterns are different from those three genes. Therefore, the analysis of the transcriptome in H3.1/3.2 KD embryos will be useful to comprehensively understand the regulation of ZGA. Moreover, comparing this transcriptome with that of embryos in which DNA replication is inhibited, it will lead to a comprehensive understanding of the involvement of H3.1/3.2 in the change of gene expression in DNA replication-depend manner at the two-cell stage.

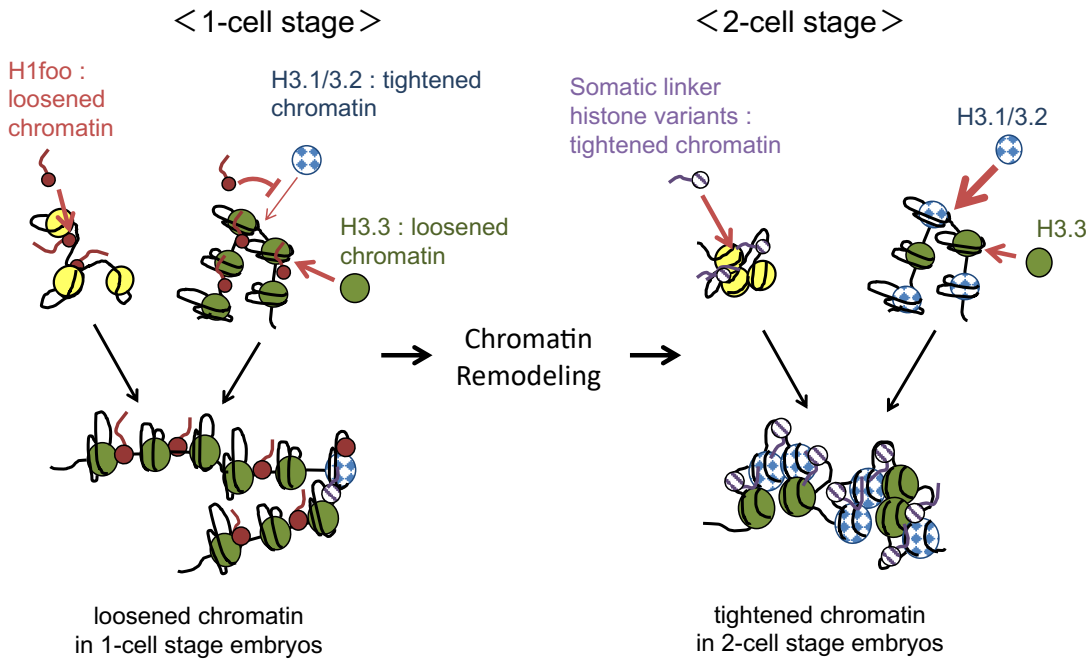

Figure. C&P. The involvement of histone variants in the chromatin remodeling during the one- and two-cell stages.

In one-cell stage embryos, the level of nuclear deposition of H1foo and H3.1/3.2 are high and low, respectively. H1FOO has weaker ability to condense the chromatin than other variants, while H3.1/3.2 is associated with the condensed chromatin. H1foo represses the chromatin deposition of H3.1/3.2, which contributes in part the low level of H3.1/3.2 in one-cell stage embryos. Taken together, it is suggested that these mechanisms are involved in the loosened chromatin structure in one-cell stage embryos. At the two-cell stage, the nuclear deposition of H1FOO dramatically decreases, while that of somatic linker histone variants and H3.1/3.2 increase. The tightened chromatin structure is thus established at the two-cell stage.

MATERIALS AND METHODS

Synonym name and expression of linker histone H1 variants

The synonym and symbol name of each linker histone variant are used as follows: (H1a: *H1f1*, H1b: *H1f5*, H1c: *H1f2*, H1d: *H1f3*, H1e: *H1f4*, H1f0: *H1f0*, H1fx: *H1f10*, H1foo: *H1f8*, H1t: *H1f6,* H1t2: *H1f* and Hils1: *Hils1*). Synonym names are used throughout this thesis. The relative expression levels of various linker histone H1 variants were analyzed by using RNA sequencing data (Abe et al., 2015).

