様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

科学研究費助成事業　　研究成果報告書

令和　元 年　 ６ 月　 ４ 日現在

機関番号：８２４０１
研究種目：若手研究(B)
研究期間：2017～2018
課題番号：１７Ｋ１８３５８

研究課題名（和文）Detecting combinatorial histone modification dynamics at single nucleosome resolution for key genes in Epithelial-Mesenchymal-Transition

研究課題名（英文）Detecting combinatorial histone modification dynamics at single nucleosome resolution for key genes in Epithelial-Mesenchymal-Transition

研究代表者

チャン　レンチエン（Chang, Jen-Chien）

国立研究開発法人理化学研究所・生命医科学研究センター・研究員

研究者番号：１０７９２１３７

交付決定額（研究期間全体）：（直接経費）　　2,600,000円

研究成果の概要（和文）：ヒト細胞は、それぞれの細胞種ごとに異なるゲノム領域が特定の組み合わせで用いられることにより、特有の形態および機能を有する。本研究では、健康な細胞状態を維持するためのメカニズムの一つであるヒストン修飾に焦点を当てた。従来の解析手法では、同一ヌクレオソーム上に存在するヒストン修飾のコンビネーションを正確に同定することができなかった。そこで本研究では、内在性クロマチンの複数のヒストン修飾を同時に検出する、高感度単一分子イメージングアッセイを開発した。さらに、上皮間葉転換（EMT）の各段階で認められる特徴的なクロマチン状態に特異的な候補遺伝子リストを同定した。国内外の学会等で本成果に係る発表を行った。

研究成果の学術的意義や社会的意義
The developed method for measuring single nucleosomes has reached an unprecedented resolution and expanded the toolbox for epigenetics research. Furthermore, the biological process EMT studied here is critical for cancer metastasis. Understanding its mechanism is the basis to improve human health.

研究成果の概要（英文）：Each human cell type has a unique morphology and function enabled by the usage of specific combinations of different parts of the genome. This research focused on histone modifications, which is one of many molecular levels that are tightly regulated to maintain a healthy cell status. Multiple histone modifications can co-occur on the same nucleosome and function together; however, conventional method cannot identify the accurate combinatorial histone modification in a heterogeneous cell population. In this research, a highly sensitive single-molecule imaging assay was developed to simultaneously detect multiple histone modifications on single nucleosomes extracted from cells. Moreover, to understand Epithelial-Mesenchymal Transition (EMT), a list of candidate genes with varied chromatin states during EMT was identified for further investigation. The results have been presented in several domestic and international conferences.

研究分野：Epigenetics

キーワード：nucleosome　histone modification　chromatin state　single-molecule imaging　EMT　enChIP　epigenetics　bivalent promoter

１．研究開始当初の背景

(1) What is combinatorial histone modification?

The fundamental repeating unit of chromatin in eukaryote cells is the nucleosome, which consists of DNA wrapped around core histones containing two copies of the proteins H2A, H2B, H3 and H4. While the DNA carries the genetic code of trait inheritance, epigenetics mechanisms can affect how and which sets of gene are expressed and therefore control phenotype. Epigenetic mechanisms include DNA methylation, histone modifications (HM), non-coding RNAs, and physical organization of chromosomal structures. These epigenetic features combine to form specific "epigenetic states," which are cell-type-specific and dynamic. Modulation of the epigenome is important to normal cellular differentiation and development, and dysregulation of the epigenetic states can lead to diseases. For example, it has been shown that tumor formation can be induced by perturbing epigenetic states without modifying genomic sequences. Therefore, being able to assay the complex epigenomic features will help in understanding the basic biological processes as well as disease mechanism.

Individual HMs are identified and correlated to particular functional genomic elements. For example, trimethylation at lysine 4 of histone H3 (H3K4me3, similar abbreviation hereafter) is usually found to mark active promoter region. The histone code hypothesis further states that the combinations of HMs can implicate unique biological outcomes. It is supported by the fact that various chromatin mediators were found to contain multiple binding domains for combinations of histone modifications. Moreover, some specific PTM combinations and the protein complex recognizes them are also associated to breast cancer. In addition, active histone mark H3K4me3 is found to coexist with a repressive H3K27me3 in the so-called "bivalent domain" at developmentally regulated genes in embryonic stem cells. Also, active enhancer regions are often marked by H3K4me1 and H3K27ac. All these evidences suggest that <u>histone modifications do not function alone, but rather in combinations</u>.

