Chromatin structure, pluripotency and differentiation

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Abstract

The state of cell differentiation in adult tissues was once thought to be permanent and irreversible. Since Dolly's cloning and, more recently, the generation of induced pluripotent stem cells (iPSCs) from differentiated cells, the traditional paradigm of cell identity has been reexamined. Much effort has been directed toward understanding how cellular identity is achieved and maintained, and studies are ongoing to investigate how cellular identity can be changed. Cell-specific transcription patterns can be altered by modulating the expression of a few transcription factors, which are known as master regulators of cell fate. Epigenetics also plays a major role in cell type specification because the differentiation process is accompanied by major chromatin remodeling. Moreover, whole-genome analyses reveal that nuclear architecture, as defined by the establishment of chromatin domains, regulates gene interactions in a cell-type-specific manner. In this paper, we review the current knowledge of chromatin states that are relevant to both pluripotency and gene expression during differentiation. Information about the epigenetic regulation of gene expression in iPSCs or naïve embryonic stem cells, compared with their differentiated derivatives, will be important as a practical consideration in the long-term maintenance of pluripotent cell cultures for therapeutic purposes.

Keywords: induced pluripotent stem cell, human embryonic stem cell, chromatin, pluripotency, development, histone methylation, histone acetylation, epigenome, chromosome territories

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Introduction

Since the first reprogramming of adult fibroblasts into induced pluripotent stem cells (iPSCs),[1,2] much effort has been directed toward characterizing the transcriptional networks that control the expression of genes that regulate pluripotency. Transcriptional regulation during reprogramming is achieved by a core regulatory circuit of genes, such as Sox2, Oct4 and Nanog, which can control their own transcriptional activity through a positive regulatory loop, the activation of genes responsible for maintaining embryonic stem cell (ESC) pluripotency and the repression of genes that promote ESC differentiation. These core genes regulate transcription by recruiting RNA polymerase II and by their association with other transcription factors and chromatin regulators (for a review, see Refs.[3,4]).

Importantly, gene expression programs are activated in the context of chromatin; therefore, analyzing the epigenetic regulation of gene expression is essential for elucidating mechanisms of cell-lineage determination. However, relatively little is known about the structure and nuclear organization of chromatin in pluripotent stem cells and how it changes during cell differentiation and reprogramming. Initial imaging and biochemical studies of chromatin architecture in mouse embryonic and neural progenitor cells (NPCs) clearly showed that chromatin is reorganized during differentiation.[5] More recently, the construction of chromatin landscapes in stem cells and their differentiated derivatives has been enabled by the development of chromatin immunoprecipitation followed by whole-genome sequencing (ChIP-seq).[6] We now know that the structure of chromatin at promoters and transcription regulatory sequences correlates with their active, repressed or poised transcriptional status, which, in turn, correlates with the state of cell differentiation.[7] These studies have helped to identify cell-type-specific transcriptional networks and to elucidate cellular developmental potential.

Moreover, there is growing evidence that the regulation of gene expression relies not only on local DNA and/or histone epigenetic modifications but also on higher order chromatin structure and nuclear spatial organization. Locus-specific chromatin conformation changes upon gene activation facilitate the interaction between cis-regulatory transcriptional elements and gene promoters.[8] Gene activation is correlated not only with chromatin decondensation but also with gene re-positioning inside the nucleus, as has been shown for genes involved in pluripotency, such as Nanog and Oct4.[9,10] At a larger scale, individual chromosomes

are organized into discrete domains within the interphase nucleus.[11] The nuclear arrangement of chromosome territories (CTs) and specific subregions is not random; rather, it is correlated with transcriptional regulation. Open/active chromatin and closed/inactive chromatin are spatially segregated to form genome-wide compartments, which, in turn, regulate gene interactions within and between domains.[12] Therefore, it is not surprising that the processes that regulate interphase nuclear architecture occur in patterns that are specified by both the cell type and the stage of differentiation.

Herein, we review the current knowledge of chromatin states that are relevant to pluripotency and differentiation. Gene regulation by transcription factors and DNA methylation are also essential for the maintenance of these states and for the transformative processes, but they are not reviewed here. Understanding ESC and iPSCs is of current interest in biomedicine because of their capacity to self-renew and develop into any adult tissue in response to specific cues. Both of these cell types have become integral tools in the rapidly growing field of regenerative medicine.

Chromatin structure

In eukaryotes, DNA is organized into a highly ordered nucleoprotein assembly called chromatin, whose fundamental unit is the nucleosome. The nucleosome is composed of an octamer of the four core histones (H3, H4, H2A and H2B, organized into two dimers, H3-H4 and H2A-H2B) with 146 bp of DNA wrapped around them.[13] Histones are subject to multiple post-translational modifications (PTMs), including acetylation, methylation, ubiquitylation, phosphorylation and sumoylation. These PTMs determine open and closed chromatin conformations, which, in turn, regulate the differential access and recruitment of transcription factors and other regulatory chromatin binding proteins to DNA.[14]

The most studied histone marks involved in stem cell biology and differentiation are the methylation (me) and acetylation (ac) of specific lysine residues of histones H3 and H4, such as H3K9ac, H3K9me2–3, H3K4me3, H3K27me3 and H3K36me3. The studies show that the whole-genome epigenetic status (epigenome) of pluripotent and fully committed cells is different[6] and that reprogramming of somatic cells with the Yamanaka cocktail (Oct4, Sox2, Klf4 and c-Myc, referred to as OSKM) results in the acquisition of pluripotent features that are very similar to those observed in human ESCs. However, the chromatin landscapes of ESCs and iPSCs are somewhat different, which indicates that full reprogramming will require further research.

