

Open Chromatin, Epigenetic Plasticity, and Nuclear Organization in Pluripotency

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Pluripotent embryonic stem cells (ESCs) are considered to have open and accessible chromatin relative to differentiated cells. However, as many studies supporting these conclusions relied on ESCs grown in serum, it has been suggested that some of these features are the result of culture conditions, particularly as more recent work using GSK3/MEK inhibitors (“2i”) to mimic “ground-state” conditions of the pre-implantation blastocyst observed some altered epigenetic features. Here, we systematically review chromatin and epigenetic features in 2i- and serum-grown conditions to come to a clearer picture of what are genuine characteristics of pluripotency and what open chromatin features predict pluripotency.

Introduction

Upon fertilization, the totipotent zygote begins its continuous journey to become an embryo. At around day 3 post fertilization (in mouse), the embryo separates into the outer trophectoderm cells, which will ultimately form the placenta, and the inner cell mass (ICM) to form the pre-implantation blastocyst (Smith, 2017). The ICM then segregates into the pluripotent epiblast stem cells (EpiSCs) and the hypoblast cells, the latter of which contributes to the extraembryonic endoderm. The ICM and the pre-implantation epiblast cells are pluripotent, develop into the embryo proper and are thus capable of generating cell types of the three germ layers: ectoderm, mesoderm, and endoderm. Once the blastocyst is implanted in the uterus wall, gastrulation begins, forming the three early germ layers of the developing embryo. Pluripotency of the ICM and epiblast cells is thus a very transient stage during development, between the formation of the blastocyst and implantation. By contrast, when ICM/epiblast cells are grown *in vitro*, forming colonies of embryonic stem cells (ESCs), this pluripotent stage is preserved (Figure 1). These ESCs not only maintain their potential to become all cell types *in vitro* and *in vivo* but can also self-renew for many generations when properly maintained, providing great promise for regenerative medicine.

While many of the key transcription factors (TFs) and signaling networks in pluripotent cells have been identified, how these cells maintain their dual capacity to self-renew and differentiate into all cell types is still largely unknown. However, it has become apparent over the past years that chromatin and epigenetics play a major role in these processes. Chromatin dynamics, structure, and function was shown to be distinct in pluripotent cells, compared with differentiated cells (Gaspar-Maia et al., 2011). These distinct features, which we describe in detail below, led to the “open chromatin” hypothesis (Gaspar-Maia et al., 2011; Gasser, 2002; Giadrossi et al., 2007; Meshorer and Misteli, 2006), which posits that ESCs, possess a plastic and dynamic chromatin state in order to maintain an unlimited potential to differentiate.

More recently, it was suggested that at least some of these features, e.g., promiscuous transcription, bivalent domains, and DNA methylation, may be attributed to the growth conditions used for culturing ESCs (Marks et al., 2012). Especially pertinent in this regard are the high serum concentrations (usually around 15%) that are normally used for culturing undifferentiated ESCs, especially when grown in the absence of mouse embryonic fibroblasts (MEFs). The cells may be sensing multiple, simultaneous, and often opposing signals from the numerous factors present within the serum, some of which may be unknown or not entirely characterized. This results in a “confused” state, which may explain some of the unique features attributed to pluripotent stem cells (Figure 1). Indeed, when mouse ESCs were grown in the presence of GSK-3 α/β and MEK1/2 inhibitors (“2i”) instead of in high serum concentrations, mimicking the so-called “ground state” of pluripotency (Ying et al., 2008), they displayed some altered epigenetic features and transcriptional output that resembled those found in the pre-implantation ICM (Marks et al., 2012).

Here, we re-examine the epigenetic, nuclear, and chromatin features that were attributed to undifferentiated, pluripotent ESCs and attempt to resolve whether these are genuine characteristics of pluripotency or whether they are a corollary of the growth conditions used to grow the cells. We focus on mouse ESCs not only to avoid species confusion but also because human ESCs (hESCs), until recently, were considered to be developmentally equivalent to mouse post-implantation EpiSCs, and conditions supporting a more naive state in human ESCs were only quite recently worked out. In addition, it is not yet clear whether naive hESCs are comparable to serum-grown mouse ESCs, or to 2i-grown ground-state mouse ESCs (Ying and Smith, 2017). However, toward the end, we summarize the state of affairs of human ESC chromatin, albeit more briefly, and compare the two species. By and large, both mouse and human ESCs, be it ground-state, serum-grown, or “primed,” show open chromatin features that distinguish them from somatic cells, even if such open chromatin features are less pronounced than in earlier pre-implantation stages (Wu et al., 2016).



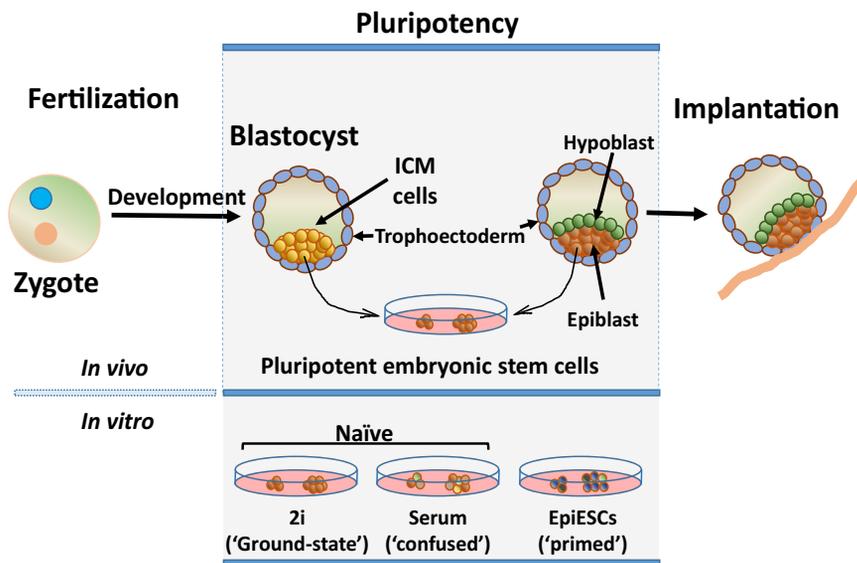


Figure 1. Pluripotency Is Transient *In Vivo* but Lasting in Embryonic Stem Cells