(2) Why we need a new method for measuring combinatorial histone modifications?

Chromatin Immunoprecipitation followed by next-generation sequencing (ChIP-seq) is the most common tools in epigenomics. ChIP-seq experiment measures the genomic location of HM (or DNA-binding proteins) in a given sample. Thousands of genome-wide histone modification maps in various cell types has been generated by large international consortiums such as ENCODE and Roadmap Epigenomics. These data serve as an important reference and pave the roads for many researches. However, ChIP-seq has two limitations: [1] it can only analyze one HM at a time, and [2] it measures the average states among cell populations. Whether the "chromatin states" found by computational association studies of multiple ChIP-seq data-sets really represent the true combinations of HMs on the same nucleosome is still unknown. As shown in Fig. 1, the overlapped peak regions identified by comparing two independent ChIP-seq experiments can be a result of cell-to-cell heterogeneity. Therefore, <u>being able to directly measure each single nucleosome is the key to understand the true combination of histone modifications and their biological meaning.</u>

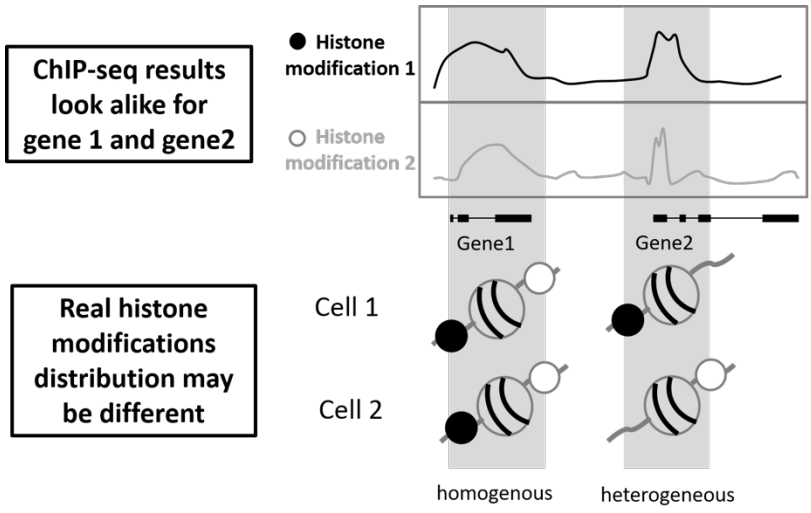

Figure 1: Current difficulties of ChIP-seq: it cannot distinguish combinatorial heterogeneity of two or even more histone modifications.

Several efforts have been put forward to attack this challenge, such as sequential-ChIP, mass spectrometry, and Single Chromatin Analysis in Nanochannels. However, none of them can simultaneously probe more than two histone modification on a single nucleosome with

its genomic information. Therefore, this Kakenhi project was set to develop a new method for detecting combinatorial histone modification.

(3) What is EMT and why I applied the new method to it?

Epithelial to Mesenchymal Transition (EMT) is a phenomenon that cell loses its adhesive nature and turn into a more migratory and invasive state. It happens in the normal cell process such as embryonic development, but is also linked to cancer metastasis and progression which arouse attention in recent years. Many studies have found several key genes such as ZEB1 and SNAI1 which regulates EMT; however, due to the highly plastic and heterogeneous nature of this process, its intermediate epigenetic states and the point of irreversibility are not yet understood. By probing the combinatorial epigenetic modifications for key genes at single nucleosome resolution, I expect to provide deeper understanding to the molecular mechanism of EMT.

２．研究の目的

The research purposes for this Kakenhi project were:

Aim 1: (a) Developing a method to measure combinatorial histone modifications for single nucleosomes extracted from cells. (b) Combining CRISPR-pull-down to enrich targeted nucleosome from single genomic locus.

Aim 2: Investigating the intermediate states critical in EMT process, by comparing bulk ChIP-seq and the new method.

３．研究の方法

This research was based on a method developed in the lab - Single-molecule imaging of multiple histone modifications on a single nucleosome.