Histone acetylation

Histone acetylation occurs at multiple lysine residues and is controlled by histone acetyltransferases (HATs) and histone deacetylases (HDACs), which add or remove, respectively, acetyl groups to histones. HATs can be grouped into three major families: GNAT, Myst and p300/CBP.[15,16] Similarly, HDACs are classified into class I, with HDAC1–2 members; class II, with HDAC4–7 and 9–10; class III, which includes Sirtuins; and class IV, with HDAC11 as the only member.[17]

The strong correlation between gene activation and histone acetylation was noticed many years ago.[18] Indeed, the analysis of the nucleosome structure of a 30 Mb chromatin region of the human genome indicated that both H3 and H4 acetylation levels were enriched at active promoters and enhancers.[19] An increasing number of studies support that histone acetylation plays a major role in pluripotency maintenance. Overall, the total nuclear levels of H3K9ac are higher in stem cells than in differentiated cells.[20] Indeed, studies using chemical inhibitors of HDACs show that acetylation is important for stem cell renewal and somatic reprogramming. The HDAC inhibitor butyrate promotes stem cell survival and enhances somatic reprogramming of both human and mouse cells by increasing H3K9ac and the expression of pluripotency genes.[21,22] Similarly, valproic acid (VPA), another HDAC inhibitor, also enhances the somatic reprogramming of mouse embryonic fibroblasts by promoting the activation and repression of certain pluripotency and lineage-specific genes, respectively, even in the absence of c-myc.[23] Additionally, VPA induces global H3K9 acetylation, but its effects are more pronounced at promoters that show high DNAse I hypersensitivity and in which HAT p300 is already present. In the same line of evidence, a genome-wide analysis of HDAC1 occupancy in mouse ESCs showed that HDAC1 binds to actively expressed genes, including genes that are involved in pluripotency, such as Nanog, Sox and Oct4.[24] In addition, targeted genes are transcriptionally up-regulated in HDAC1-depleted cells, which suggests that HDAC1 plays a role in limiting their transcriptional expression.[24] These results are consistent with a genome-wide mapping of HATs and HDACs in human CD4+ T cells, which shows that both enzymes are simultaneously enriched at the promoters and enhancers of active genes.[25] In contrast to these results, the inhibition of class I and class II HDACs with trichostatin A (TSA) promotes differentiation by down-regulating pluripotency genes and up-regulating lineage-specific genes.[24,26] The deletion of HDC1 or HDC2 does not affect mouse ES cell self-renewal or promote unscheduled differentiation into embryoid bodies. However, HDC1-depleted embryoid bodies exhibit an enhanced differentiation toward the mesoderm and neuroectoderm cell lineages compared with wild-type embryos.[27] Overall, these results suggest that fine tuning the histone acetylation status at promoters is essential for proper transcriptional regulation. Therefore, two factors, the balanced activity of HATs and HDACs at specific loci, might be essential for pluripotency maintenance and the launching of lineage-specific transcriptional programs.

Other important HDACs that are up-regulated in ESCs are members of the Sir2 family of NAD+ deacetylases, or Sirtuins. Mammals have seven Sirtuins, which are denoted SirT1 to 7. These proteins are major players in sensing redox cellular stress and coordinating an adequate response, which ranges from changing chromatin structure to altering mitochondria metabolic function.[28] Sirtuins also have an

important role in the maintenance of genome integrity and in the control of cell-cycle progression.[29] However, little is known about the function of most Sirtuins during development. We know that SirT1 expression is relatively elevated in human ESCs, in which transcriptionally repressed genes that are known to be involved in development have deacetylated H3K9 at their promoters. Upon differentiation, SirT1 protein levels decrease, and its targeted genes are expressed.[30] Consistently, resveratrol, a known activator of SirT1, enhances mouse embryonic fibroblast cell reprogramming.[31] In addition, Sirt1 controls the expression of Oct4 and Nanog, which are hallmarks of pluripotency, possibly through an unknown indirect mechanism because Sirt1 does not bind to their promoters.[30] This observation is consistent with a previous study that showed that SirT1 is involved in stem cell pluripotency maintenance in response to endogenous oxidative reactive species through inhibition of p53 nuclear translocation and p53-dependent Nanog repression.[32] A role for SirT1 in hematopoietic lineage differentiation has also been proposed because SirT1-depleted ESCs have impaired early differentiation of hematopoietic progenitors.[33] In addition, embryonic and adult hematopoietic progenitors that are deficient in SirT1 are reduced in number under hypoxic conditions *ex vivo*.[34] Overall, these results suggest that SirT1 may regulate the proliferation and differentiation of stem cells in response to cellular redox levels, which may have important consequences in tissue renewal.

Histone methylation

Histone methylation at specific lysine and arginine residues is probably the epigenetic modification which has the greatest diversity of effects. Mono-, di- or tri-methylation of histones is related to a wide variety of genome functions, such as the activation or repression of transcription, DNA damage response via the recruitment of DNA repair proteins and replication progression by the firing of replication origins.[14] At the level of transcriptional regulation, H3K4me1 and 3 are associated with the activation of gene expression, H3K36me3 is associated with transcriptional elongation, and H3K9me3 and H3K27me3 are associated with silenced genes.[35] Remarkably, in ESCs, most gene promoters that present the H3K27me3 repressive mark are also tri-methylated at H3K4, forming what are called bivalent domains[7] (discussed in detail in Section Chromatin landscapes of differentiated and embryonic stem cells). Indeed, both marks have been linked with impaired embryogenesis or cell differentiation.

In mammals, H3K4 methylation is catalyzed by the family of SET1/MLL histone methyltransferases. Each methyltransferase complex contains one of the catalytic subunits (SET1A, SET1B, MLL1, MLL2, MLL3 and MLL4) and all the shared core subunits (Ash2, RbBp5, Wdr5 and Dpy30), which are required for proper methylation activity (for a review, see Ref.[36]). Deletion of the catalytic subunits SET1, MLL1, MLL2, MLL3 and MLL4 does not affect H3K4me3 levels, which indicates functional redundancy, and deletion of only the core subunits leads to a significant reduction in this epigenetic mark. This observation might explain why it is still not clear whether H3K4me3 promotes ESC renewal, the induction of cell lineage specification or both, despite accumulated evidence of the importance of H3K4me3 in stem cell biology. The Wdr5 core subunit is important for maintaining both global and locus-specific levels of H3K4me3 in ESCs. Wdr5 associates with the transcriptional factor Oct4, which results in Wdr5 recruitment to pluripotency-associated genes to promote H3K4me3 and to maintain the self-renewal state of ESCs. Consistently, deletion of the Wdr5 protein in ESCs results in a loss of pluripotency and cell differentiation.[37] In addition, Wdr5 activity is required for the proper somatic reprogramming of mouse embryonic fibroblasts induced by OSKM transduction.[37] In contrast to these results, the reduction of H3K4me3 levels by knocking down the Dpy-30 core subunit impairs the activation of early lineage-specific genes and results in a prolonged pluripotency state. Interestingly, Dpy-30 deletion does not affect the transcriptional levels of the pluripotency genes Nanog, Oct4, Klf4 and Sox2, despite causing a significant reduction in H3K4me3 levels at their promoters.[38] Overall, these reports indicate that the regulation of H3K4me3 is complex and that further research will be required to establish the specific role of different SET1/MLL proteins in pluripotency and differentiation.

