博 士 論 文 の 要 約

博士の専攻分野の名称： 博　士（食資源学）　　氏名　　沖宗　慶一

学 位 論 文 題　　名

Development of a nucleosome assembly method using a wheat germ cell-free co-expression system
（コムギ胚芽無細胞タンパク質共翻訳によるヌクレオソーム再構成法の確立）

Background

In the field of chromatin research, the biochemical and structural approaches have provided important evidence to fill the knowledge gap determined in physiological studies. In eukaryotes, a genome DNA is compacted in the cell nuclei as a form of chromatin. The basic building block of the chromatin is a nucleosome, composed of four histones, H2A, H2B, H3, and H4, and tightly wrapped by ~1.7 times DNA. In yeast, the correlations between chromatin structure and gene regulation are well described, in which genes in the regions with the highly compacted chromatins (closed) are depressed, while those within the less compacted chromatins (opened) are actively expressed. The recent advent of the next generation sequencing together with chromatin immunoprecipitation (ChIP-seq) using anti-histone antibodies enables the mapping of nucleosome density in a whole genome, corresponding to either silent or active gene regions in other eukaryotes, including human, animals, plants, and others. Several factors are known to contribute to the alteration of chromatin structure and function, mainly through genetic and ChIP-seq approaches. For example, a set of posttranslational modifications (PTMs) on the histone proteins serves as a marker for packed chromatin regions, noncanonical histones (histone variants) can be found in the transcriptional start sites in the opened chromatin regions, and others. Thus, somehow, regions of chromatins are assembled into either opened or closed structures by chromatin assembly factors and chromatin remodeling factors in the cells. However, molecular mechanisms of how these chromatins are assembled, how histone variants contribute to change chromatin structure, and how PTMs of dozens of combinations result in different chromatin functions are still unknown.

To understand chromatin function and structure, it is important to analyze nucleosome biochemically. A salt dialysis-based nucleosome assembly has been widely used for structural and biochemical studies, yet several technical limitations exist. For example, histone proteins needed to be prepared using the recombinant method, which results in insoluble products and subsequent refolding is necessary. Furthermore, assembled nucleosomes are stable such that they may be irrelevant products for biochemistry. Recently, a cell-free wheat germ co-expression chromatin assembly methods were developed in our laboratory, which solved the above limitations (Okimune et al., 2020, BMC

biotechnology; Endo et al., 2020, FEBS Open bio; Okimune et al., 2021, FEBS Open bio). By using the method, a highly purified form of assembled human chromatins was prepared by optimizing several purification steps, and structures were determined by Cryo-EM analysis.

Methods

In vitro co-expression and chromatin assembly method was utilized to reconstitute three different types of human chromatins containing a canonical histone H3.1, and variants H3.3 and H3.X (Fig. 1). Core histone-coding mRNAs were synthesized individually by *in vitro* transcription using SP6 RNA polymerase in the presence of a template plasmid pEU vector carrying each histone gene. Transcripts were measured at 260 nm and 280 nm and the RNA concentration was estimated at 260 nm followed by RNA purification (Okimune et al., 2020, BMC biotechnology). Co-expression chromatin assembly was performed using a wheat germ cell-free protein synthesis platform and chromatins assembled on the 3x601 Widom nucleosome positioning sequences, which were confirmed by electrophoresis mobility assay (EMSA), SDS-PAGE, and Micrococcal Nuclease (MNase) analyses (Okimune et al., 2021, FEBS Open bio). The purification of chromatins was performed by Mg^{2+}-precipitation (Endo et al., 2020, FEBS Open bio) followed by sucrose gradient ultracentrifugation in the presence of paraformaldehyde (GraFix) (Kastner et al., 2008, Nature Methods). Purified chromatins were subjected to Cryo-EM analysis using Krios G4 (Thermo Fisher Scientific) in the Faculty of Pharmaceutical Sciences, Hokkaido University. Cryo-EM images were used for three-dimensional small particle reconstitution using RELION software (https://relion.readthedocs.io/en/release-5.0/). The 3D models were built and refined using Coot with Phenix software (https://phenix-online.org/download/other.html).

Figure 1. Multiple amino acids sequence alignment of three human histone H3s. Three human histones, H3.1, H3.3, and H3.X were sequence aligned, in which blue showed H3.X specific amino acid and green showed Variants-specific amino acids.

Results and Discussion

Reconstituted and purified H3.1-containing chromatin were analyzed by EMSA, SDS-PAGE, and MNase assays (Fig. 2). Results suggested that H3.1-containing chromatin was in the form of high purity and four core histones were in a stoichiometry. Furthermore, around 150 bp DNA was determined to be wrapped around core histone octamer, altogether suggesting that reconstituted and purified H3.1-containing chromatin is ready for structural study. Analogously, H3.3 and H3.X-containing chromatins were reconstituted and purified.