I have set up the required TIRF microscope with single-molecule sensitivity, basic experimental protocol, and the analysis procedure for imaging multiple histone modifications on a single nucleosome as shown in Fig 2. Chromatin is extracted from the cell and fragmented by Micrococcal Nuclease (MNase). Mononucleosome fraction is enriched by ultracentrifuge in glycerol gradient followed by fractionation. We check by gel electrophoresis to ensure there is no contamination of polynucleosome. The extracted nucleosome is ligated to a dsDNA adapter conjugated with biotin and Alexa-488 dye. The complex is immobilized to a slide surface coated with PEG-biotin-neutravidin. The surface is enclosed in a house-made minifluidic channel or temperature controlled Bioptech microfluidics system. Fluorescent antibodies are injected in the chamber (< 1nM), allowing dynamic binding with their target HMs, and images are taken every 15 min (until equilibrium, ~6h depending on antibodies) using a house constructed 3-color single-molecule TIRF microscope. Next, antibodies are washed by buffer and replace for HM3 and HM4, and imaged. The imaging cycle is repeated until all modifications of interest are imaged. Finally, quantitative single-molecule colocalization analysis are done by customized program written in ImageJ Macro and Icy to extract the HM co-occurrence and exclude the false positive signal.

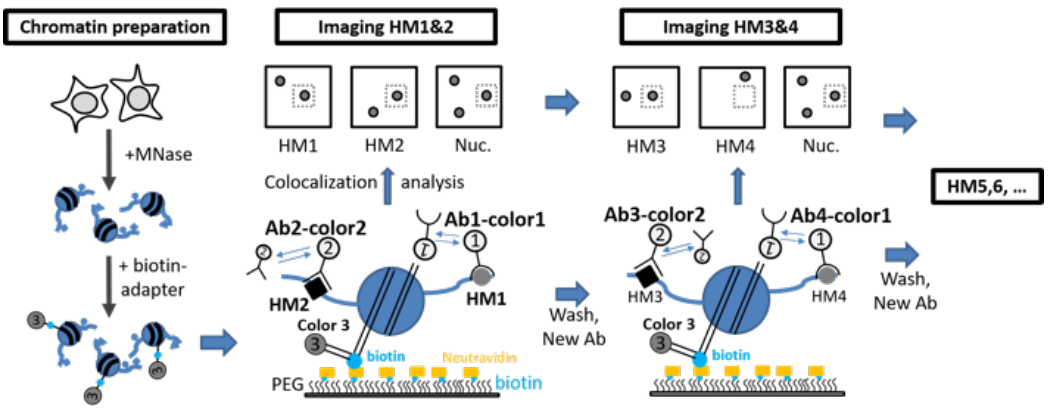

Figure 2: Single-molecule imaging of multiple histone modifications on single nucleosomes.

4．研究成果

(1) Optimization of single-molecule imaging protocols

Based on the original methods described, we have further optimized the protocol. The number of cells required for sample preparation is less than 100k, and in one microfluidics channel we can obtain data for more than 100k nucleosome in less than 6 hours imaging time. An example imaging data is shown in Figure 3, where in the same view we are able to capture single nucleosomes with all 4 combinations of two histone marks (H3K27ac and H3K4me3) from MCF10A cell.

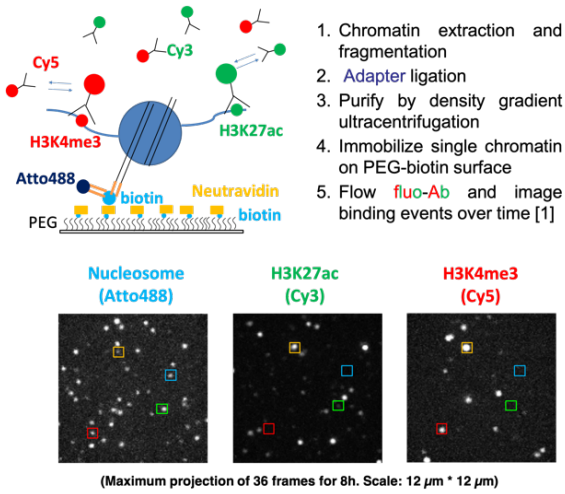

Figure 3: Example data for simultaneously detecting H3K4me3 and H3K27ac on the single nucleosome.

