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Review

Large chromatin domains in pluripotent and differentiated cells

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Pluripotent stem cells are able to proliferate unlimitedly and to generate all somatic cell types, thus holding a great promise in medical applications. Epigenetic modifications are believed to play crucial roles in regulating pluripotency and differentiation. Recent genome-wide studies on mammalian systems have revealed several types of large chromatin domains which are associated with higher-order organization of the genome. The elucidation of genomic distribution and dynamics of these domains have shed light on the mechanisms underling pluripotency and lineage commitment.

Keywords pluripotent stem cell; epigenetics; chromatin

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Introduction

Pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), are able to propagate themselves infinitely (self-renewal) and to differentiate into all types of somatic cells (pluripotency). The dual capacities have made PSCs ideal systems for biological researches as well as promising tools in cell-based therapy, disease modeling, and drug screening [1]. ESCs are derived from inner cell mass (ICM) in the pre-implantation embryo [2–4], whereas iPSCs are reprogrammed from somatic cells by forced induction of pluripotency-associated transcription factors [5–7] or RNA molecules [8,9]. Although iPSCs were thought as ESC-like, recent evidence has indicated that the two types of PSCs may be different in genetic and epigenetic stability [10].

Epigenetics is the study of mitotically heritable information which is independent of DNA sequence alterations [11]. Epigenetic mechanisms are crucial for normal development, and aberrant epigenetic programming has been linked to the occurrence of diseases. Furthermore, epigenetic modifications are sensitive to environmental cues and are reversible by the treatment of epigenetic drugs. Thus, epigenetic has become an 'epicenter' in modern medicine [12]. While core pluripotent factors (OCT4, NANOG, and SOX2) and their regulatory circuitries are thought to govern pluripotency [13,14], epigenetic mechanisms play important roles in maintaining pluripotency and in fixing the identities of differentiated cells. During cellular differentiation and reprogramming, cells obtain new phenotypes which are stable during cell divisions, yet their DNA sequences remain largely unchanged. Furthermore, knocking-out of epigenetic machineries resulted in embryonic lethality *in vivo* and impaired ES cell differentiation *in vitro* [15], pointing to the importance of epigenetic modifications in development. Hence, the elucidation of epigenetic signatures will help us to better understand the molecular mechanisms underlining self-renewal, differentiation, and reprogramming.

In the nucleus, DNA and its associated proteins are packed into chromatin. The nucleosome, the fundamental unit of chromatin, consists of an octamer of core histones (two each of H2A, H2B, H3, and H4), and 147 bp of DNA wrapped around the core histone particle. The N-terminal tails of histone often experience covalent modifications which are involved in multiple biological processes including transcriptional regulation, DNA damage repair, and alternative splicing [16]. The functional relevance of a modification relies on the type of modification and the position it occur. Histone acetylation is associated with open chromatin and gene activation, the characteristics of euchromatin. However, histone methylation is functionally versatile: methylation on H3K4, H3K36, and H3K79 are euchromatic marks, whereas those occurred on H3K9, H3K27, and H4K20 are generally associated with repressive chromatin. Furthermore, methylation on same positions may have different biological outcomes according to the amount of methyl group. For example, di- and trimethylation of H3 lysine-9 (H3K9me2 and H3K9me3) are repressive marks, whereas H3K9me1 is associated with active chromatin. The detailed genomic distribution pattern of these marks had been discussed elsewhere [17–19].

Epigenetic modifications including histone marks can be involved in multiple layers of epigenetic regulation. In the first layer, modifications on regulatory regions such as promoters and enhancers can change chromatin structure locally, thereby affecting the expression of adjacent genes. Most of epigenomic studies have focused on this layer. In higher layers, large chromatin blocks can co-regulate gene expression, form higher-order chromatin, and influence chromosome positioning. Recently, the researchers have started to explore this attractive field. In the last decade, with the advent of high-throughput technologies including microarray and next-generation sequencing, analyzing epigenetic modifications in genome scale (epigenomics) has become prevalent. Extensive epigenomic experiments have been performed to decipher epigenetic profiles in PSCs and their differentiated counterparts. Here we highlight recent advances on chromatin signatures in PSCs and differentiated cells, with focuses on large chromatin domains which affect higher-order chromatin and nuclear architecture.