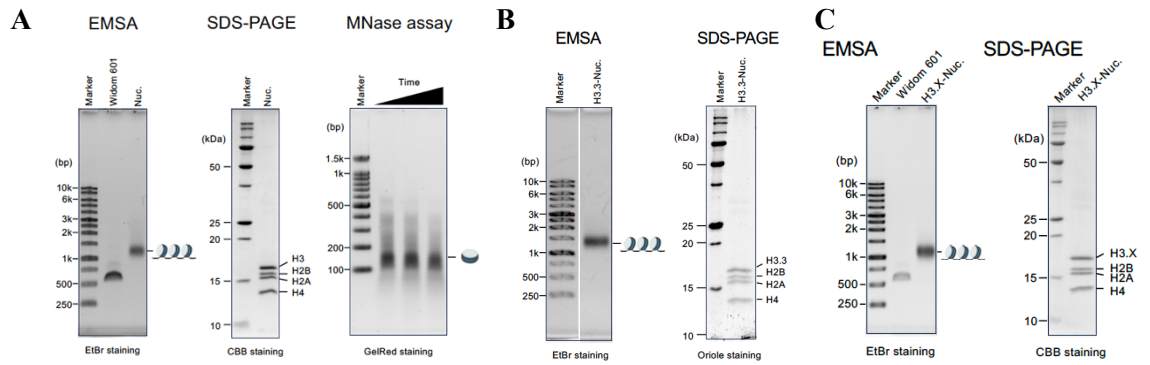

Figure 2. Assessment of reconstituted and purified H3.1-, H3.3-, and H3.X-containing chromatins. H3.1-containing chromatin was analyzed by EMSA, SDS-PAGE, and MNase assays (**A**). H3.3- and H3.X-containing chromatins were analyzed by EMSA and MNase assays (**B** and **C**, respectively).

Three-dimensional structures of H3.1-, H3.3-, and H3.X-containing chromatins were determined by Cryo-EM analysis with 2.88Å, 2.95Å, and 3.26Å, respectively (Fig. 3). As expected, the core of nucleosomes occupied by four core histones are tightly wrapped by around 150 bp double-stranded DNA. Structural results support that the chromatins reconstituted by co-expression chromatin assembly method are in agreement with the structures determined by previous studies.

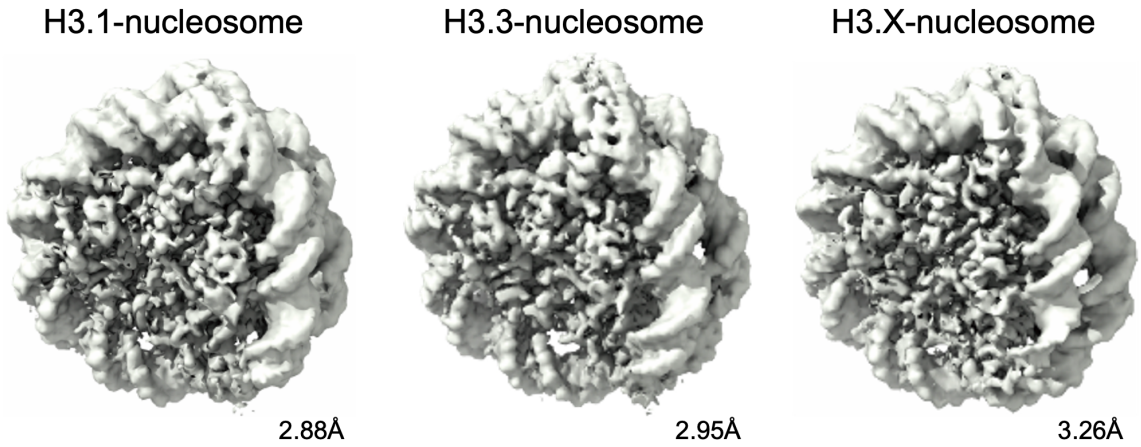

Figure 3. Three-dimensional structures of H3.1-, H3.3-, and H3.X-containing chromatins analyzed by Cryo-EM structural determination.

Conclusion

In vitro reconstituted chromatins under a near physiological condition were structurally determined for the first time. The obtained structures seemed to be consistent with the previously reported three-dimensional structures of chromatins, which were reconstituted by a conventional salt dialysis methodology. Since the method described herein enables the obtaining of functional chromatins, it will be possible to reconstitute chromatin and nuclei protein interaction in the future research.