(2) Nucleosome extraction from a single genomic locus

In order to gain insight of combinatorial state of HMs at a single gene locus, CRISPR/Cas9 system was proposed to target and enrich nucleosome from pre-determined genomic region for imaging. To test this idea, *in vitro* Engineered DNA-binding molecule-mediated chromatin immunoprecipitation (*in vitro* enChIP) followed by DNA sequencing was performed. Three guide RNAs are designed and pooled to target *CDH1* promoter in MCF10A cell. After immunoprecipitation, the DNA is amplified for the next-generation sequencing (one lane, Illumina Hiseq 2500). The sequencing result is shown in Fig. 4, reads are successfully enriched at *CDH1* promoter as expected, whereas the gRNA(-) control has no enrichment. In the meantime, we also identified genomic regions interacting with *CDH1* promoter. These regions overlapped with ATAC-seq peaks and/or differentially accessible peaks during EMT, indicating their potential regulatory roles.

Since our goal is to purify the nucleosome from targeted locus for imaging, we calculated the signal-to-noise ratio, and found that there are 364 reads falling in *CDH1* promoter peak, out of ~ 33M total unique reads. Although the enrichment over gRNA(-) control is over 3-folds, at the single nucleosome level only one in 10^5 can give true signal. We therefore concluded it is not practical to proceed the single nucleosome imaging with this strategy.

Another route to obtain genomic information after nucleosome imaging currently undergoing is to integrate on-site single-molecule sequencing. This concept has been recently demonstrated by another group. However, the sequencing throughput is yet low. Further improvement has to be made for practical usage.

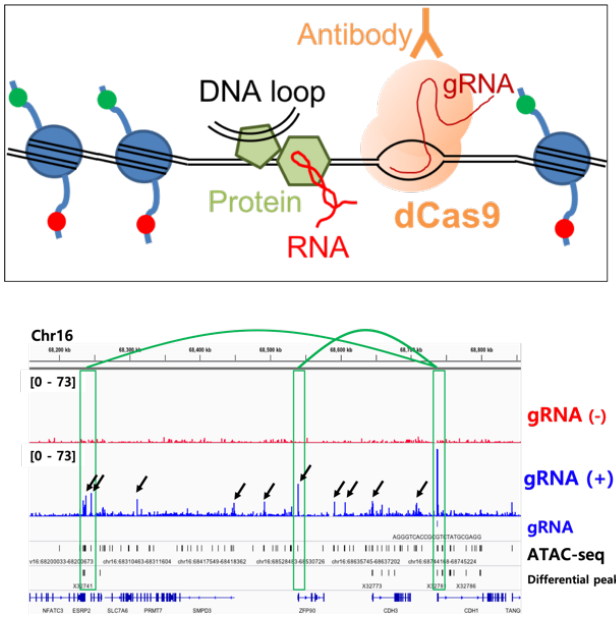

Figure 4: in vitro enChIP-seq targeting CDH1 promoter in MCF10A.

(3) Intermediate chromatin states critical in EMT process

The second aim in this project is to investigate the intermediate chromatin states critical in EMT. An inducible EMT model system (by Zeb-1 overexpression) in MCF10A cell line is established and provided by the collaborator Dr. Kazuhide Watanabe, RIKEN IMS. When Doxycycline is added in the medium, Zeb 1 will be overexpressed and cells undergo EMT in less than 48 hours (Fig.5, upper cell images).

Bulk ChIP-seq for six histone modifications (H3K4me1, H3K4me3, H3K9me3, H3K9ac, H3K27ac, H3K27me3) at 0h and 24h after EMT induction were conducted. After basic read processing, genome mapping, peak calling, and quality control, chromatin states analysis (ChromHMM) was performed to model combinatorial histone modifications as shown in Fig. 6.

By systematic comparison of the chromatin states at 0h and 24h, as well as integration with EMT-related genes identified by CAGE, we generated a list of genes with intermediate chromatin state transition during EMT, including transcription factor *FOXC2* (E gene, expression down-regulated in EMT) and *MAFB* (M gene, opposite to E gene) (Fig. 5 & 6). These candidate genes may have critical roles for EMT and worth follow-up investigation.