Following fertilization, the zygote (left) develops over a course of several days into the blastocyst (middle, left), which comprises the outer trophoectoderm cells (light blue) and the inner cell mass (ICM, yellow). The ICM consists of pluripotent cells, which later segregate into the pluripotent inner epiblast cells (middle right, dark orange) and the outer hypoblast cells (green), which will contribute to extraembryonic endoderm. Once the embryo is implanted in the uterus wall (right), the pluripotent cells begin to develop into the three germ layers (ectoderm, mesoderm, endoderm), and pluripotency is gradually restricted. When ICM cells or epiblast stem cells are successfully cultured *in vitro*, they become embryonic stem cells (ESCs) and maintain their pluripotency and their capacity to self-renew. Ever since their initial derivation, in 1981, mouse ESCs have been grown in the presence of fetal calf serum (FCS), routinely used to culture cells but which contains many undefined molecules, giving rise to undefined and even conflicting signaling pathways resulting in a somewhat heterogeneous (“confused”) culture system. A few years later, in 1988, leukemia inhibitory factor (LIF), a cytokine

that activates STAT3 signaling, was found to prevent ESC spontaneous differentiation and maintain its pluripotency. While serum with LIF is still the most commonly used culture system for mouse ESCs, in 2008, a combination of GSK3 and Mek1/2 inhibitors were shown to better mimic the ground-state pluripotency of the developing embryo, resulting in a homogeneous pluripotent culture.

Mouse Embryonic Stem Cells

Genome Organization in Pluripotency: Fewer Contacts and Some Long-Range Interactions

At the chromosome level, pluripotent mouse ESCs already possess an established nuclear organization and chromosome territories (Mayer et al., 2005), although chromosome intermingling, which is frequent in differentiated cells, is particularly low in serum-grown ESCs (Maharana et al., 2016). In line with this, DamID, which maps lamina-associated domains (LADs), demonstrated that LADs are already present in serum-grown ESCs, and the lion’s share of these domains remains stable during differentiation (Peric-Hupkes et al., 2010). An established higher-order structure in mouse ESCs was confirmed by mapping topological-associated domains (TADs) using Hi-C approaches (Dixon et al., 2015; Dixon et al., 2012), as well as chromosome conformation capture carbon copy (5C) in combination with high-throughput sequencing (Phillips-Cremins et al., 2013).

Promoter capture Hi-C technology was used to generate chromosomal interaction maps for all annotated promoters in the mouse genome using a collection of custom-designed biotinylated RNA “baits.” This so-called CHi-C technique was used to compare mouse serum-grown ESCs with fetal liver cells, both of which displayed a strong bias toward cell-type specific interactions. Supporting this view, gene promoters interacting with more than 10 enhancers specifically in ESCs were enriched in developmental pathways and embryogenesis, whereas those in fetal liver cells were enriched in metabolic functions (Schoenfelder et al., 2015). These results suggest that while serum-grown ESCs possess, as we have seen, an established chromosome and genome organization, they also show remarkable ESC-specific, developmentally related contacts. A recent study that used ultra-deep Hi-C sequencing in serum-grown mouse ESCs and in neuronally differentiated progeny (both neural progenitor cells [NPCs] and neurons) found that overall, the number

of compartment borders decreases, while compartment size increases, during ESC differentiation and that intra-TAD contacts become stronger, while inter-TAD contacts become progressively depleted (Bonev et al., 2017). Merging 3D organization with epigenetic profiles, the authors found that Polycomb-mediated interactions were among the strongest interactions in undifferentiated ESCs, becoming progressively disrupted upon differentiation, except for a small subset of genes, which displayed enhanced interactions, correlating with Ring1B binding. Overall, this comprehensive study showed that ESC differentiation entails changes in all levels of genome organization, including, most notably, the Polycomb and TF interaction network. In other words, while the large-scale architecture seems to be overall similar in all cells, ESCs tend to also exploit higher level regulation *in trans*, mediated chiefly by PRC proteins (Figure 2).

A study directly comparing higher-order genome organization in 2i- versus serum-grown mouse ESCs found that while the overall organization and number of interactions were similar between the two states, a group of extremely long-range promoter-promoter interactions (ELRIs) was found to display a time-dependent loss during the transition from serum to 2i conditions (Joshi et al., 2015). This was accompanied by transcriptional rewiring and reduction of H3K27me3 around these ELRIs in 2i conditions, which were required for this genomic reprogramming event. Interestingly, 2i-LIF conditions were shown to be necessary to reinstate several interactions that failed to reestablish during the reprogramming process from NPCs to induced pluripotent stem cells (iPSCs) (Beagan et al., 2016), again suggesting that serum provides a confusing environment as opposed to the ground-state environment of the MEK/GSK3 pathways inhibitors (Figure 2). Another recent study, which directly compared chromatin loops in 2i-grown ESCs, serum-grown ESCs, EpiSCs, and NPCs, found a widespread gain of structural loops, especially at CTCF sites, during the exit from pluripotency (Pekowska et al.,

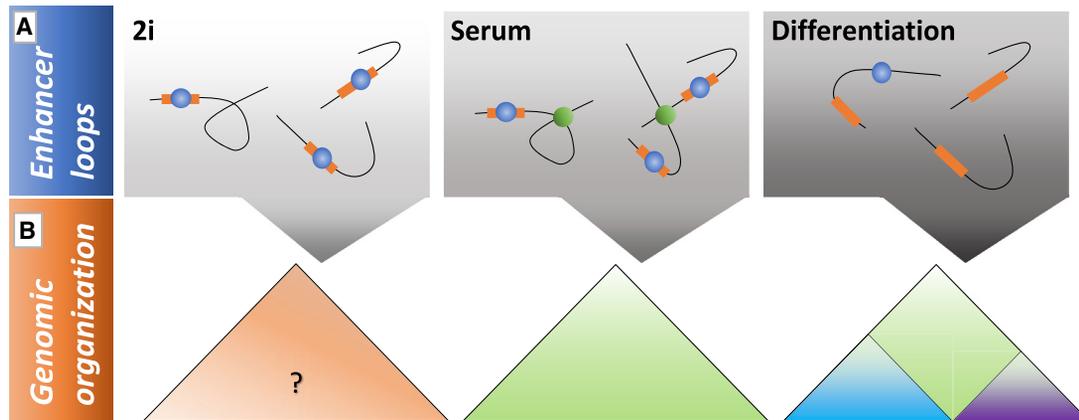


Figure 2. Changes in Higher-Order Genome Organization during ESC Differentiation

(A) ESCs, either in ground-state (left) or serum conditions (middle), possess distal enhancers (orange boxes), which colocalize with architectural proteins (CTCF, cohesin, condensin, blue circles) and interact mainly with pluripotency TFs. Upon differentiation (right), these long-range contacts are decreased. In addition, in serum-grown ESCs, extremely long-range intra- and inter-chromosomal polycomb complex (green circles) mediated interactions are present. These interactions are largely absent in 2i ESCs and in differentiated cells.
(B) While the global genomic organization is established in the pluripotent state and remains throughout differentiation, several notable changes are observed: the number of compartment borders decreases while compartment size increases during ESC differentiation, and inter-TAD contacts become progressively depleted while intra-TAD contacts become stronger (bottom right).

