論文内容の要旨

論文題目 **Involvement of histone H2A variants replacement in the genome remodeling during mouse pre-implantation development**

（マウス着床前初期胚におけるゲノムリモデリングへのヒストン **H2A** 変異体置換の関与）

氏名：那順 布和

Introduction

After fertilization, terminally differentiated oocytes are transformed into totipotent zygotes, and then begin to differentiate at the late pre-implantation stage (Fig. 1). During this period, the gene expression pattern changes substantially, which involves genome-wide chromatin remodeling. This remodeling has also been thought to occur when somatic cell nuclei are transferred into enucleated oocytes in which gene expression profile of the somatic donor nuclei is changed to sustain early embryonic development. However, the mechanism underlying chromatin remodeling is largely unknown.

In recent years, emerging evidences have shown that chromatin structure is remodeled by the changes in the composition of nucleosome by replacing the canonical histones with their variants. Histones are the main structural proteins that package eukaryotic DNA into chromatin and a variety of variants for histones H2A, H2B, and H3 have been identified in eukaryotes. Among these nuclear core histones, H2A has the largest number of variants, i.e. H2A.Z, H2A.X, macroH2A and H2A.Bbd for canonical histone H2A. Nucleosomes containing variant histone H2A.Z are unstable and more mobile than ordinary nucleosomes. H2A.Z nucleosomes can change the higher order chromatin structure and chromatin fibers having H2A.Z may have a relaxed conformation. On the other hand, nucleosomes containing variant histone macroH2A are resistant to chromatin remodeling and thought to be repressive to gene expression. The chromatin containing H2A.X is required for DNA repair mechanism. Furthermore, H2A.X is extraordinarily abundant in the chromatin of embryonic stem cells (ES cells), suggesting that it is involved in the maintenance of their pluripotent state. Thus, the functions of H2A variants during various cellular processes in differentiated cells have been well documented, however, few studies have investigated their roles in germ cells and embryos. In this study I investigate their dynamic changes and the underlying mechanisms as well as their functions during pre-implantation development.

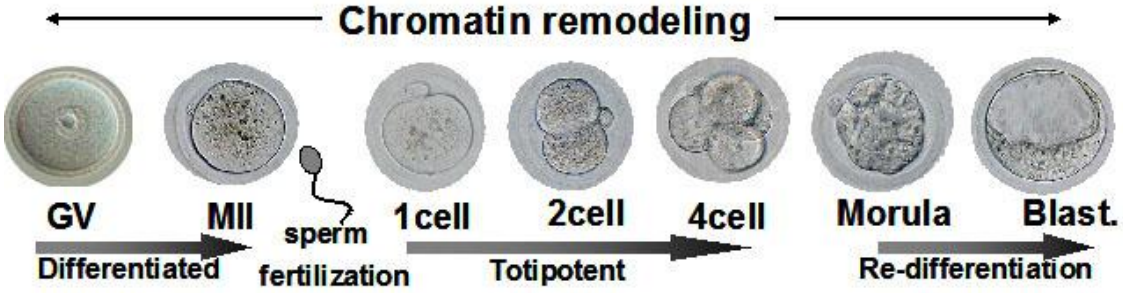

Fig.1.Oocyte maturation and early embryo development during which chromatin remodeling occurs

Results and Discussions

1. Changes in the nuclear deposition of histone H2A variants during pre-implantation development

First, I examined the changes in nuclear deposition of canonical histone H2A and variants histone H2A.Z and H2A.X during oogenesis and pre-implantation development using specific antibodies. Immunofluoresence analysis demonstrated that all of these three histones were readily detected in the nuclei of full-grown and MII stage oocytes. However, in the pronuclei of 1-cell embryos after fertilization, a weak and no signal of H2A and H2A.Z, respectively, was detected, whereas a strong signal of H2A.X was still observed. At

the later developmental stages, nuclear deposition of H2A.X was decreased while H2A and H2A.Z were increased. MacroH2A has been reported to have similar dynamics with H2A.Z and disappeared from chromatin after fertilization. This decline in H2A and the depletion of H2A.Z and macroH2A after fertilization were confirmed by generating transgenic mouse lines carrying Flag epitope-tagged H2A, H2A.Z, and macroH2A.