Functional Heterogeneity in PSC Cultures

As retaining properties of pluripotency, PSCs obtained from current culture conditions are generally regarded as homogenous cell population. However, recent evidence has demonstrated that the PSC cultures contain functionally distinct subpopulations. For example, at least two major subpopulations have been identified in mouse ESC cultures: one is ICM like, and the other is epiblast like. While the pluripotent gene *Oct4* is equally expressed in both states, the ICM-like cells have higher expression levels on the stem cell markers such as *Nanog*, *Rex1*, *SSEA1*, and *Stella* [20–23]. Similarly, human ESC cultures can also be divided into multiple subsets by two surface markers, *c-KIT* and *A2B5* [24]. More importantly, these subsets in both mouse and human appear differentiation bias and carry different epigenetic marks on some of the stem cell genes. As sharing the same culture conditions, iPSCs can be speculated to contain subpopulations as well. Thus, these studies have indicated that *in vitro* PSC cultures are functionally heterogenous cell populations. As epigenomic studies cannot be conducted at a single-cell level, one should use cautions to explain data from these mixed populations.

Open Chromatin Structure in Pluripotent Cells

The chromatin of PSCs, unlike that of differentiated cells, appears some interesting features including more open conformation, looser binding with its associated proteins, and higher physical softness [25–28]. By using electron spectroscopic imaging, a recent study examined the dynamics of chromatin structure from one-cell to early post-implantation embryos, and found that the chromatin of eight-cell embryos and ICM cells are highly dispersed and indistinguishable from that of ESCs. However, trophectoderm and primitive endoderm cells displayed higher chromatin compaction levels, thus providing *in vivo* evidence for more open chromatin in PSCs [29]. Consistent with these observations, global transcription in both coding and non-coding regions was found in ESCs, but the transcription pattern became more limited upon differentiation [30]. Taken together, such evidence supports the notion that ESCs have a higher plasticity compared with the differentiated cells.

Bivalent Domains

Histone 3 lysine-4 trimethylation (H3K4me3) and lysine-27 trimethylation (H3K27me3), catalyzed by trithorax-group and polycomb-group (PcG) proteins, respectively, are classic epigenetic marks essential for development. H3K4me3 is associated with gene activation, whereas H3K27me3 is linked to gene repression. Intriguingly, the two functionally opposite modifications are highly co-enriched on the promoters of key developmental genes in both mouse and human ESCs, but less co-exist in differentiated cells [31–34]. In addition, genome-wide distribution pattern of H3K4me3 and H4K27me3 are almost indistinguishable between human ES and iPS cells [35]. Genes marked by these 'bivalent domains' [32] are largely silenced in pluripotent cells. On differentiation, some bivalent domains selectively resolve to monovalent marks of either H3K4me3 or H3K27me3, resulting in gene activating or remaining silencing in a lineage-specific manner. It was proposed that bivalent domains reflect a chromatin state 'poised' for activation, in agreement with a higher plasticity in stem cells [32].

Furthermore, whole-genome chromatin immunoprecipitation (ChIP)-seq analysis of histone modifications identified >2000 of bivalent domains in mouse ESCs, most of which resolve to monovalent status in lineage-committed cells [36]. Interestingly, the majority of bivalent domains are located on CpG-rich promoters, whereas CpG-poor promoters are generally absent of both H3K4me3 and H3K27me3. Genome-wide mapping of PcG complexes (PRC1 and PRC2) in mouse ESCs indicated that bivalent domains can be separated into two classes: the first class overlaps both PRC1 and PRC2 and the second class overlaps only PRC2. Bivalent domains of the first class are evolutionarily conserved and highly associated with developmental regulator gene promoters [37]. RNA immunoprecipitation-seq experiments have identified >9000 transcripts physically associated with PRC2.