2018), supporting the increase in intra-TAD contacts mentioned above (Bonev et al., 2017).

Taken together, these results demonstrate that while both 2i- and serum-grown ESCs have an overall established and quite similar 3D genomic architecture, several extremely long-range contacts are present only in serum conditions and the TAD compartment structure changes (Figure 2; Table 1). Functionally, this suggests that while such long-range interactions might be dispensable for pluripotency of cultured ESCs, they may reflect the situation in the developing embryo, suggesting that the required rewiring likely takes place when ESCs are transplanted into blastocysts. In addition, both 2i- and serum-grown ESCs have a significantly lower number of chromatin loops compared with EpiSCs and differentiated cells, suggesting that loop formation may restrict developmental potential.

Global Chromatin Structure in Ground State versus Serum: Nucleosome “Clutches” Define Pluripotency

Imaging-based approaches demonstrated that serum-grown ESCs have a distinct organization of chromatin from that observed in differentiating cells. The classical DAPI-dense condensed HP1-positive, H3K9me3 heterochromatin foci appear to be more diffuse in ESCs (Aoto et al., 2006; Meshorer et al., 2006), and they increase in number and compaction during differentiation (Fussner et al., 2011; Kobayakawa et al., 2007; Meshorer, 2007; Meshorer et al., 2006) (Figure 3A), suggesting a global rearrangement of centromeres. A direct side-by-side comparison of 2i-grown, serum-grown, and primed EpiSCs shows an increase in H3K9me3 foci from 2i- to serum-grown to EpiSCs and, somewhat unexpectedly, H3K27me3-positive DAPI domains in 2i-grown cells (Tosolini et al., 2018). Especially visible is the redistribution of the heterochromatin protein HP1 β from an almost completely diffuse pattern in nuclei of undifferentiated ESCs to the gradual accumulation in heterochromatin foci during differentiation (Mattout et al., 2015) (Figure 3B). Electro-spectroscopic imaging (ESI) revealed a more homogeneous chromatin structure in pluripotent cells both *in vitro* (Efroni

et al., 2008; Fussner et al., 2011) and *in vivo* (Ahmed et al., 2010), while early ESC differentiation entails the formation of blocks of peripheral condensed heterochromatin (Hiratani et al., 2010). The transition from widely dispersed 10 nm fibers to peripheral heterochromatin organization was also observed during ESC differentiation to EpiSCs, a process that, interestingly, did not require DNA methylation (Hassan-Zadeh et al., 2017). The nuclear lamina itself, which is largely, although not completely (Eckersley-Maslin et al., 2013), devoid of Lamin A in ESCs (Melcer et al., 2012), is also less organized and more wrinkled in ESCs (Meshorer and Misteli, 2006; Pagliara et al., 2014) (Figure 3C). The examination of DAPI-stained images from recent studies reporting 2i growth conditions showed an overall global nuclear architecture similar to that observed in serum (Guyochin et al., 2014), and ESI imaging revealed a highly homogeneous chromatin structure in 2i-grown reprogrammed fibroblasts (Fussner et al., 2011). In one example, MAD2L2 was shown to maintain an open chromatin state in 2i-grown ESCs (Rahjouei et al., 2017). ESCs depleted for MAD2L2 showed an elevated level of heterochromatin and spreading of H3K9 methylation (Rahjouei et al., 2017). The pluripotency factor Nanog was also shown to regulate pericentromeric heterochromatin organization in mouse ESCs (Novo et al., 2016). *Nanog*^{-/-} ESCs displayed chromocenter organization that was indistinguishable from differentiated cells. Importantly, global chromatin organization and chromocenter clustering were shown to be unaffected when the ESCs were switched to 2i conditions, implying no difference in the global arrangement of chromatin between these conditions.

An elegant imaging study directly compared serum-grown and 2i-grown mouse ESCs using stochastic optical reconstruction microscopy (STORM) super-resolution microscopy (Ricci et al., 2015). The authors found that nucleosomes are arranged in discrete nanodomains in interphase nuclei of mouse ESCs, which they termed “clutches.” Interestingly, 2i-grown ESCs had a lower number of nucleosomes per clutch as compared

Table 1. Chromatin Features in Serum- and 2i-Grown ESCs, Compared with Differentiated Cells.

		Serum	References	2i	References
Genome organization	chromosome territories	no change	(Mayer et al., 2005)	–	–
	chromosome intermingling	lower	(Maharana et al., 2016)	–	–
	LADs	no change	(Peric-Hupkes et al., 2010)	–	–
	TADs – global	no change	(Dixon et al., 2012; Joshi et al., 2015)	no change	(Joshi et al., 2015)
	TADs – finer resolution	weaker / higher intra-/ inter-tad contacts	(Bonev et al., 2017)	–	–
	long-distance interaction	higher	(Phillips-Cremins et al., 2013)	highest	(Joshi et al., 2015)
Chromatin structure	heterochromatin foci, <i>light microscopy</i>	fewer, less compact	(Aoto et al., 2006; Kobayakawa et al., 2007; Meshorer et al., 2006)	comparable to serum	(Guyochin et al., 2014; Novo et al., 2016; Rahjouei et al., 2017)
	chromatin distribution, <i>electrospectroscopic imaging</i>	more homogeneous	(Ahmed et al., 2010; Efroni et al., 2008; Hiratani et al., 2010)	more homogeneous	(Fussner et al., 2011)
	HP1 β distribution	more diffuse	(Mattout et al., 2015)	–	–
	nuclear lamina	wrinkled, less organized	(Meshorer and Misteli, 2006; Pagliara et al., 2014)	–	–
	nucleosome clutches, <i>super-resolution microscopy</i>	fewer nucleosomes	(Ricci et al., 2015)	fewest nucleosomes	(Ricci et al., 2015)
Chromatin accessibility	MNase digestion	more accessible	(Morozumi et al., 2016)	–	–
	DHS-seq	more accessible	(Deng et al., 2013)	–	–
	FAIRE-seq	more accessible	(Murtha et al., 2015)	even more accessible	(Murtha et al., 2015)
	RED-seq	more accessible	(Chen et al., 2014)	–	–
	ATAC-seq	more accessible	(Simon et al., 2017; Xu et al., 2017)	comparable to serum	(Hendrickson et al., 2017; Wu et al., 2016)
The epigenome	DNA methylation	comparable	(Melcer et al., 2012)	much lower	(Marks et al., 2012)
	active histone modifications	slightly higher	(Efroni et al., 2008; Hezroni et al., 2011; Morozumi et al., 2016; Qiao et al., 2015; Yellajoshiyula et al., 2011)	slightly higher	(Rahjouei et al., 2017)
	suppressive histone modifications	slightly lower	(Ahmed et al., 2010; Efroni et al., 2008; Liu et al., 2015; Loh et al., 2007; Sridharan et al., 2013)	slightly lower	(Fussner et al., 2011)
	H3K27me3	slightly lower	(Juan et al., 2016)	lower at developmental genes	(Marks et al., 2012)
	γ H2AX	higher	(Banáth et al., 2009)	higher	(Rahjouei et al., 2017)
Transposable elements	DNA methylation	comparable (~80%)	(Walter et al., 2016)	much lower (<20%)	(Walter et al., 2016)
	H3K27me3	high only on ERV-I group	(Leeb et al., 2010)	higher on ERVs from all groups	(Walter et al., 2016)
	H3K9me3	high on most ERVs	(Karimi et al., 2011)	high on most ERVs	(Walter et al., 2016)
X inactivation	Xist expression	much lower, heterogeneous	(Kobayashi et al., 2016)	much lower, homogeneous	(Sousa et al., 2018)
Replication timing	asynchrony	higher	(Rivera-Mulia et al., 2018)	higher	(Rivera-Mulia et al., 2018)
		comparable	(Masika et al., 2017)	lower	(Masika et al., 2017)