In contrast, H3K27 di- and tri-methylation are mediated by Polycomb Repressive Complex 2 (PRC2) (for a review, see Ref.[39]). Unlike the SET1/MLL complex, the individual deletion of any of the PRC2 proteins results in severe phenotypes during embryogenesis. Interestingly, deletion of the PRC2 components in mouse ESCs does not affect pluripotency and, instead, results in impaired lineage commitment upon external stimuli.[40,41] These results are consistent with the localization of PRC2 at the promoters of known developmental regulators. Therefore, impaired lineage commitment upon external stimuli in PRC2-depleted cells might be the consequence of improper transcriptional silencing of genes that are not related to the specific differentiation pathway.

Chromatin landscapes of differentiated and ESCs

Much information about the epigenome has been obtained in recent years by using high-throughput DNA sequencing approaches, such as ChIP-seq. Importantly, ChIP-seq has helped to define the epigenetic landscapes of pluripotent and differentiated cells by facilitating the description of specific histone marks at all of the nucleosomal positions of the genome. These cell-type-specific epigenetic landscapes clearly demonstrate that epigenetic changes accompany cell fate/differentiation transitions and correlate with the transcriptional program of the cell.

At present, many reports support the idea that pluripotent cells have epigenetic features that are associated with an open chromatin state on a genome-wide scale. Indeed, quantitative imaging and biochemical studies have shown that euchromatin, which is measured by the amount of active H3 and H4 acetylation as well as H4K36me2 and H3K4me3, is increased in ESCs and iPSCs compared with differentiated cells, whereas heterochromatin, which is measured by the amount of H3K9me3, is reduced.[5,20,42–45]

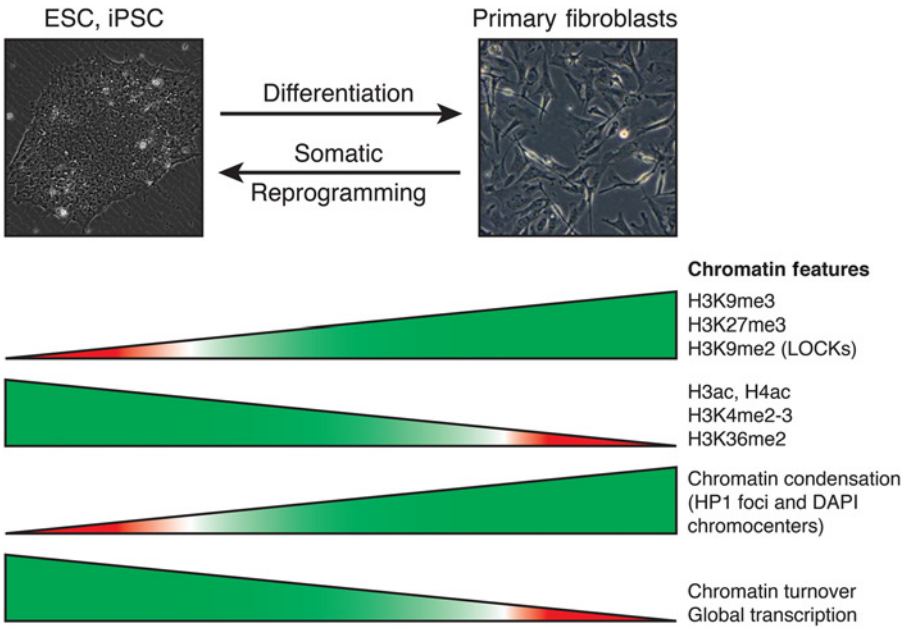

Figure 1 Differential chromatin features of pluripotent and differentiated cells. The stem cell state is associated with active epigenetic marks (H3 and H4 acetylation, H3K4 and H3K36 methylation), together with increased turnover of proteins that bind to chromatin and high global transcription levels. Conversely, the differentiated state is associated with chromatin condensation and global heterochromatinization (such as increased levels of HP1 foci and chromatin domains marked with repressive H3K9me2–3 and H3K27me3 marks). Above photos are of a human iPSC colony (left) and fully differentiated primary fibroblasts (right). iPSC, induced pluripotent stem cell

In addition, heterochromatin is less compacted in ESCs and iPSCs than in differentiated cells, as indicated by the decreased volume of constitutive heterochromatin chromocenters visualized after DAPI staining.[42,43] Moreover, structural heterochromatin proteins, such as HP1, are hyperdynamically bound to DNA in ESCs, as indicated by fluorescence recovery after photobleaching (FRAP) assays[5] (Figure 1).

In contrast to stem cells, cellular development is associated with a general heterochromatinization process. ChIP-seq experiments using the H1 human ESC lineage, differentiated IMR90 fibroblasts and CD4+ T cells demonstrated a cell-type-specific expansion of repressive H3K9me3 and H3K27me3 domains during differentiation.[46] In addition, differentiated human and mouse cells acquire large regions of H3K9 dimethylation or LOCKs (large organized chromatin K9-modifications).[47] Importantly, H3K9me2 domains are linked to tissue-specific chromatin architecture, as discussed in section Nuclear domains.