Notably, 21% of bivalent domains in mouse ESCs show PRC2-interacting transcripts, suggesting that RNAs may regulate stem cell fate by recruiting PcG complexes to bivalent domains [38].

Recently, Hong *et al.* analyzed H3K4me3 and H3K27me3 in fractionated human ESC subsets, and found that some lineage-specific genes showing bivalency in unfractionated ESCs are actually monovalent in the functionally distinct cell subpopulations, although pluripotent genes (*OCT4* and *NANOG*) are comparably expressed in these subsets. These data suggested that the observation of bivalent domains may reflect the heterogeneity of ESC cultures [24]. Nevertheless, further investigations, especially large-scale analysis of fractionated PSCs, are needed to confirm this observation.

Large Heterochromatin Domains

Due to the 'gene-center' tradition in this field, most of epigenomic studies have focused on small regions of histone modifications on regulatory elements such as promoters and enhancers. Nevertheless, recent literatures have started to expand the scope to explore broader modifications on other parts of the genome. Using native ChIP coupled with microarrays (NChIP-chip) and a novel statistical strategy designed to find large domains, Wen *et al.* [39] analyzed the genome-wide distribution of histone 3 lysine-9 dimethylation (H3K9me2), a mark of facultative heterochromatin, in undifferentiated mouse ESCs, *in vitro* differentiated ESCs, and two primary tissues (liver and brain). The authors found surprisingly large stretches of H3K9me2 modifications termed as large organized chromatin K9-modifications (LOCKs), which affect up to 46% of the genome. Genomic regions with LOCKs are generally gene poor and with low density of CpG dinucleotides, and the boundaries of LOCKs enrich for the binding of CCCTC-binding factor (CTCF), the major insulator in mammalian genome. LOCKs are highly conserved between human and mouse and are strongly associated with domain-wide gene regressing in a tissue-specific manner. The formation of LOCKs relies on G9a, a histone methyltransferase depositing H3K9. More importantly, using the same statistical criteria that defined tissue specific LOCKs, they showed that both the genome coverage and domain size increase from undifferentiated ESCs to *in vitro* differentiated cells (coverage, 4% vs. 31%; average size, 43 vs. 93 kb). The authors further proposed that LOCKs may facilitate the epigenetic memory of cell-type-specific higher-order chromatin in differentiation and development [39].

By reanalyzing the original datasets of Wen *et al.*, Filion and Steensel [40] claimed that there is no fundamental difference between H3K9me2 domains in undifferentiated ESCs and differentiated cells. Of note, the two-state hidden Markov model algorithm they used may have omitted quantitative differences of LOCKs between the two cell types [41]. Recently, Lienert *et al.* [42] mapped H3K9me2 on promoters and chr19 in mouse ESC and neurons differentiated from ESCs, and they claimed that H3K9me2 modifications cover >50% of the chr19 genome in both cell types with only a 5% increase in neurons [42]. Interestingly, quantitative differences of H3K9me2 have also been detected in the Lienert's study, as indicated by quantitative polymerase chain reaction (PCR) results in their Figure 2(B) [42]. Furthermore, it is known that neuronal lineages have higher plasticity, as also observed by Wen *et al.*, which shows that LOCKs are much less present in brain (10%) than in liver (46%), indicating that the dynamics of H3K9me2 in differentiation may be lineage specific.