Collection and culture of oocytes and embryos

Fully grown oocytes were obtained from the ovaries of 8- to 12-week-old B6D2F1 female mice 45–48 h after the injection of 7.5 I.U. pregnant mare serum gonadotropin (PMSG; ASKA Pharmaceutical, Tokyo, Japan). The ovary was removed from the mouse, placed in HEPES-buffered K+-modified simplex optimized medium (KSOM) (Lawitts et al., 1993) containing 0.2 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich, St. Louis, MO, USA) and punctured with a 30-gauge needle. Only fully grown oocytes were collected. Spermatozoa were obtained from the cauda epididymis of adult ICR male mice (SLC, Shizuoka, Japan) and cultured in HTF medium (Quinn et al., 1984) for *in vitro* fertilization. Metaphase II (MII) stage oocytes were obtained from the ampulla of the oviduct of 3-week-old B6D2F1 female mice injected with 6 I.U. PMSG followed 48 h later with 7.5 I.U. of human chorionic gonadotropin (ASKA Pharmaceutical, Tokyo, Japan). The obtained MII stage oocytes were inseminated with spermatozoa that had been pre-cultured for 2 h. The embryos were transferred to fresh KSOM medium, washed several times to remove surrounding cumulus cells, and cultured in an atmosphere of 5% CO_2 and 95% air at 38°C. All of the procedures using animals were

reviewed and approved by the University of Tokyo Institutional Animal Care and Use Committee and were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

Reverse transcription and real-time quantitative polymerase chain reaction (qPCR)

Mll stage oocytes and embryos were collected. RNA extraction, reverse transcription followed by real-time qPCR were performed as described previously (Abe et al., 2015, Kawamura et al., 2012). The primers and conditions used in PCR are shown in Table. M&M. Rabbit α-globin was used as an external control.

Vector construction

I constructed a vector encoding H1foo (H1foo-polyA pcDNA3.1), H1a (H1a-polyA pcDNA3.1), H1c (H1c-polyA pcDNA3.1) and H1d (H1d-polyA pcDNA3.1) as described below.

I amplified the H1foo coding sequence from the pEGFP-C3-H1foo construct, which had been previously prepared, and H1a coding sequence from cDNA derived from the testis of B6N male mice, and H1c coding sequence from cDNA derived from the brain of B6N female mice by PCR with PrimeSTAR Max DNA Polymerase (Takara, Shiga, Japan). The primers used in PCR were as follows.

H1foo primers: 5'-ATGGCTCCTGGGAGTGTCTCCAGTG-3' (forward), 5'-TCAGGCCTGAGTGTTCTCAGGGGTC-3' (reverse);

H1a primers: 5'-ATGTCGGAGACGGCGCCAGTCGCTC-3' (forward), 5'-TCACTTTTTCTTGGGCGCTGCCTTC -3' (reverse);

H1c primers: 5'-ATGTCTGAGGCTGCTCCTGCTGCCC-3' (forward), 5'-CTACTTTTTCTTGGCTGCAACCTTC-3' (reverse).

The amplified PCR products were treated with Ex-taq (Takara) and purified with the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). The purified PCR products were cloned into the pCRII-TOPO vector by a TOPO TA cloning kit (Life Technologies, Carlsbad, CA, USA) following the manufacturer's protocol to generate pCRII-TOPO-H1foo, pCRII-TOPO-H1a and pCRII-TOPO-H1c. The products were transformed into DH5α (9057, Takara, cell density: 1–2 × 10^9 bacteria/mL). The plasmids were purified with the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich) and verified by DNA sequencing. pCRII-TOPO-H1foo, pCRII-TOPO-H1a and pCRII-TOPO-H1c were digested with EcoR1 and separated by electrophoresis. Their inserts were purified with the Wizard SV Gel and the PCR Clean-Up System (Promega). eGFP-polyA pcDNA3.1 (Yamagata et al., 2005) was digested with EcoR1 and treated with alkaline phosphatase to remove the sequence coding enhanced green fluorescent protein. The GFP-polyA pcDNA3.1 vector was separated by electrophoresis and purified with the Wizard SV Gel and PCR Clean-Up System. The purified vector and H1foo, H1a or H1c inserts were ligated with Ligation High (Toyobo) following the manufacturer's protocol. The product was transformed into DH5α. The plasmid was purified with the GenElute™ Plasmid Miniprep Kit and verified by DNA sequencing. I amplified the H1d coding sequence from genomic DNA of B6N female mice by PCR with KOD-Plus-polymerase (Toyobo, Osaka, Japan). PCR product were purified with the Wizard SV Gel and PCR Clean-Up System (Promega), digested with BamH1 and EcoR1, thus separated by electrophoresis. Their inserts were purified with the Wizard SV Gel and the PCR Clean-Up System (Promega) and cloned into eGFP-polyA

pcDNA3.1 digested with BamH1 and EcoR1 as mentioned above. The primers used in PCR were as follows.