(Continued on next page)

Table 1. Continued

	Serum	References	2i	References
Nuclear plasticity	higher	(Bhattacharya et al., 2009; Meshorer et al., 2006; Morozumi et al., 2016)	-	-
core histone dynamics	higher	(Melcer et al., 2012; Meshorer et al., 2006)	-	-
linker histone dynamics	higher	(Deng et al., 2013; Melcer et al., 2012)	-	-
HMG protein dynamics	higher	(Christogianni et al., 2017; Dialynas et al., 2007; Meshorer et al., 2006; Stixová et al., 2011)	-	-
HP1 dynamics	higher	(Bhattacharya et al., 2009)	-	-
Lamin B dynamics	higher	(Bošković et al., 2014; Meshorer et al., 2006)	-	-
H3.3 global dynamics	similar	-	higher around TSSs	(Schlesinger et al., 2017)
H3.3 turnover	-	(Yildirim et al., 2014)	-	-
MacroH2A2 turnover	higher at promoters	-	-	-

All descriptions (“lower,” “higher,” etc.) are in relation to differentiated cells.

with serum-grown ESCs, which displayed a more heterogeneous distribution, as expected. ESC-derived NPCs had the highest number of nucleosomes per clutch (Figure 4). This suggested that nucleosome clustering is a feature of the developmental state of the cells, a hypothesis that was tested successfully, enabling the prediction of pluripotency in a variety of iPSCs (Ricci et al., 2015). Taken together, these results suggest that the global chromatin structure of serum- and 2i-grown ESCs is very similar and that 2i conditions support and even enhance an open chromatin configuration, chromocenter clustering, and heterochromatin distribution (Table 1). Thus, the “open chromatin” hypothesis of ESCs is strengthened by these imaging-based results, suggesting once again that pluripotency entails unique chromatin features compared with differentiated cells and, importantly, that global chromatin organization predicts pluripotency and function.

Chromatin Is Globally More Accessible in Pluripotent Cells

An open chromatin conformation should entail higher accessibility of chromatin to either enzymatic digestion, e.g., micrococcal nuclease (MNase), DNase I, etc. or to methods that integrate elements within the genome, e.g., assay for transposase-accessible chromatin sequencing (ATAC-seq). Several studies over the past decade indeed demonstrated higher chromatin accessibility in ESCs. Global higher accessibility to MNase digestion was shown for serum-grown ESCs compared with differentiating cells (Morozumi et al., 2016). In one thorough study, which mapped genome-wide DNase-I hypersensitive sites (DHSs), a striking reduction of DHSs was revealed from ~55,000 sites in serum-grown ESCs to ~30,000 in NPCs (Deng et al., 2013). Interestingly, the high mobility group protein HMGN1 was found to be largely responsible for maintaining accessible DHSs in ESCs. Deletion of HMGN1 in ESCs resulted in a dramatic drop of DHSs by almost 70%, adding to the growing list of factors that act to maintain an accessible chromatin in ESCs (Alajem et al., 2015; Gaspar-Maia et al., 2009; Ho et al., 2009; Melcer et al., 2012; Morozumi et al., 2016; Novo et al., 2016; Wan et al., 2013; Yellajoshiyula et al., 2011). Although enzymatic digestion of chromatin is usually regarded as a measure of chromatin accessibility, quantitative conclusions usually require different enzyme concentrations. Therefore, DHSs as such, may not directly reflect regions of accessible chromatin but rather TF binding sites. In this case, the results above should be interpreted as demonstrating a higher number of measured TF-bound events in ESCs rather than accessible chromatin *per se*, although if we assume that ESCs do not require a larger number of TFs to operate, TF binding may in turn also reflect more accessible chromatin *in vivo*. Be it as it may, the consistent differences between pluripotent and differentiating cells support the model that posits that ESCs keep all options open.

An alternative method to DHSs is formaldehyde-assisted identification of regulatory elements (FAIRE) (Giresi et al., 2007). Comprehensive mapping of open chromatin regions using FAIRE-seq in 2i-grown ESCs, EpiSCs, NPCs, and MEFs revealed a marked difference between the two pluripotent cell lines, ESCs and EpiSCs, and the two differentiated cell types (Murtha et al., 2015). ESCs and EpiSCs displayed a greater percentage of their open chromatin at promoters and exons, compared with the NPCs and MEFs, and overall, as expected, ESCs had the