Interestingly, ESC ChIP-seq experiments have also revealed that many genes that are essential for developmental regulation are epigenetically marked by the simultaneous presence of H3K4me3 and H3K27me3 at their promoters;[6,7,48] histone marks that are associated with active and silenced promoters, respectively.[2,49,50] These promoters also present low levels of the active histone mark H3K9ac.[51] This 'bivalent' histone profile is associated with CpG-rich promoters, correlates with low promoter activity and is thought to be a mechanism to silence important developmental genes in ESCs while keeping them poised for future repression or activation.[7] Approximately 16% and 22% of the known genes present bivalent histone markings in human and mouse ESCs, respectively.[6,48] Examples of genes with bivalent domains are PAX6, GATA4, GATA6, CDX2 and HOX genes. When differentiation occurs, the epigenetic marks at bivalent promoters resolve according to the developmental function and fate of the genes that they control. Genes that have functions that are unrelated to the specific differentiation pathway typically resolve to monovalent H3K27me3 or lose both marks and become repressed, whereas genes involved in the differentiation process, such as genes involved in neurogenesis in NSCs, resolve to monovalent H3K4me3 and become active.[6,7] Initially, bivalent domains were suggested to be unique to stem cells because bivalent marking is a key mechanism for controlling the expression of developmental regulators in ESCs. However, a few bivalent domains are also observed in differentiated cells. A recent report proposed that at the molecular level, bivalent histone marks are maintained by the Utf1 protein, which competes with PRC2 for DNA binding at CpG-rich bivalent promoters while also recruiting proteins that are involved in mRNA degradation. These two opposing functions of the Utf1 protein are important for preventing non-reversible silencing of developmentally regulated genes and, at the same time, promoting the degradation of non-specific transcripts.[52]

It is important, however, to acknowledge that the above-described epigenetic landscapes have been obtained from ESCs cultured in the presence of serum plus the cytokine LIF (leukemia inhibitory factor). LIF promotes stem cell renewal by activating the Stat3 signaling pathway, but the presence of serum introduces multiple uncontrolled factors. In past years, the refinement of cell culture conditions for ESCs has helped to elucidate the molecular players that promote cell renewal and the inhibition

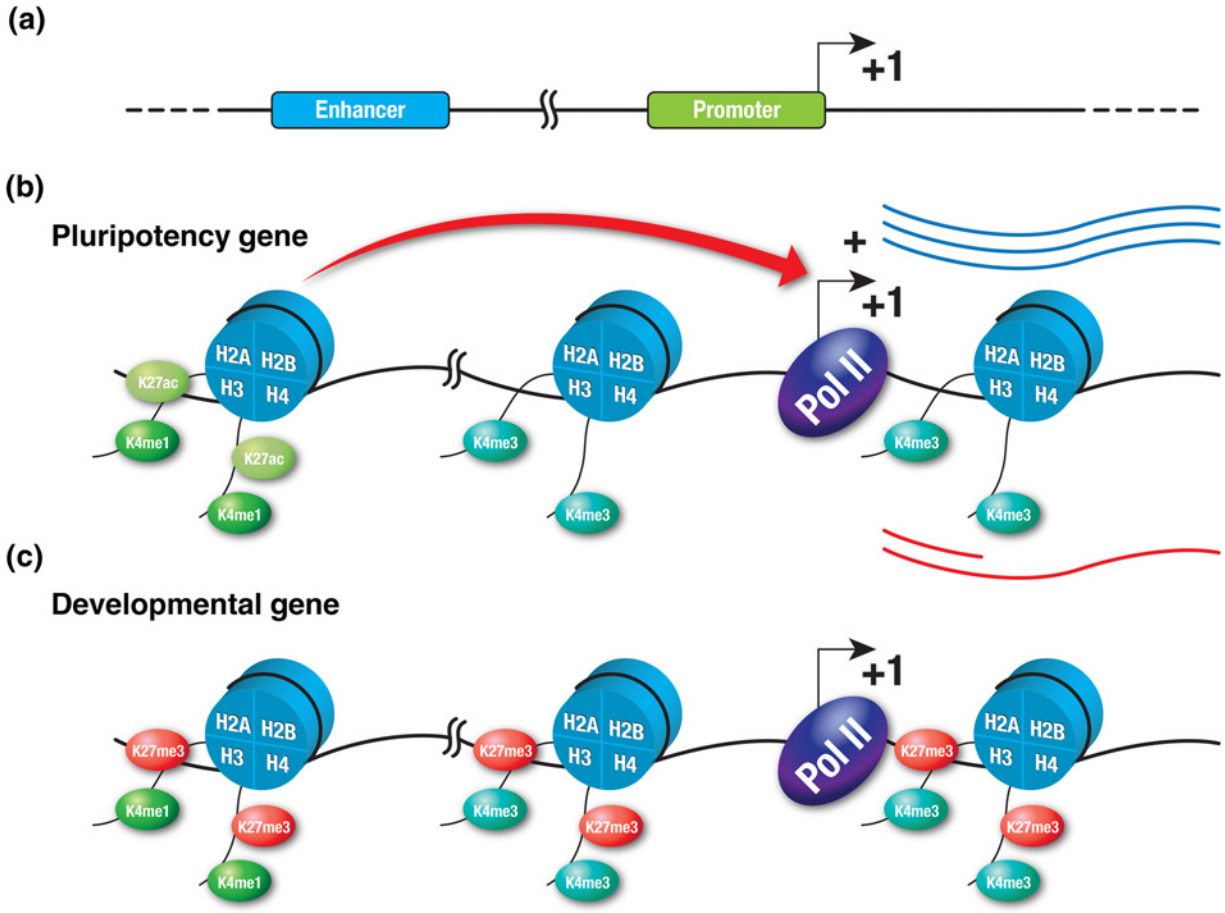

Figure 2 Chromatin structure of pluripotency and lineage-specific genes in embryonic stem and induced pluripotent stem cells. (a) Schematic representation of the cis-acting DNA elements that regulate gene expression: promoter (green) and a distal enhancer (blue), further described in (b,c). (b) Representation of nucleosomes and cell-type-specific histone modifications surrounding the transcription start site (+1) of an active pluripotency gene. Arrow represents association of the enhancer and promoter by chromatin looping: RNA polymerase II (Pol II), RNA products (blue lines). (c) Same as (b), but for a transcriptionally poised developmental gene with bivalent domains. Preloaded RNA polymerase II (Pol II) at the promoter may result in low level transcription of the gene (long red line) and/or proximal-promoter paused polymerase resulting in short RNA products (short red line)