H1d primers: 5'- CGGGATCCCGATGTCCGAGACCGCTCCCGCGGCGC-3' (forward), 5'- GGAATTCCCTACTTCTTGCGAGGGGCAGCCTTC -3' (reverse).

The vectors were digested with Xba1 (for H1foo, H1a, H1c, H1d-polyA pcDNA3.1) or Not1 (for pCRII-TOPO-eGFP-H2B constructed in previous study (Ooga et al., 2016)) and purified with phenol/chloroform/isoamyl alcohol for *in vitro* transcription (Nippon Gene, Tokyo, Japan). This linearized vector was transcribed with mMESSAGE mMACHINE T7 (for H1foo, H1a, H1c, H1d -polyA pcDNA3.1; Life Technologies) and mMESSAGE mMACHINE SP6 (for pCRII-TOPO-eGFP-H2B; Life Technologies) following the manufacturer's protocol. eGFP-H2B cRNA was poly A tailed using a poly A tailing kit (Life Technologies) and purified with LiCl precipitation solution (Life Technologies) and diluted with RNase-free water (Takara).

Microinjection

Fully grown oocytes

Fully grown oocytes were transferred to HEPES-buffered KSOM medium containing 0.2 mM IBMX and injected with 10 pL Stealth RNAi^{TM} small interfering RNA (siRNA) against H1foo or both H3.1 and 3.2 (H1foo, H3.1/3.2; 10 μM; Life Technologies) or Stealth^{TM} RNAi Negative Control Duplexes (10 μM; control siRNA; Life Technologies) using a narrow glass capillary (GC100 Tf-10, Harvard Apparatus, Holliston, MA, USA). Fully grown oocytes were co-injected with 10 pL siRNA of H1foo (10 μM), H3.1/3.2 (10μM) or siControl (10 μM) and cRNA of eGFP-H2B (500 ng/μL) for FRAP analyses in H1foo or H3.1/3.2 KD embryos. Microinjection was performed under an inverted

microscope (Eclipse TE300; Nikon, Tokyo, Japan) using a micromanipulator (Narishige., Tokyo, Japan) and a microinjector (IM300; Narishige). The injected oocytes were washed with α-MEM (Gibco-BRL, Grand Island, NY, USA) without IBMX and incubated in α-MEM for 16 h (H1foo KD) or 18 h (H3.1/3.2 KD) in an atmosphere of 5% CO_2 and 95% air at 38°C to allow *in vitro* maturation. Only MII stage oocytes with the first polar body were collected and inseminated with spermatozoa after 16 or 18 h of incubation. The siRNA sequence against H1foo or H3.1/3.2 was as follows:

H1foo; 5'-AAGCCAGGUCCAAGUUGCCGCAGAA-3'

H3.1/3.2; 5'-GUGCGCGAGAUCGCGCAGGACUUCA-3'

Mll stage oocytes

Obtained Mll oocytes from 3-week-old B6D2F1 female mice were moved to α-MEM medium containing 300 μg/mL hyaluronidase (Sigma-Aldrich) and incubated for 5 min at 38 °C to remove surrounding cumulus cells. After removing surround cumulus cells, oocytes were washed with α-MEM and injected with 10 pL cRNA of each linker histone variants (H1a, H1d; 500 ng/μL) using a narrow glass capillary. Injected Mll oocytes were washed with α-MEM medium and inseminated with spermatozoa.

One-cell stage embryos

One-cell stage embryos at 4 hours post insemination (hpi) were transferred to KSOM medium containing 300 μg/mL hyaluronidase (Sigma-Aldrich) and incubated for 5 min at 38 °C to remove surrounding cumulus cells. Then the embryos were transferred to KSOM medium, washed several times, and co-injected with 10 pL cRNA of H1foo, H1a, H1c (1,000 ng/μL) using a narrow glass capillary to overexpress the linker histone variants at 4–7 hpi. The embryos were co-injected with 10 pL cRNA of H1foo or H1c

(1,000 ng/μL) for FRAP analyses in H1foo or H1c overexpressing embryos. The injected embryos were washed and further incubated with KSOM.