In summary, the core issue of these debates is whether the quantitative differences of LOCKs are due to detection bias or reflecting underling biological complexity. Given recent observations that ESC cultures are functionally heterogenous, one should be very careful to explain the weaker signals seen in the datasets of ESCs. Further investigations on fractionated ESC populations should provide key evidence to address these debates. Furthermore, a global reduction of H3K9me2 LOCKs and domain-wide increase of H3K4me2 in GC-rich LOCKs are observed during epithelial-to-mesenchymal transition (EMT), a process acquiring stem cell traits [43], suggesting that quantitative difference of LOCKs may be functionally relevant. Interestingly, this epigenomic reprogramming is dependent on the function of histone demethylase lysine specific demethylase 1, which may have dual roles in repressing and activating by forming different complexes during EMT [43].

In addition, other types of repressive histone marks, H3K27me3, and lysine-9 trimethylation (H3K9me3), are also found to form broad domains in mammalian genomes. Pauler *et al.* [44] applied an algorithm named broad local enrichments to analyze ChIP-chip data generated in mouse embryonic fibroblasts on chr17, and they found large blocks of H3K27me3 in both silenced genes and intergenic regions. This observation was confirmed by reanalyzing published ChIP-seq datasets using the same algorithm [44]. Furthermore, genome-wide analysis in human ESCs and fibroblasts using ChIP-seq shows that the genome coverage of H3K27me3 is 4% each in ESCs, but increases to 12% (H3K27me3) and 16% (H3K9me3) in fibroblasts [45]. The sizes of domain also increase for both makers. Introduction of fibroblast into iPSCs is associated with substantial reduction of these repressive domains [45]. Collectively, these data demonstrate that the process of stem cell differentiation may accompany with large-scale expansion of heterochromatin domains, although its generality is awaited for further determination in other lineages.

Lamina-Associated Domains

It has long been recognized by electron microscopy that heterochromatin usually locates on nuclear periphery in interphase nucleus [46]. Furthermore, artificially tether of genes on nuclear lamina leads to heritable gene repression [47], suggesting an important role of lamina association in epigenetic regulation. Guelen *et al.* [48] constructed a genome-wide map of chromatin−lamina interactions in human fibroblasts using the DamID approach. These authors defined ∼1300 lamina-associated domains (LADs), with sizes of 0.1–10 Mb, affecting ∼40% of the human genome. These domains are gene poor and low transcribed. Insulator CTCF is found to enrich on the boundaries of LADs. These data indicated a domain-like organization of human genome in the interphase nucleus. Further genome-wide mapping of LADs was expanded to mouse ESCs, *in vitro* differentiated neural progenitor cells (NPCs), and terminally differentiated astrocytes [49]. Similar to the human data, they identified ∼1100–1400 LADs with sizes from 40 kb to 15 Mb in each cell types, covering ∼40% of the mouse genome. These LADs show relatively small differences, overlapping by 73%–87%, between the three stages of differentiation. Interestingly, the authors also noticed lower signal of LADs compared with that of differentiated cells, suggesting that lamina association in ESCs may be less robust or more variable among individual cells of ESC populations [49]. It is known that nuclear membrane of ESCs contains only lamin B but not lamin A/C, whereas the differentiated cells have both types of lamin, consistent with more fluidic nuclei in ESCs. It would be interesting to explore whether lack of lamin A/C affects membrane−chromatin interacting in ESCs.

Intriguingly, LOCKs and LADs mark similar genomic locations as 82% of placenta LOCKs overlapping LADs in fibroblasts. However, causal relationships underling the correlation remain elusive, as knocking-down of G9a did not change the overall positioning toward nuclear rim in mouse ESCs [50].

Partial DNA Methylation Domains

Methylation at the fifth-position of cytosine (5 mC) is the most studied epigenetic modification, which plays a vital role in gene regulation and genome organization. Although DNA methylation has been studied for decades, the genome-wide distribution of 5 mC has remained unclear until recently. Lister *et al.* [51] described the first nucleotide-resolution maps of methylated cytosines in human ESCs and fetal fibroblasts by genome-wide bisulfite sequencing (MethylC-seq). Surprisingly, ∼25% of methylated cytosines in human ESCs occur in the non-CpG contexts, but only 0.02% is found in fibroblasts, suggesting that ESCs may use a unique system for methylation. While CpG methylation in promoters is associated with gene repression, methylation on gene bodies is found to be positively correlative to gene expression in fibroblasts but not in ESCs.