of differentiation. It has been shown that a combination of LIF together with MEK and GSK protein inhibitors, a condition called '2 inhibitors' or simply '2i,' can substitute for serum. The result is the formation of purely undifferentiated ESC colonies, which are more representative of the ground state of pluripotency.[53] Indeed, a recent study has demonstrated that the culture environment affects both the transcriptome and epigenome signatures of mouse ESCs.[54] Bivalent promoters at developmental genes are observed in serum and 2i-grown ESCs, but they are much less represented under 2i conditions because of a reduction in the H3K27me3 histone mark abundance (1000 genes in 2i versus 3000 genes in serum-containing culture conditions). In addition, this study reveals that RNA polymerase pausing is higher under 2i conditions than in the presence of serum and suggests that promoter-proximal pausing may play a major role in poising lineage-specific genes as well as H3K4me3/H3K27me3 histone marks.

Although the transcription of coding genes is initiated at gene promoters, distal DNA elements, such as enhancers, silencers, insulators and locus control regions (LCRs), are also crucial for the correct spatial and temporal expression of genes. On a genome-wide scale and in several cell types, the chromatin signature of active enhancers is associated with H3K4me1 and H3K27ac,[19,55,56] including active hESC enhancers.[56–58] Indeed, the simultaneous presence of H3K4me1/H3K27ac in human ESCs is found at enhancers that regulate genes that are known to be expressed in the embryo epiblast. Interestingly, enhancers for genes active in early development have a specific histone profile. H3K4me1 is present, H3K27ac is absent, and instead, the polycomb repressive mark H3K27me3 is enriched. Moreover, these are poised enhancers and they control the expression of genes that regulate development, which also present bivalent histone domains at their promoters. These results suggest that not only promoters but also distal regulatory elements are subject to similar chromatin regulation (Figure 2).

In conclusion, ESCs and iPSCs are characterized by a more open and plastic chromatin organization that is thought to be important not only for pluripotency but also for differentiation upon developmental cues. However, the relevance of these findings to stem cell biology *in vivo* remains unclear. It is clear that the culture environment affects the pluripotent status of ESCs, which correlates with a specific chromatin signature. Furthermore, currently used human

ESC lines are thought to be in a later stage of embryonic development when compared with mouse ESCs. Despite substantial advances in our knowledge about stem cell biology and reprogramming in recent years, these results highlight the need to reevaluate chromatin landscapes using ESCs that better represent the inner cell mass of the blastocyst. Nevertheless, the description of distinctive epigenomics landscapes has helped to reinforce the idea that the epigenome is tightly connected to cell fate.

Nuclear architecture

The spatial compartmentalization of nuclear processes, such as transcription, replication and DNA repair, supports the notion that the nuclear architecture provides a framework for the higher-order regulation of genome function (for reviews, see Ref.[59]). Fluorescence *in situ* hybridization analysis of three-dimensionally preserved nuclei (3D-FISH) showed that chromatin is compartmentalized in the interphase nucleus: chromosomes in interphase cells occupy distinct, non-overlapping territories.[11] The nuclear position of CTs and subregions is correlated with replication timing, gene density and GC content.[60–65] A classic example is the gene-rich chromosome 19, which is frequently positioned close to the nuclear center, whereas the gene-poor chromosome 18 is closer to the nuclear periphery.[11,60] These observations have been extended to other human chromosomes and several cell types, including ESCs, with similar conclusions (for a review, see Refs.[66,67]).

More recently, genome-wide patterns of chromatin interaction obtained by spatially constrained chromatin ligation followed by parallel deep sequencing (Hi-C) confirmed the existence of CTs and non-overlapping 1 Mb-sized open and closed chromatin domains.[12] These domains would act as unit blocks of dynamic but non-random chromatin geometry inside the nucleus. The ultrastructure of these domains is still poorly understood, but it is specified in a cell-type- and cell-differentiation-specific manner and, therefore, is of great importance to our understanding of stem cell genomic regulation.

Chromatin looping

Gene activation has been associated with chromatin decondensation and chromatin looping out from the CT (i.e. higher-order chromatin structural changes). Prominent examples include MHC,[68] epidermal differentiation complex,[69] and ANT3 [70] and the Hox genes.[71] These reports together suggest that chromatin looping may be the universal functional unit of genome organization in the nucleus. Indeed, a recent study based on experimental data generated by FISH on a scale of 0.5 to 10 Mb proposed that chromatin folding can be explained by the formation of loops broadly ranging in size.[72] Such an organization is especially important in transcriptional regulation because it can control the accessibility of the transcriptional machinery and regulatory factors to specific genes.[73] These results underscore the importance of a defined nuclear organization for the maintenance of a cell type-specific transcription pattern. This type of organization may be of particular importance with regard to the maintenance of ESC pluripotency. The radial organization of the chromosomes, which is arranged by the relative gene density, appears to be maintained between stem cells and differentiated cells. However, genes that are known to be essential to pluripotency adopt preferential external positions relative to their CTs (a characteristic of active genes) in ESCs when compared with their differentiated derivatives.[9,10]