It is possible that the amounts of H2A variant proteins stored in oocytes change after fertilization, leading to changes in the levels of nuclear deposition. To address this hypothesis, we examined the expression of histone H2A and its variants by RT-PCR (Fig. 2). However, these transcripts were expressed at similar levels

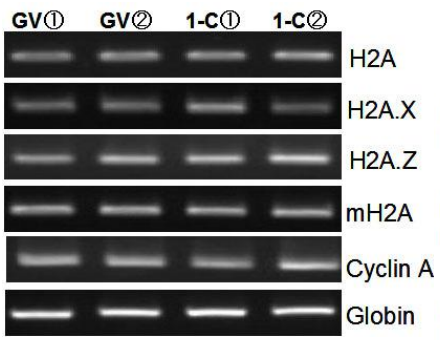

Fig. 2. RT-PCR analysis of histone H2A and its variants expression before and after fertilization. Cyclin A2 and Globin are served as control.

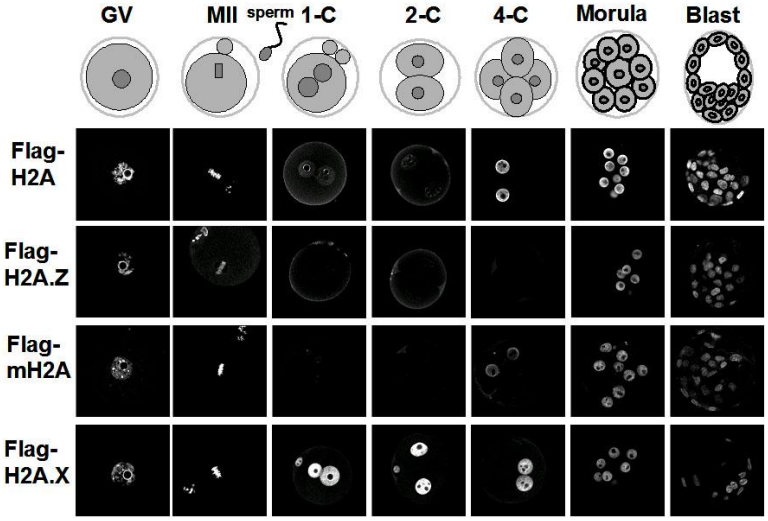

Fig. 3. Nuclear incorporation of H2A and its variants in the oocytes and pre-implantation embryos. Flag-tagged H2A, H2A.Z, macroH2A (mH2A) and H2A.X mRNAs were microinjected into the GV and MII oocytes or one blastomere of 2-cell embryos and the nuclear incorporations of H2A variants were detected by anti-Flag antibody.

before and after fertilization. Furthermore, in immunoblotting analyses performed using anti-H2A.X and H2A.Z antibodies, levels of histone proteins changed little after fertilization. Thus, it is likely that the mechanisms regulating H2A variant incorporation is dramatically altered after fertilization.

Histones are dynamically assembled and removed from chromatin and the equilibrium determines their abundance. Thus, it is possible that the decrease in H2A, H2A.Z and macroH2A deposition in the nucleus after fertilization may have been caused by a decrease in the incorporation rate of these proteins into chromatin. Therefore, microinjection experiments with mRNAs encoding the Flag-tagged proteins were conducted to examine the incorporations of exogenously expressed histone variants. In agreement with the results obtained by the immunocytochemical and the transgenic mouse analyses, Flag-H2A.X was abundantly incorporated into the nucleus after fertilization, whereas Flag-H2A incorporation was limited, and Flag-H2A.Z and Flag-macroH2A showed minimal incorporation into the nucleus. Active nuclear incorporation of all of these proteins occurred at the late pre-implantation stage (Fig. 3).