Another unpredicted result in the Lister's paper is the finding of large continuous regions showing less methylation in fibroblasts compared with ESCs, which are termed as partially methylated domains (PMDs) [51]. The PMDs comprise ∼40% of genome regions in fibroblasts. Interestingly, gene within or near PMDs are much less expressed in fibroblasts than in ESCs. More importantly, PMDs are strongly associated with large H3K9me3 and H3K27me3 domains identified in fibroblasts [51], and the homologous regions of H3K9me2 blocks in mouse differentiated cells [41], which suggests a reverse correlation between PMDs and large heterochromatin domains.

Similar to the observation of PMDs, genome-wide mapping of cancer methylomes identified large regions that are less methylated in cancer cells than in the normal [52]. These hypomethylation blocks encompass >50% of the genome, and significantly overlap PMDs [85% overlapping, odds ratio (OR) = 6.5, $P < 10^{-16}$], LOCKs (89% overlapping, OR = 6.8, $P < 10^{-16}$), and LADs (83% overlapping, OR = 4.9, $P < 10^{-16}$) [52], which suggests that these parts of genome experience dramatic and complicated epigenetic programming in both development and disease.

Replication Timing Domains

In eukaryotic cells, DNA replication occurs in a defined temporal order in S phase, and the replication timing of a specific part of the genome is thought to associate with its chromatin organization. For example, early replication is associated with active chromatin which places on interior nucleus, whereas late replication is linked to repressive chromatin located on nuclear and nucleolar periphery. Genome-wide analysis of replication timing in mouse ESCs and differentiated NPCs demonstrated that chromosomes can be segmented into large domains of megabase sizes [53]. These 'replication domains' are relatively small and discrete in ESCs but consolidate into larger zones in neural progenitors, consistent with changes of gene expression and nuclear positioning of chromosome. The status of replication domains is correlated to the content of GC and LINE1 repeats, rather than gene density. A more comprehensive study profiled genome-wide replication domains in 22 mouse cell lines representing 10 early developmental stages [54]. About 45% of the genome is shown to change replication timing during these states. Of note, a set of early-to-late replication timing switches are seen between

ESCs and Epiblast stem cells (EpiSCs), coinciding with the appearance of compacted chromatin near the nuclear periphery in EpiSCs, and then these regions keep largely unchanged in later developmental stages. Interestingly, during the induction of iPSCs, replication timing of these regions are difficult to reprogram into ESC-like state. As expected, there is a positive correlation between early replication and active chromatin marks such as H3K4me, H3K9ac, and H3K36me3. However, the relationship between late replication and histone marks is still complicated. It seems that H3K9me2 is associated with late replication, but causal relationships remain unclear [55].

Outlook

In summary, evidences from multiple resources have all pointed to domain organization of the genome. These large chromatin domains are highly dynamic in cellular differentiation and reprogramming, and are significantly associated with large-scale gene regulation and 3D organization of chromatin. More discrete patterns of large chromatin domains seen in PSCs further support the idea of higher plasticity in pluripotent cells. Importantly, due to functionally heterogeneity in PSC cultures, extensive analysis of fractionated cell populations will provide information for the 'real' epigenomic features of pluripotent cells. Although correlations have started to be established between some of large domains such as LOCKs and LADs, their physical interactions, and causal relationships to nuclear architecture remain to be determined. The mechanisms for establishing and maintaining of these large domains in mammalian cells are still unclear. Recent identification of non-coding RNAs (ncRNAs) associated with PRC2 indicated that ncRNAs may play important roles in establishing heterochromatin domains [38,56]. Finally, the basic principles and methodologies of the chromatin domains can be also applied to the disease studies, thereby enhancing our understanding of the epigenetic basis underlying development and disease.

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