One of the best examples of the correlation between nuclear architecture and cell differentiation is the Hox gene family of developmental regulators in murine cells. Genes of the Hoxb cluster loop out from its CTs upon ESC differentiation induced by retinoic acid[71] and during mouse embryonic development.[74] These results support the notion that looping out of CTs is necessary for transcriptional activation. However, this process is not straightforward, and specific differentiation processes might have differently scheduled patterns of chromatin reorganization. Primarily, chromatin decondensation is not always linked to looping out of CTs. This pattern was clearly indicated by the work from Bickmore's lab: Using 3D-FISH, they measured the nuclear distance between the 3′ and the 5′ ends of the Hoxd cluster, which is thought to represent decondensation or unfolding of higher-order chromatin structure.[71,75] They observed that Hoxd locus decondensation in the mouse embryo occurs in both the limb bud and in the tail bud, where Hox genes are expressed, but that looping outside of the CT occurs only in the tail bud.[75] Moreover, if chromatin decondensation and looping out is correlated with increased transcriptional activity, then we should observe a bystander activation of the genes that flank the looping locus. However, genes adjacent to the Hoxb locus were not activated despite being moved outside of their CTs during the extensive CT reorganization that occurs upon ESC differentiation.[75] Furthermore, the same group showed that a looped-out transposed Hoxb1 gene did not get transcriptional activated.[76] Looping favors the association with transcription factories and an activation of gene expression, which is seen in some of the flanking genes of a human beta-globin LCR targeted to the mouse genome in an area that contains mostly housekeeping genes.[77] However, even in this case, the authors concluded that the increased transcription factor association, which is induced by the beta-globin LCR, it is not dependent on its position within the CT but instead results from local chromatin decondensation that facilitates the interaction of the LCR with flanking gene promoters. Nevertheless, and at least at the level of transcriptional regulation, these reports support the idea that chromatin looping is a mechanism that explains long-range regulatory chromatin contacts better than alternative mechanisms, such as 'walking' along the intervening chromatin until finding the promoter.

In conclusion, chromatin decondensation allows specific loci to move within CTs, including looping out, thus increasing the capability of finding a transcription-competent environment and facilitating long-range regulatory gene interactions. Therefore, chromatin decondensation and looping out might be a prerequisite for gene activation

Table 1 Summary of nuclear domains types and features

	ESC genome coverage	Domain scale	Enrichment at domain boundaries	Changes during differentiation
Replication domains	Genome-wide	200 kb to 2 Mb	Active histone marks (H3K9Ac, H3K4me3, K27Ac, K36me3)	Early to Late replication switch of selected domains
Topological domains	Genome-wide	1 Mb	CTCF, HK genes, SINE elements	NO
H3K9me2 chromatin domains (LOCKs)	Inactive chromatin	Up to 4.9 Mb	CTCF	Increased number
Lamina-associated domains (LADs)	Inactive chromatin	Up to 10 Mb	CTCF, active promoters, CpG islands	Increased number
Chromosome territories	Genome-wide	Chromosome	N/A	NO

N/A, not applicable

rather than an indicator of gene activity, which might define, as discussed above for cell-type-specific histone landscapes, the cell differentiation state and fate.

Nuclear domains

As discussed above, the nuclear arrangement of CTs and specific loci within these territories is not random. Adjacent chromosome domains that are either rich or poor in transcribed genes are spatially separate inside the CT and show little intermingling.[12,63] Thus, the nuclear position of genes in the interphase nucleus is mostly constrained by their allocation to a specific CT[64] and chromosome domain.[78] Overall, these observations suggest that in a given cell type, chromosome interactions are limited by the highly compartmentalized spatial organization of the chromatin and, therefore, gene interactions are modulated by proximity in the nucleus. A key remaining question is the nature of the mechanisms that underlie nuclear architecture. Recent methodological and technological advances in genome-wide analyses of DNA and protein interactions have provided new insights into the factors that regulate the formation of chromosomal domains. A representation of such domains is presented below and summarized in Table 1.

Replication domains

Observations that relatively gene-rich (Giemsa-light staining) chromosomal bands replicate earlier in the cell cycle compared with gene-poor (Giemsa-dark staining) bands have suggested that metaphase chromatin is arranged in distinct chromosomal bands that undergo replication in a temporally programmed way during the S-phase.[79] Those observations suggested that replication timing was a feature of the genome sequence structure. Indeed, there is growing evidence that DNA replication and nuclear architecture are correlated, as early and late replication takes place at distinct loci within the nucleus. DNA replication initiates in a small number of foci associated with intracellular laminin A/C structures. These foci disperse to hundreds of small foci during the S-phase progression to cluster over regions that contain heterochromatin by the end of S-phase.[79] Moreover, the timing of replication is correlated with the pattern of gene expression for a given cell type and stage of development: most of the constitutively expressed housekeeping genes are replicated relatively early in the S-phase, whereas many tissue-specific genes are replicated later but become early replicating in the specific cell type or tissue where they are expressed. Late replicating genes are mostly silent.[81,82]

Genome-wide analysis further revealed that replication timing is regulated at the level of large chromosome domains in a cell type-specific manner. The replication timing profile for an entire mouse ESC genome was generated by differentially labeling early and late replicated DNA, which were previously sorted by flow cytometry, and hybridizing it to a genomic oligonucleotide microarray.[81] Early and late replication time loci were found to occupy distinct chromosome compartments, referred to as replication domains that ranged from 200 kb to 2 Mb in length. In both mouse and human ESCs, these domains are very stable and reproducible.[1,83] Indeed, the ESC-specific distribution of the replication domains can be used as a fingerprint for pluripotentiality because it is maintained between ESCs and iPSCs.[84] There is a positive correlation between early replication domains and transcription activity in both mESCs and neural precursor cell derivatives, which can be determined both by analyzing the level of mRNA expression of the genes that are present in the early domains and by the presence of active epigenetic marks at their promoters, such as H3K4me3 and H3K36me3. However, the same correlation does not hold in late replicating domains, which are not enriched by silence marks such as H3K9me3.[82,83] A concentration of active histone modifications is found at the boundaries of the early replication domains, such as H3K9Ac, K4me1–3, K27Ac and K36me3. The authors argued that the unexpected enrichment of active marks at the boundaries of early domains may protect these transcriptionally active domains from silencing by heterochromatin spreading from neighboring territories.[84]