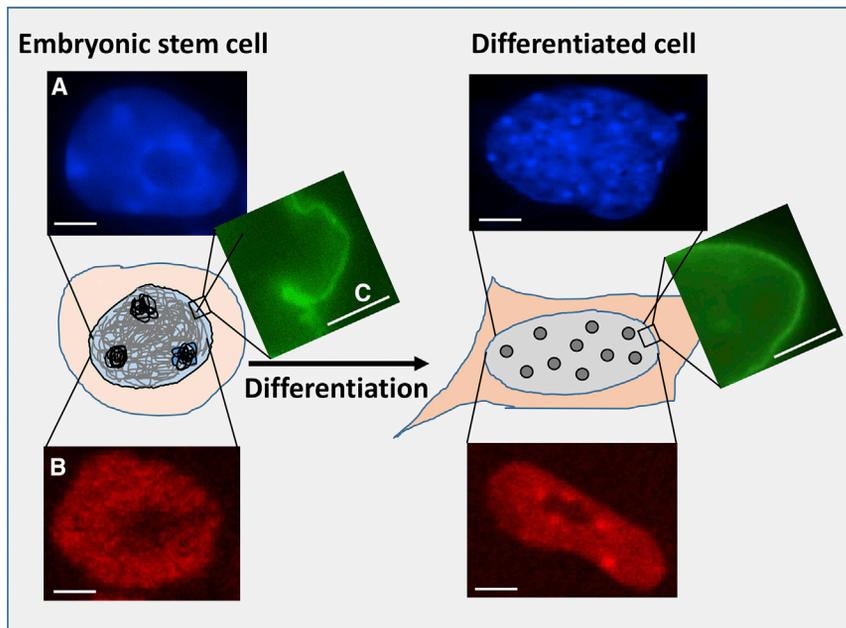


Figure 3. Heterochromatin and Nuclear Lamina Rearrangement during Embryonic Stem Cell Differentiation

A schematic diagram depicting a representative ESC (left) and a differentiated cell (right) is shown in the middle. The black circles represent heterochromatin foci, which are larger and more dispersed in ESCs and more numerous and condensed in differentiated cells.

(A) Representative DAPI stained (blue) undifferentiated (left) and differentiated (right) mouse ESC.

(B) HP1 β rearrangement during ESC differentiation. HP1 β (red) is dispersed in undifferentiated ESCs (left) and becomes concentrated in heterochromatin foci (right).

(C) Nuclear lamina rearrangement during ESC differentiation. Shown are representative immunofluorescence images of Lamin B staining (green) in undifferentiated (left) and differentiated (right) mouse ESCs. All scale bars, 2 μ m.

most accessible chromatin, followed by EpiSCs. While both DNase-seq and FAIRE-seq have been used extensively in the past several years to probe open chromatin, a limitation of these methods is that they typically identify nucleosome-depleted regions. To address this, a modified genome-wide accessibility assay—restriction endonuclease digestion of chromatin coupled to deep sequencing (RED-seq)—was developed utilizing restriction enzyme digestion and biotin adaptor ligation (Chen et al., 2014). RED-seq was able to detect nucleosome-depleted regions, akin to DNase-seq and FAIRE-seq, but in addition, it enabled the detection of chromatin accessibility within nucleosomes. Comparing ESCs and MEFs, this study reported, once again, a global open chromatin structure in serum-grown ESCs. A decrease of over 20% in the number of RED-seq open chromatin peaks were observed over both DHSs and CTCF sites. To complement such genome-wide accessibility methods, nuclease footprinting, which utilizes MNase digestion to reveal nucleosome footprints of different sizes, can be used to track changes in footprints over selected binding sites. Nuclease footprints demonstrated, for example, that the chromatin remodeling complex esBAF (Ho et al., 2009) acts to maintain open chromatin in ESCs, especially by promoting KLF4 occupancy, thus maintaining an accessible chromatin structure over KLF4 binding sites (Hainer and Fazio, 2015). ATAC-seq performed in serum-grown mouse ESCs and during early ESC endodermal differentiation identified over 85,000 open chromatin sites in ESCs (Simon et al., 2017). Following differentiation to definitive endoderm, both gain (~19%) and loss (~32.5%) of ATAC-seq peaks were identified, but globally, a net loss of over 13% in accessible sites were recorded, down to just over 70,000 sites. Although absolute quantitative conclusions should be taken with caution, these results are in line with the DHS, FAIRE-seq, and RED-seq studies discussed above. In another monoallelic ATAC-seq study, serum-grown ESCs displayed biallelic accessibility of the promoter-enriched random monoallelically accessible elements,

some of which became monoallelic in ESC-derived NPCs (Xu et al., 2017), possibly explaining, at least partially, the higher overall accessibility observed in ESCs. This coincides with the biallelic expression observed in serum-grown ESCs, which largely becomes monoallelic in NPCs (Eckersley-Maslin et al., 2014).

Although not all accessibility assays have been performed in ground-state 2i conditions (e.g., nucleosome positioning, which we do not discuss here), ATAC-seq was recently conducted in serum-grown and 2i-grown ESCs in two separate studies (Hendrickson et al., 2017; Wu et al., 2016), but which, importantly, enabled a side-by-side comparison (Hendrickson et al., 2017). Somewhat unexpectedly, the two profiles seemed almost identical, and both had a slightly higher number of ATAC-seq peaks than ICM cells. In this case, the 2i-grown cells looked more similar to serum-grown cells than to ICM cells. Taken together, all genome-wide studies aiming to characterize accessible and open chromatin in ESCs, be it serum-grown or in ground-state conditions, agree that ESC chromatin is overall more accessible, more prone to enzymatic digestion, and more amenable for transposon insertions (Table 1), demonstrating once again that open chromatin accompanies, and is very likely required for, pluripotent cells.

Changing the Epigenome: 2i Reduces Global DNA Methylation and Local H3K27me3

Perhaps the two most prominent features of ground-state pluripotency, distinguishing it from serum-grown ESCs, are the global low-level DNA methylation and the reduced H3K27 methylation in developmentally regulated genes (Marks et al., 2012). Notwithstanding, a significant fraction (> 18%) of 2i-grown cells showed chromocenter accumulation of H3K27me3, a feature never observed in serum-grown cells (Tosolini et al., 2018). Recent reports added mechanistic insights into the erasure of DNA methylation when ESCs are switched from serum to 2i, identifying several key players in the process. Examples include the *de novo* DNA methyltransferase (DNMT) expression driven by fibroblast growth factor (FGF) signaling (Ficz et al., 2013), maintenance DNMTs regulated by UHRF1 (von Meyenn et al., 2016), and p53, which restricts the