Immunocytochemistry

H1FOO, H1D, H3.1/3.2, H3.3 and LaminB1: The embryos were fixed in 3.7% paraformaldehyde (PFA) and 0.2% Triton X-100 in PBS for 20 min (H1FOO, H1D, H3.1/3.2 and H3.3) or 40min (LaminB1) at room temperature. The embryos were washed with 1% bovine serum albumin (BSA) in PBS (PBS/BSA) and incubated with 1% BSA and 0.2% Tween 20 in PBS containing rabbit anti-H1FOO antibody (1:500 dilution; An antibody of H1FOO against a KLH conjugated 12-mer peptide (CRVRKAKTPENT) was raised by Scrum; Tokyo, Japan), rabbit anti-H1D antibody (1:500 dilution; An antibody of H1D against a KLH conjugated 12-mer peptide (CTKAKKAAPRKK) was raised by Scrum; Tokyo, Japan), rabbit anti-LaminB1 antibody (1:400 dilution; ab16048; Abcam, Cambridge, MA, USA), or 1% BSA and 0.2% Tween 20 in PBS containing mouse anti-H3.1/H3.2 (1:500 dilution; CE-039B; Cosmo Bio, Tokyo, Japan) and rat anti-H3.3 (1:100 dilution; CE-040B; Cosmo Bio) antibodies overnight at 4°C. The embryos were treated with secondary Alexa Fluor 568 donkey anti-rabbit antibody (1:100 dilution; A10042; Life Technologies) for anti-H1FOO or H1D, Alexa Fluor 647 goat anti-rabbit antibody (1:100 dilution; A31573; Life Technologies) for anti-LaminB1 or Alexa Fluor 488 goat anti-rat antibody (1:100 dilution; 4416S; Cell Signaling Technology, Danvers, MA, USA) for anti-H3.3 and Alexa Fluor 647 goat anti-mouse antibody (1:100 dilution; A21236; Life Technologies) for anti-H3.1/H3.2 for 1h at room temperature. The cells were washed with PBS/BSA and mounted on glass slides with Vectashield (Vector Laboratories,

Burlingame, CA, USA) containing 3 μg/mL 4',6-diamidino-2-phenylindole (DAPI; Dojindo Laboratories, Kumamoto, Japan). H3.1/3.2 and H3.3 were co-immunostained.

γH2AX, H3K4me3: Embryos were fixed in 3.7% PFA in PBS for 20 min at room temperature. The cells were washed with PBS/BSA and permeabilized with 0.5% Triton X-100 in PBS for 15 min at room temperature. They were washed with PBS/BSA and incubated with 1% BSA and 0.2% Tween 20 in PBS containing mouse anti-γH2AX antibody (1:100 dilution; #07-164; Millipore, Billerica, MA) or rabbit anti-H3K4me3 antibody (1:100 dilution; #07-473; Millipore) overnight at 4°C. The cells were washed with PBS/BSA and then incubated with PBS/BSA containing the Alexa Fluor 488 donkey anti-mouse antibody (1:100 dilution; Life Technologies) for anti-γH2AX or Alexa Fluor 647 goat anti-rabbit antibody (1:100 dilution; A31573; Life Technologies) for anti-H3K4me3 for 1 h at room temperature. The cells were washed with PBS/BSA and mounted on glass slides and prepared as described above.

H3K9me2: Embryos were fixed in 3.7% PFA in PBS for 60 min at room temperature. The cells were washed with PBS/BSA and permeabilized with 0.5% Triton X-100 in PBS for 15 min at room temperature. They were washed with PBS/BSA and incubated with 1% BSA and 0.2% Tween 20 in PBS containing mouse anti-H3K9me2 antibody (1:100 dilution; ab1220; Abcam) overnight at 4°C. The cells were washed with PBS/BSA and then incubated with PBS/BSA containing the Alexa Fluor 488 donkey anti-mouse antibody (1:100 dilution; Life Technologies) for 1 h at room temperature. The cells were washed with PBS/BSA and mounted on glass slides and prepared as described above.

To wash away free proteins in the nucleoplasm, embryos were permeabilized with Triton X-100 before fixation following the method described by Hajkova et al.,

(2010). The linker histone overexpressing embryos were treated with rabbit anti-H1FOO antibody (1:500 dilution; Scrum), rabbit anti-H1C antibody (1:500 dilution; ab181977; Abcam) or mouse anti-H3.1/3.2 antibody (1:100 dilution; Cosmo Bio) overnight at 4°C. Alexa Fluor 568 donkey anti-rabbit antibody (1:400 dilution; Life Technologies) for anti-H1FOO and anti-H1C or Alexa Fluor 647 goat anti-mouse antibody (1:100 dilution; Life Technologies) for anti-H3.1/H3.2 were applied for 1 h at room temperature. The cells were washed with PBS/BSA and mounted on glass slides and prepared as described above.