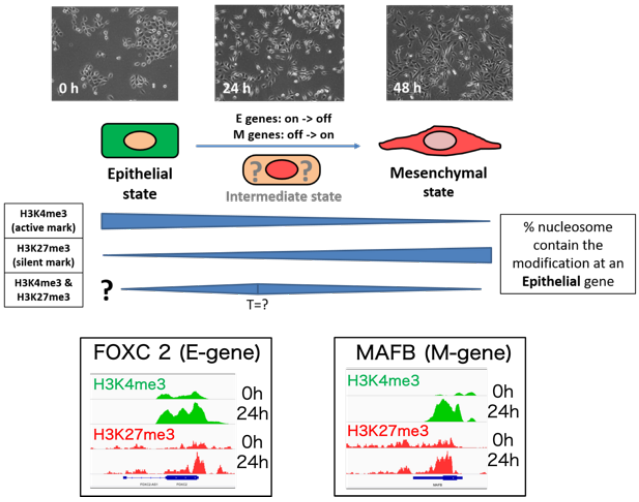

Figure 5: Time course ChIP-seq for an inducible EMT model system. Two critical transcription factor shows bivalent chromatin states at intermediate time points.

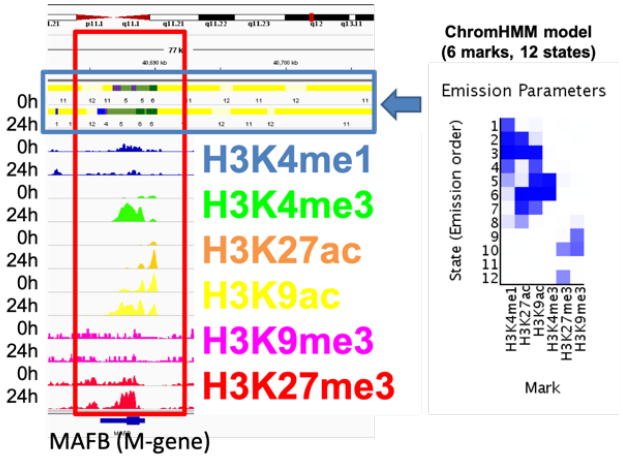

Figure 6: Chromatin states learned from 6 histone marks for 0h and 24h at *MAFB* locus.

5．主な発表論文等

〔雑誌論文〕（計 0 件）

〔学会発表〕（計 6 件）

① Jen-Chien Chang et. al., Towards chromatin state detection at higher resolution, contents, and throughput, Human Genome Meeting, 2019.

② Jen-Chien Chang et. al., Single-molecule detection of combinatorial histone modifications for key genes in Epithelial-Mesenchymal-Transition, The 41st Annual Meeting of MBSJ, 2018.

③ Jen-Chien Chang et. al., Single-molecule detection of combinatorial histone modifications for key genes in Epithelial-Mesenchymal-Transition, The 56th Annual Meeting of The Biophysical Society of Japan, 2018.

④ Jen-Chien Chang et. al., Mapping combinatorial epigenetic modifications at single nucleosome resolution, The 62nd Annual Meeting of Biophysical Society, 2018.

⑤ Jen-Chien Chang et. al., Mapping combinatorial epigenetic modifications at single nucleosome resolution, The 12th International Workshop on Advanced Genomics, 2017.

⑥ Jen-Chien Chang et. al., Mapping combinatorial epigenetic modifications at single nucleosome resolution, Consortium of Biological Sciences (ConBio) 2017, 2017.

〔図書〕（計 0 件）

〔産業財産権〕

○出願状況（計 0 件）
○取得状況（計 0 件）

〔その他〕
ホームページ等
https://orcid.org/0000-0002-6986-8479
http://www.ims.riken.jp/labo/68/research.html

６．研究組織

(1)研究分担者
研究分担者氏名：
ローマ字氏名：
所属研究機関名：
部局名：
職名：
研究者番号（8 桁）：

(2)研究協力者
研究協力者氏名：
ローマ字氏名：

※科研費による研究は、研究者の自覚と責任において実施するものです。そのため、研究の実施や研究成果の公表等については、国の要請等に基づくものではなく、その研究成果に関する見解や責任は、研究者個人に帰属されます。