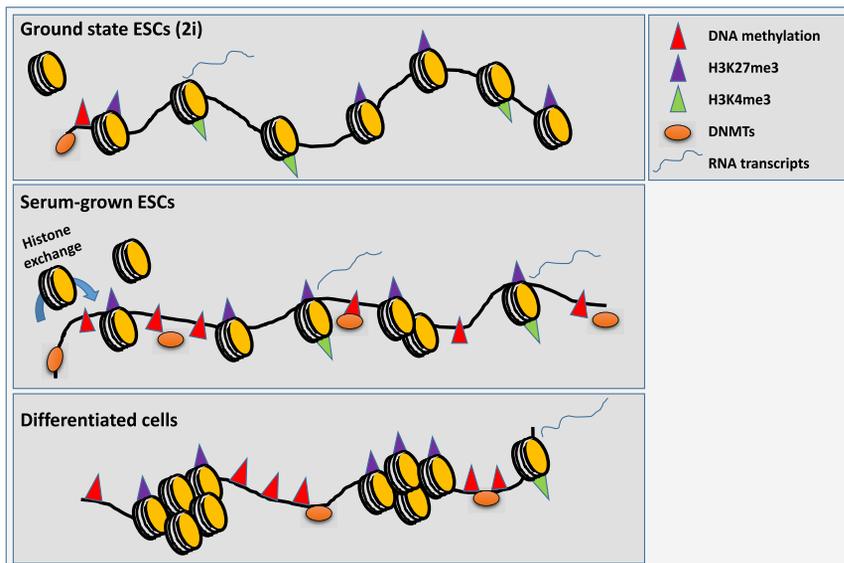


Figure 4. Several Distinct Epigenetic Features in Undifferentiated and Differentiated ESCs

Shown are schematic diagrams of chromatin in ground-state (2i) ESCs (top), serum-grown ESCs (middle), and differentiated cells (bottom). Nucleosomes “clutches” rearrange during differentiation. Ground-state ESCs show the lowest number of nucleosomes per clutch, serum-grown ESCs show an intermediate number, and differentiating cells display the highest number of nucleosomes per clutch. DNA methylation (red triangles) is globally reduced in ground-state ESCs compared with both serum-grown ESCs and differentiating cells, both of which have elevated levels of DNMTs (orange circles). H3K27me3 (purple triangles) is reduced locally at developmental genes in ground-state ESCs, while in serum-grown ESCs, it marks, together with H3K4me3 (green triangles), “bivalent,” developmentally regulated genes, some of which are resolved during differentiation.

expression of the *de novo* DNMTs while inducing the expression of TET1 and TET2, which promote DNA demethylation (Tovy et al., 2017). The demethylation process was shown to be completed within about 7 days, but interestingly, most of the demethylation occurred rapidly, already within the first 24 h after the switch to 2i conditions (Ficz et al., 2013). Conversely, the transition from 2i back to serum conditions is accompanied by upregulation of *de novo* DNMTs and downregulation of PRDM14, the latter of which both represses *de novo* DNMTs and promotes Tet activity on target genes (Ficz et al., 2013; Kalikan et al., 2017). Recently, using whole-genome bisulfite sequencing (WGBS) and single-cell profiling, DNA methylation heterogeneity was described during the transition from 2i- to serum-grown ESCs (Rulands et al., 2018). Interestingly, the methylation state of many enhancer loci within individual ESCs is synchronized and coupled with nascent transcription, resulting in an oscillating pattern of methylation, which is faster than cell fate decisions and autonomous of the cell cycle. Functionally, this may suggest an important role for the heterogeneous states observed under serum conditions for rapid cell fate decisions during the exit from pluripotency. Curiously, despite these widespread global differences in DNA methylation between the serum- and 2i-grown conditions, the differentiation potential of these two states was shown to be overall similar (Marks et al., 2012), suggesting that the acquired DNA methylation in serum conditions can be easily reprogrammed. Recent work, however, showed that prolonged inhibition of Mek1/2 signaling results in aberrant DNA hypomethylation of several imprinted loci and loss of H2A.X, resulting in impaired developmental potential (Choi et al., 2017; Yagi et al., 2017). Replacement of Mek1/2 inhibitors with Src inhibitors (a2i) preserved global and local (imprinted control regions) DNA methylation as well as developmental potential while maintaining a ground-state-like transcriptional state (Choi et al., 2017). Based on the epigenetic similarity between 2i ESCs and ESCs grown in the presence of GSK3 and Src inhibitors, it is likely that the latter would display an open chromatin structure characteristic of both serum- and

2i-grown ESCs. Together, it appears that while DNA methylation is perhaps the most distinguishing feature between 2i- and serum-grown ESCs, its function seems to be redundant in undifferentiated ESCs.

ESC chromatin was shown to be enriched with activity-related histone modifications in both serum (Efroni et al., 2008; Hezroni et al., 2011; Morozumi et al., 2016; Qiao et al., 2015; Yellajoshyula et al., 2011) and ground-state (Rahjouei et al., 2017) conditions and, curiously, also with γ H2AX, the phosphorylated form of the DNA-damage related H2A.X, suggested to reflect their open chromatin nature (Banáth et al., 2009; Rahjouei et al., 2017). ESCs were also shown to be relatively depleted for heterochromatin-associated histone marks, especially histone modifications marking constitutive heterochromatin, i.e., H3K9me3 (Loh et al., 2007; Wen et al., 2009). Specifically, ESC differentiation was shown to entail heterochromatin spreading into “large organized chromatin K9 domains” (LOCKs) (Wen et al., 2009). Although the statistical validity of these particular data was later challenged (Lienert et al., 2011), several other studies employing either imaging-based examinations (Ahmed et al., 2010; Efroni et al., 2008; Mattout et al., 2011) or biochemical and epigenomic assays (Liu et al., 2015; Loh et al., 2007; Sridharan et al., 2013), confirmed the paucity of heterochromatin-related histone modifications (i.e., H3K9me2; H3K9me3) in both serum- and 2i-grown mouse pluripotent cells by high-throughput mapping. Interestingly, a recent study demonstrated H3K9me3-independent inaccessible chromatin formation and silencing of lineage-specifying genes in 2i-grown ESCs by a complex consisting of ADNP and CHD4 as well as the two HP1 proteins HP1 β and HP1 γ (Ostapcuk et al., 2018). Both HP1 β and HP1 γ were already previously shown not to be associated with heterochromatin and H3K9me3 and to assume a more genic distribution in ESCs (Mattout et al., 2015). Overall, the constitutive heterochromatin content in pluripotent cells, both 2i- and serum-grown, seems lower than in differentiated cells, supporting the view that ESCs keep their options open by refraining from irreversible silencing mechanisms. Which

alternative silencing pathways operate, especially in 2i conditions, largely remain to be identified.