The samples were observed under a Carl Zeiss LSM5 exciter laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany) and Olympus FV300 laser scanning microscope (Olympus, Tokyo, Japan). Fluorescence intensity of the H3 variants, H3K9me3 and H3K4me3 were measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

5-bromo-2'-deoxyuridine (BrdU) assay

DNA replication was analyzed by BrdU incorporation in DNA as described by Aoki et al. (Aoki et al., 1999) with slight modifications. The embryos were incubated with KSOM containing 10 μM BrdU (90139520; Roche, Basel, Switzerland) for 1 h at 38°C. After fixation, the embryos were washed with PBS/BSA and washed again with 0.05% Tween-20 in PBS. After the treatment with 2N HCl for 1 h, the embryos were washed with PBS/BSA in PBS, moved and incubated in 0.1 M Tris-HCl (pH 8.5) and 0.02% Triton X-100 in PBS for 15 min at room temperature. Mouse anti-BrdU antibody (1:100 dilution; 11170376001, Roche Diagnostic, Indianapolis, IN, USA) and rabbit anti-H3K9me3 antibody (1:1000 dilution; 04–772; Millipore) were used. The latter was

used to distinguish the parental origin, male or female, of the pronucleus in one-cell stage embryos. Alexa Fluor 488 donkey anti-mouse antibody (1:100 dilution; Life Technologies) for anti-BrdU and Alexa Fluor 647 goat anti-rabbit antibody (1:250 dilution; Life Technologies) for anti-H3K9me3 were used as secondary antibodies. The cells were washed with PBS/BSA and mounted on glass slides and prepared as described above.

FRAP analyses

The samples for FRAP analysis were observed under a Carl Zeiss LSM5 exciter laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany) and Olympus FV300 laser scanning microscope (Olympus, Tokyo, Japan). FRAP analyses were performed as previously described (Ooga et al., 2016) with slight modifications. Excitation of the laser at 488 nm was set to 2–3% (Carl Zeiss) or 0.2% (Olympus) at ordinary time, and fluorescence intensity was set to about 2,000. I took three photos at five second intervals before photo-bleaching, which was performed for ten seconds (Carl Zeiss) or one second (Olympus) with excitation of the 488 nm laser set to 100% (Carl Zeiss) or 3% (Olympus). A total of 12 pictures were taken every five seconds.

Name	Forward primer	Reverse primer	Tm (°C)	Cycle
Real-time quantitative PCR				
Rabbit α-globin	CACCGAGGACAGTTTCCATT	CCAGTTACCAGGTGCTGGAT	60	
H1a	CTACTGCCACGGAGAAACCT	GAAGAAACTGCCTGCACGATG	60	
H1b	AAGCCTAAGACCGCTAAGCC	TTGCTGTCGCAAGCAAACC	60	
H1c	GGCGGCTGTGACCAAGAAA	CTTTAGACGCGCTCTTGACCT	60	
H1d	CAAGGCTCCCAAGGCTAAGG	GCACCACTCTACTTCTTGCGA	60	
H1d	GTCAAGAAGAAGGCCCGCAA	CCTTGGTGATGAGTTCGGACA	60	
H1f0	AGAGCCACTACAAGGTGGGT	GTCACTAGGCGCTTGATGGA	60	
H1fx	ATGGTTCGACCAGCAGAACG	CACCTGCAGTAGCGTATCGT	60	
H1foo	AGTGTCTCCAGTGTTTCTTCCTC	CAGCATTGTTGGGTTCCTTTGG	60	
H1t	GCTGATTCCTGAGGCCCTTT	TTGAGGGCCAGCTTGATACG	60	
Eif1a	AAGAAGTCTGAAGGCCTATG	CAGAGAACTTGGAAGGTAGC	60	
Zfp352	AAAGCCTTGATCCTCAGGTG	GCCGAAGAGTTTTTCTGAGG	60	
Zscan4d	CCTAGGGAGCTACCAGGGTT	AGGCTCCTGGCATGTTTGAA	60	
LINE-1	AGTGCAGAGTTCTATCAGACCTTC	AACCTACTTGGTCAGGATGGATG	60	
Wsb1	CTCCAAGGCAAGTCCCTAGC	TGTAAAGGGCGCTCTGTCTG	60	
Timd2	CAGGTGCCGTGGAGTCTCATAC	GCTGGTATCGACTGTTCCTCTGATG	60	
Sord	ATCGGGTTGCCATCGAG	GAGGTTTCCATCATCCGGG	60	

Table. M&M. PCR primers and conditions.

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