2. Dynamics of histone H2A variants during reprogramming in somatic cell nuclear transferred embryos

At present, somatic cell nuclear transfer (SCNT) is the only one experimental approach which can reprogram somatic nucleus to achieve totipotency. During this process, genomic reprogramming also occurs and previous works suggested that chromatin structure of transferred somatic nuclei may be remodeled after SCNT. As shown above, the deposition of histone H2A and its variants are dynamically changed after fertilization. Therefore, I also investigated chromatin incorporation and deposition of histone H2A, H2A.X and H2A.Z in the first cell cycle of the nuclear transferred embryos in which genomic reprogramming takes place.

First, we transplanted the condensed chromatin of ES cells expressing Flag-tagged histone H2A, H2A.X or H2A.Z into enucleated MII stage oocytes, respectively, and chased the changes in nuclear depositions of Flag-H2A variants after activation. As shown in Fig. 4A, signals for Flag-H2A, Flag-H2A.X and Flag-H2A.Z were readily detected in the reconstructed embryo when they had been incubated for 5 h without activation. However, signal for Flag-H2A or Flag-H2A.Z was faint or even undetectable while only Flag-H2A.X is

abundant in the transplanted nucleus of reconstructed embryo which had been activated and examined 5 h later. These results demonstrated that histone H2A and H2A.Z were rapidly lost from the chromatin of transplanted ES cell nucleus in activated embryos while only H2A.X remained.

Oocytes have a large pool of chromatin-free histones. To examine whether or not these oocyte-stored H2A variants are replaced with the original chromatin histones in the transplanted nucleus, MII stage oocytes were microinjected with mRNAs coding Flag-H2A, Flag-H2A.X or Flag-H2.Z. One hour after microinjection, they were enucleated and transferred with wild type ES cells and activated. As shown in Fig. 4B, Flag-H2A.Z signal was undetectable and only faint Flag-H2A was detected in the transplanted nucleus while Flag-H2A.X signal was readily detected 5 h after activation. These results demonstrated that only H2A.X was actively incorporated into the transplanted nuclei while the incorporation of H2A was limited and that of H2A.Z was even negligible. Taken together, only H2A.X is abundant while H2A and H2A.Z were declined or even disappeared from the transplanted nuclei. These were consistent with my findings in natural fertilization and developing process, which further suggested that abundant deposition of histone H2A.X and decline or disappearance of histone H2A and H2A.Z is required for the genome remodeling process.

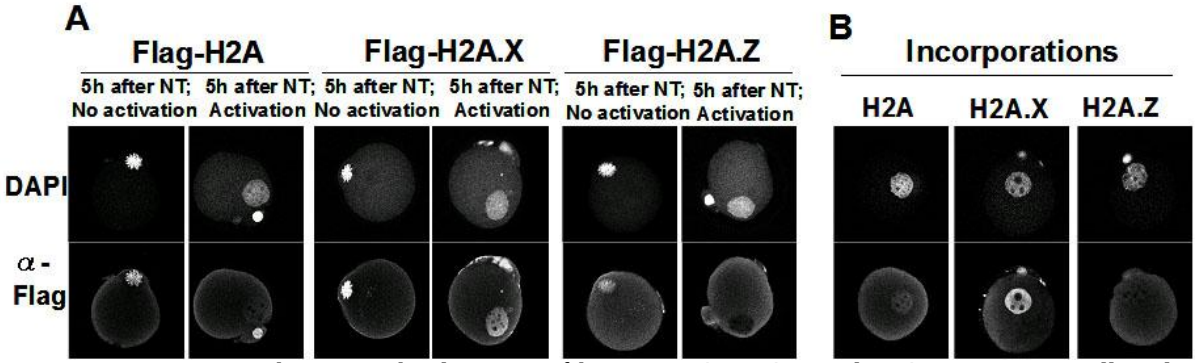

Fig. 4. Dynamic changes in the depositon of histone H2A, H2A.X and H2A.Z in somatic cell nuclear transferred embryos. (A) Chase of Flag-tagged histone H2A, H2A.X and H2A.Z in the nuclei of ES cells after they were transplanted into enucleated oocytes. (B) Incorporations of oocyte-stored histone H2A, H2A.X and H2.Z into transplanted nuclei.