Polycomb-associated “facultative” heterochromatin, i.e., H3K27me₃, was also shown to be relatively low in serum-grown ESCs and to increase during differentiation (Juan et al., 2016). Interestingly, in the same study, H3K27me₂ was found to display the opposite trend, suggesting that H3K27me₂ is partially methylated into H3K27me₃ during ESC differentiation. In 2i conditions, H3K27me₃ is significantly depleted in developmentally regulated genes (Juan et al., 2016; Marks et al., 2012), but by contrast to the promiscuously transcribing serum-grown ESCs (Efroni et al., 2008; Lin et al., 2012; Nie et al., 2012; Percharde et al., 2017), ground-state ESCs do not show permissive transcription (Marks et al., 2012) and do not express satellite repeats (Tosolini et al., 2018) as serum-grown ESCs do (Efroni et al., 2008; Tosolini et al., 2018), suggesting that transcription in 2i-grown ESCs is blocked by means other than H3K27me₃, H3K9me₃, or DNA methylation.

In serum-grown ESCs, H3K27me₃ was shown to be present together with H3K4me₃ in many developmentally regulated genes, forming the so-called bivalent chromatin (Azuara et al., 2006; Bernstein et al., 2006; Mikkelsen et al., 2007). Bivalent chromatin is an elegant concept. It not only explained how ESCs silence their “active-chromatin-enriched” genome but also provided a mechanism for rapid activation or suppression of developmentally regulated genes during differentiation (Harikumar and Meshorer, 2015; Voigt et al., 2013). The demonstration that 2i-grown ESCs are largely devoid of H3K27me₃ in developmental genes challenged the bivalency idea in ground-state ESCs. In addition, all early studies were conducted on cell populations. Thus, even if we are convinced that bivalent domains exist within the same cell, it was essentially impossible, until recently, to determine whether the conflicting histone marks are present on the same chromosome, on the same nucleosome, and on the same histone molecule itself (Harikumar and Meshorer, 2015). Whether this placement is even important for their function should also be considered (Hu et al., 2013). An interesting biochemical study suggested that the H3K4me₃ and H3K27me₃ marks are present within the same nucleosome on separate histone molecules (Voigt et al., 2012). A more recent elegant approach using immobilization of single nucleosomes provided the final proof that the bivalent unit is the nucleosome itself (Shema et al., 2016). Since the latter work used serum-grown mouse ESCs, it indeed provided evidence for the presence of bivalency and its enrichment in undifferentiated ESCs (Shema et al., 2016) but did not resolve whether 2i grown ESCs completely lose this feature.

Taken together, these studies show that serum- and 2i-grown ESCs differ significantly in their global DNA methylation profiles and local H3K27me₃ profiles. Low-level DNA methylation and reduced H3K27me₃ at developmental genes is a hallmark of ground-state pluripotency and resembles the ICM. While, as we have seen above, the two states share an open and accessible chromatin structure, such differences may account for the lower number of clutches (Ricci et al., 2015) or the higher number of long-distance interactions (Joshi et al., 2015) observed in 2i-grown ESCs, discussed above. Be that as it may, while both DNA methylation and H3K7me₃ are associated with heterochromatin and silencing, serum-grown, rather than ground-state,

ESCs show transcriptional promiscuity (Efroni et al., 2008) and expression heterogeneity (Singer et al., 2014), suggesting that other silencing mechanisms are at play restricting permissive transcription in ground-state ESCs (Hainer et al., 2015).

Differences in Transposable Element Regulation between Serum and 2i

Nearly half of the mammalian genome is derived from trophectoderm (Goodier and Kazazian, 2008), including members of the long terminal repeat (LTR)-containing endogenous retroviruses (ERVs), and the non-LTR-containing retrotransposons (LINE-1 and SINE-1). These genetic elements are considered a major driving force in vertebrate evolution, and many such introduced retroviral genes, non-coding RNAs, or promoters are being used by the genome of their hosts (Schlesinger and Goff, 2015). Hence, the expression of these elements must be tightly regulated during embryonic development to balance the potentially damaging effects of widespread retrotransposition against the benefits of promoting genetic diversity. Transposon reactivation is thus an inherent danger in cells that lose epigenetic silencing during developmental reprogramming. ERV activity is controlled by multiple epigenetic mechanisms including DNA methylation, repressive histone modifications, and small RNAs (Rowe and Trono, 2011). In somatic tissues, DNA methylation and H3K9me_{2/3} have been shown to be responsible for trophectoderm silencing (Hutnick et al., 2010). In ESCs, while some ERVs are expressed in the absence of members of the polycomb complex (Leeb et al., 2010) or the H3K9me₃ methyltransferase SETDB1, a second group is mainly regulated by DNA methylation (Karimi et al., 2011). The silencing of such potentially hazardous genomic elements was suggested to be the main role of DNA methylation in mammalian cells (Bestor et al., 2015; Walsh et al., 1998). Therefore, it was not clear how trophectoderms would be regulated in 2i-grown cells, where DNA methylation is drastically diminished. Inspection of the DNA methylation landscape using WGBS in ESCs shifted from serum- to 2i-grown conditions, revealed that around 20% of DNA methylation is refractory to demethylation. DNA methylation is retained mostly on imprinted genes, major satellite repeats, and intracisternal A particles (IAP) repetitive elements (Ficz et al., 2013; Habibi et al., 2013). Interestingly, 2i-grown cells treated with vitamin C showed a transient significant increase in 5hmC and a progressive loss of 5mC. These responses were completely abolished in cells lacking TET activity. Akin to *in vivo* blastocysts, IAP elements and imprinted regions were resistant to vitamin-C-mediated demethylation (Blaschke et al., 2013). In the absence of DNA-methylation-mediated silencing, trophectoderms were shown to be silenced by suppressive histone modifications after a short wave of trophectoderm activation in 2i-LIF and vitamin-C conditions (Walter et al., 2016). Thus, it seems that specific repressive histone marks can secure the control of trophectoderms during early development and in ground-state ESC culture, in times of extensive DNA demethylation (Figure 5A). The idea that other chromatin-based modifications (e.g., H3K27me₃) can substitute for DNA methylation in silencing likely represents the rule, rather than the exception, for ground-state ESCs of some trophectoderm subgroups.