Most importantly, upon the differentiation of ESCs to neural progenitors cells (NPCs), chromatin domains reorganize from early to late (EtoL) and late to early (LtoE) replication timing. The basic unit of replication timing change is on the order of 400–800 kb and has an unusual combination of GC content and gene density, such as a high percentage of GC content but a low gene density, and enrichment for LINE-1 elements that are usually observed in late replicating heterochromatin regions. Interestingly, a switch of the replication timing correlates with changes in the subnuclear position of the chromatin domains, as measured by 3D-FISH. The exchange of nuclear compartments that parallel switches in replication timing was

confirmed by chromatin interaction maps.[83] Indeed, early and late replication domains exquisitely resemble the open (A) and closed (B) chromosome compartments, which are defined by chromatin interactions determined using HiC analysis, as described by Lieberman-Aiden *et al.* (2009).[12] Moreover, the domains that switch their replication timing from EtoL during differentiation are the most difficult to reprogram in terms of both replication timing and gene expression.[82] To confirm these observations, the same group further studied two EtoL replication switching domains that are resistant to reprogramming, the Dppa2/4 and Rex1 domains, which are enriched for genes that have differential expression between ESCs and NPCs. These researchers observed that domains that switch from EtoL during differentiation maintain an unusually high level of chromatin decondensation. However, these domains are the most insensitive to nuclease digestion, even though early replicating loci usually correspond to DNase hypersensitive sites.[85] Overall, these results indicate that early and late replication domains are organized within separate CTs to form distinct self-interacting compartments exhibit an enrichment of genes with similarly active transcriptionally status, and they suggest that replication timing is a master regulator of genome function through the establishment of cell-type-specific chromatin architecture.

Nuclear lamina-associated domains

Slightly over 1000 lamina-associated domains (LADs) of 0.1–10 Mb have been identified in human fibroblasts with the DamID technique.[86] This technique involves fusing the DNA binding protein of interest, in this case lamin A, with DNA adenine methyltransferase (Dam). Regions of DNA that interact with the lamin recombinant protein will become methylated, and are selectively amplified and hybridized to a high-density genomic microarray. The LAD borders mark a clear transition in the average gene density, and LAD domains are characterized by low gene density, low gene expression and PolII occupancy as well as by the presence of epigenetic marks of inactive chromatin such as low H3K4me2 and high H3K27me3. These observations are in agreement with previous findings that show that low-gene-density domains are preferentially located at the nuclear periphery, which is considered to be a repressive chromatin environment.[71,74] Indeed, almost 75% of gene deserts interact at least partly with lamin B1. The authors proposed that these gene deserts may be tridimensional genome organizers because of their association with the nuclear lamina. LAD boundaries are enriched in binding sites for the mammalian transcriptional insulator CTCF,[87] which suggests that there are barriers to the spreading of heterochromatin. These boundaries are also enriched in CpG islands, suggesting that CpG islands may be barriers to heterochromatization and may serve as additional boundary marks. Importantly, the ESC core regulatory protein Oct-1 and its DNA-binding motifs are preferentially found inside of LADs; this finding suggests that Oct-1 might participate in the process of targeting LADs to the nuclear lamina. Genome-nuclear lamina interactions do not change during cell differentiation, as has been shown by comparing the LADs of mouse ESCs, NPCs and terminally differentiated astrocytes.[88] However, some of the 'stemness' genes in non-ESCs are strongly bound to the nuclear lamina. These results indicate that even if the primary structure of LADs is remarkably similar between stem cells and their differentiated derivatives, the differentiation process modulates genome-lamina interactions at the level of individual transcription units. Nevertheless, the discovery of LADs provided one of the first mechanistic explanations for how nuclear architecture is established and maintained, namely, by anchoring specific chromatin domains to the nuclear lamina.

Large histone H3 lysine 9 dimethylated (H3K9me2) chromatin blocks or LOCKs

ChIP-on-chip assays, which involve chromatin immunoprecipitation (ChIP) with an antibody against H3K9me2 followed by hybridization to genomic microarrays (chip), revealed that in undifferentiated tissues, there are large H3K9me2-modified regions (up to 4.9 Mb) that are associated with tissue-specific gene repression.[47] Genes in liver-specific LOCKs are not expressed in the liver but instead are largely expressed in the brain tissue, and *vice versa.* Moreover, the loss of G9a, the major H3K9 dimethyltransferase, results in the overexpression of genes that are present in the tissue-specific H3K9me2-modified regions. Importantly, LOCKs occupy only 4% of the genome in both mouse and human ESCs compared with 31% in their differentiated derivatives, which indicates that LOCK distribution and abundance changes upon differentiation. These results are consistent with the reported whole-genome decrease in transcription levels that parallels ESC differentiation.[5] Interestingly, in differentiated cells, LOCKs overlap with LADs, and similar to LADs, the boundaries of H3K9me2-modified regions are enriched in CTCF binding sites. In conclusion, H3K9me2 domains appear to be important for the establishment of nuclear architecture by facilitating the nuclear lamina association of tissue-specific repressed chromatin domains.

Topological domains

A recent report of chromatin interactions determined by HiC of human and mouse stem cells and terminally differentiated cell types identified thousands of Mb-sized local chromatin interaction domains called 'topological domains'.[89] Similar to other described nuclear domains, the interactions between intradomain loci are more frequent than those between interdomain loci even if the genomic distances are the same. Similar to the above-mentioned domains, these observations suggest that a general feature of all the domain boundaries is to act as barriers for the spreading of heterochromatin and, therefore, to compartmentalize transcriptionally active and inactive chromatin regions. Interestingly, the topological domain boundaries are also enriched with SINE transposable elements and housekeeping and tRNA genes, further suggesting that high levels of transcriptional activity might also contribute to boundary formation. This observation is consistent with the chromatin structure found at the boundaries of early replicating domains, which are enriched in epigenetic marks that are characteristic of active chromatin. In contrast,