3. The preferential incorporation of H2A.X into chromatin in early pre-implantation embryos involves its C-terminal domain

As described above, although the expression levels of all histone variants examined did not significantly change before and after fertilization, histone H2A and H2A.Z were rarely incorporated, whereas H2A.X was abundantly incorporated into the chromatin after fertilization. Therefore, I speculated that H2A.X sequences following residue 120 may play a role in H2A.X incorporation into chromatin after fertilization, since these C-terminal 23 amino acids (XC23) are unique among H2A variants.

To test this hypothesis, I first deleted the C-terminal 23 amino acids from H2A.X (H2A.XqC; Fig. 5A). When MII-stage oocytes were microinjected with H2A.XqC mRNA and then fertilized, only a weak Flag signal was detected in the nuclei of 1- and 2-cell embryos. Indeed, its signal intensity was much lower than that of intact H2A.X (Fig. 5B). Furthermore, although the wild type forms of H2A and macroH2A were poorly incorporated into chromatin after fertilization, the chimeric

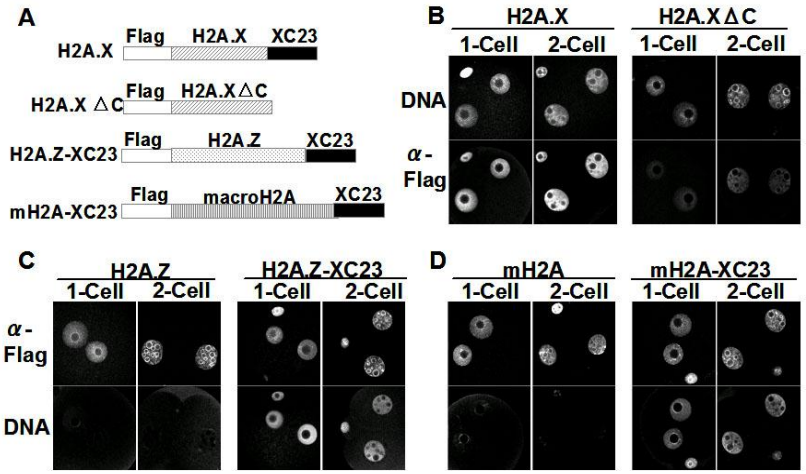

Fig.5. Various constructs encoding H2A variants with or without C-terminal 23 amino acids of H2A.X (A) and their incorporations into the nuclei of the embryos (B)-(D).

proteins H2A-XC23 and mH2A-XC23, formed by the respective fusion of H2A and macroH2A with the C-terminal 23 amino acids of H2A.X (Fig. 5A), were abundantly incorporated both in 1- and 2-cell embryos (Fig .5C and D). Taken together, these results suggest that the C-terminal 23 amino acids of H2A.X are responsible for its active chromatin incorporation after fertilization.

To investigate the biological significance of the absence of H2A.Z from the chromatin after fertilization, I examined the effect of H2A.Z-XC23 incorporation on development. MII-stage oocytes were microinjected with mRNAs encoding H2A.Z-XC23, H2A.X, or H2A.Z, and their developmental rates were examined after fertilization. Although ~90% of the embryos that had been microinjected with H2A.X or H2A.Z mRNA cleaved into 2-cell embryos at 17 hour post insemination (hpi), only 66% of H2A.Z-XC23 mRNA-microinjected embryos cleaved (Fig. 6A). The embryos that had not cleaved at 17 hpi were not arrested at the 1-cell stage but seemed to be delayed in their development, because the percentage of cleaved embryos increased to ~90% at 24 hpi.

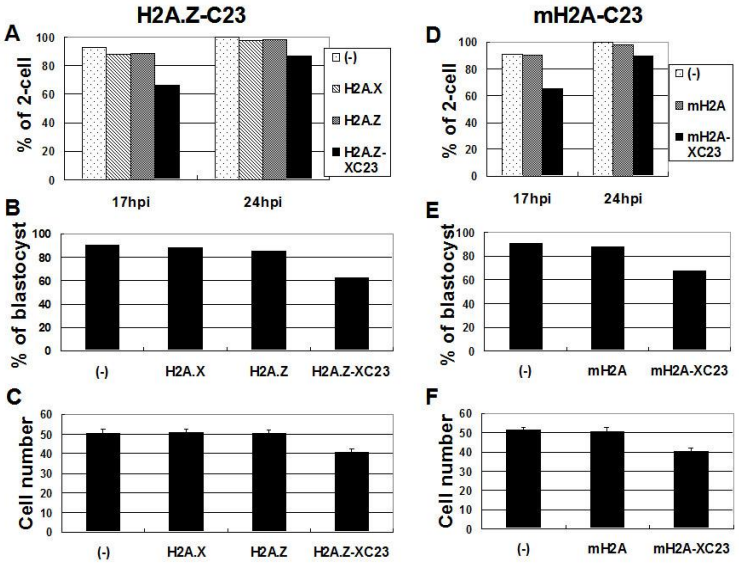

Fig.6 Nuclear incorporation of H2A.Z-C23 and mH2A-C23 cause developmental delay.

This delay in the early phase of development affected the rate of development to the blastocyst stage. Only 62% of embryos that had been microinjected with H2A.Z-XC23 mRNA developed to the blastocyst stage, whereas ~90% of the embryos microinjected with H2A.X or H2A.Z mRNA did (Fig. 6B). Even in the embryos that had developed into blastocysts, the number of cells was significantly lower in the H2A.Z-XC23 mRNA-microinjection group than in the other ones (Fig. 6C). Thus, H2A.Z-XC23 incorporation into chromatin has a detrimental effect on developmental progression. The nuclear incorporation of macroH2A-XC23 also showed similar effects on pre-implantation development (Fig. 6D, E & F). These results suggest that the absence of H2A.Z and macroH2A from the chromatin in early pre-implantation embryos is required for normal development.

Conclusion

In conclusion, chromatin deposition of histone variants changes dynamically during chromatin remodeling process not only after fertilization but also after somatic cell nuclear transfer. A summary of these changes and possible chromatin compositions are shown in Fig. 7. Formation of a unique chromatin structure due to the active incorporation (H2A.X) and elimination (H2A, H2A.Z and macroH2A) of particular variants seems to facilitate the remodeling event. Notably, a lack of epigenetic marks of active or inactive genes at the early pre-implantation stage due to a lack of H2A.Z or macroH2A, and an abundance of H2A.X, in the chromatin may be involved in establishing and maintaining totipotency and the high plasticity necessary for genome remodeling.

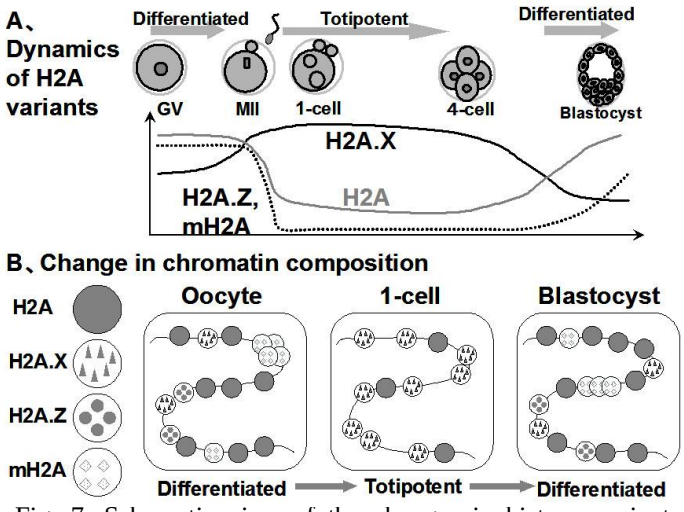

Fig. 7. Schematic view of the changes in histone variants nuclear abundance during oogenesis and pre-implantation development.