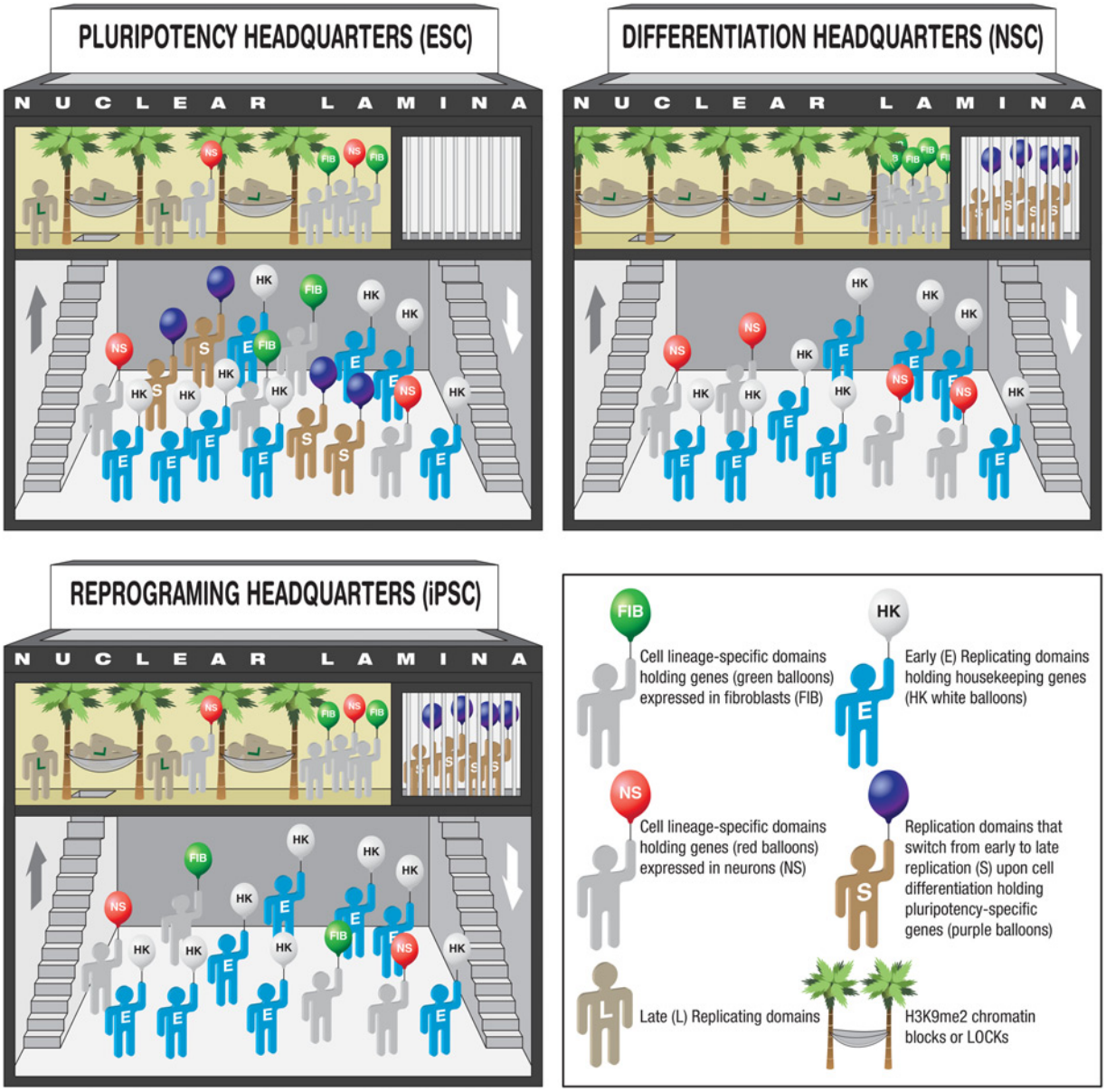

Figure 3 Model of nuclear compartmentalization into chromatin domains. Illustration of the nucleus ('headquarters') of an embryonic stem cell (ESC), a neural stem cell (NSC) and an induced pluripotent stem cell (iPSC), which exemplifies pluripotent, differentiated and reprogramed cell states, respectively. Different chromatin domains are illustrated by different classes of characters, as indicated. The nuclear periphery and interior are represented as the upstairs and downstairs floors, respectively. In all cell types, early replicating (gene rich) and late (gene poor) replicating regions occupy distinct chromatin domains (L and E characters) and are localized in different nuclear compartments (floors), as represented. Upon differentiation, some of the early replicating domains switch their timing of replication and nuclear compartment (S characters). These domains are the most difficult to reprogram as both early replication timing and gene expression are considered a pluripotency chromatin signature. In differentiated cells, there is a global nuclear reorganization of chromatin that parallels the silencing of non-lineage-specific genes by their association with the nuclear lamina, which is considered a transcriptionally repressive environment. The global decrease in transcription during differentiation parallels the increase of repressive epigenetic marks in chromatin (H3K4me2 modified domains-illustrated as hammocks), which overlap with lamina-associated domains. Upon cellular reprogramming, nuclear architecture reverts in most part to the undifferentiated state, with the remarkable exception of the early to late replicating domains, suggesting that these domains acquired an inaccessible and irreversible chromatin structure in differentiated cells (represented being in the jail)

topological domain boundaries are largely invariant despite differences in chromatin structure and genomic replication timing between cell types. However, using a smaller genomic bin of approximately 20 kb, more dynamic interacting regions that are enriched in cell type-specific expressed genes were found inside these large nuclear 'backbone' domains. For a comprehensive model of nuclear compartmentalization into chromatin domains see Figure 3.

Concluding remarks

Transcriptional programs that are characteristic of a specific cell type and stage of differentiation are executed within the context of chromatin and a proper understanding of such programs requires the deciphering of how chromatin is regulated at all levels, from the nucleosome to the CT. Accordingly, much research has been focused on the characterization of cell-type specific chromatin landscapes and

domains and their dynamic nature, which includes the study of how these epigenetic landscapes either propagate or are changed during the cell cycle. The major challenge now is the characterization of the molecular mechanisms that regulate chromatin structure and architecture in both a spatial and temporal manner. Hopefully, these efforts will result in the development of more efficient protocols for the generation of iPSC and cell-lineage derivatives to increase their potential use in regenerative medicine.

Author contributions: LS set the objective and provided guidance for the creation of the manuscript. LS and BV contributed with the literature search, synthesis of information and wrote the manuscript. JAT provided critical evaluation and editing of the manuscript.

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