Two Active X Chromosomes in Pluripotency

Random X inactivation, the process of dosage compensation in female mammals, takes place in the blastocyst stage embryo

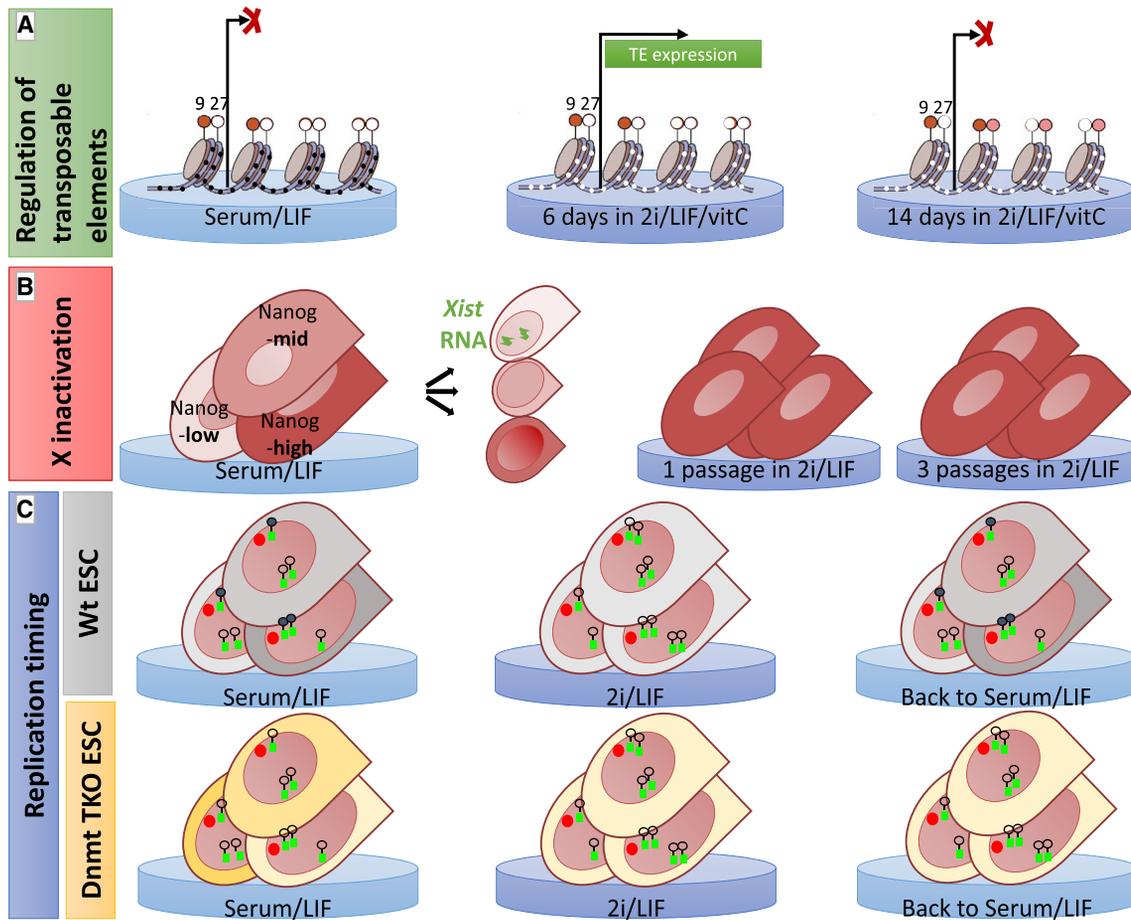


Figure 5. Transposable Elements, X Inactivation, and Replication Rimming in 2i versus Serum ESCs

(A) 2i/vitC induces a rapid loss of DNA methylation followed by a transcriptional burst of most ERVs. H3K27me3 accumulation and H3K9me3 restore trophoblast repression.

(B) Nanog and *Xist* expression correlates in serum-grown ESCs. Upon transition to 2i, Nanog is homogeneously expressed at high levels and *Xist* is repressed, reversing the initiation of the X-inactivation process.

(C) Schematic representative fluorescence *in situ* hybridization (FISH) of ESCs during S phase. The paternal allele is marked with a red probe, while the asynchronously replicating gene (*Nanog*, *Igf-1*, and *Olf1* locus on chromosome 6) is labeled green. A single dot denotes a yet-to-be replicated allele (late replication); double dots represent replicated alleles. A high percentage of single-double pattern is observed for all asynchronously replicating genes in ESCs grown in the presence of serum. In 2i, the genes are switching to synchronous replication timing—a transitory change that will revert once the 2i is removed. This reversion, or initiation of asynchronous replication, is dependent on DNA methylation and is not observed in ESCs depleted for the three DNA methyltransferases (TKO).

around the time of implantation (Augui et al., 2011). Since the process in mouse is gradual between embryonic day (E) 3.5 and E5.5 and not entirely synchronous, the blastocyst usually contains cells in different stages of X inactivation (Kobayashi et al., 2016). Regardless, in ESCs, having two active X chromosomes is considered one of the defining hallmarks of pluripotency in mammalian female cells (Kobayashi et al., 2016). X inactivation in mouse ESCs was recently evaluated in 2i versus serum conditions (Sousa et al., 2018). The authors show that 1 day after serum-grown ESCs are switched to 2i, the levels of *Xist* and other X-inactivation regulatory RNAs are downregulated, directly correlating with the upregulation of Nanog expression (Figure 5B). Unexpectedly, this effect was also observed in male ESCs. The single X chromosome in male cells was shown to go through the same initiation steps of X inactivation in the Nanog negative ESCs, as well as during the first days of differentiation in both 2i- and serum-grown ESCs. Since, as discussed

above, serum-grown ESCs are a heterogeneous population of cells, they possess mixed stages of X inactivation, although most of the cells do show two active X chromosomes (Kobayashi et al., 2016). In this regard, while 2i-grown cells may better mimic the E3.5 ICM cells, serum-grown ESCs may more faithfully represent the heterogeneous situation found in blastocysts *in vivo* (Figure 1). This idea explains why both cell types, despite some of the apparent differences described herein, are similarly pluripotent.

Replication Timing in ESCs Align with 3D Genome Organization

Mammalian DNA is replicated in a highly organized and regulated fashion, whereby the genome is divided into replication time zones that are correlated with gene expression. Active genes usually replicate early during S phase, while inactive regions replicate late (Gilbert, 2010). About half of the genome replication timing is conserved, while the other half is changing

during differentiation (Rivera-Mulia and Gilbert, 2016). Recently, it has been shown that in 2i grown ESCs, single cells of the same population maintain conserved and homogeneous replication timing patterns (Dileep and Gilbert, 2018). Interestingly, a tight correlation was observed between replication timing domains and the TADs and LADs in different cell types (Pope et al., 2014), a feature that is also conserved at the single-cell level (Nagano et al., 2017). As mentioned earlier, these megascale 3D structural domains are established in the pluripotent state, and their boundaries remain largely conserved throughout the cell cycle and development. Accordingly, while the replication domain boundaries also appear to be stable in different cell types, approximately half of the genomic domains are switching replication timing between cell states (Rivera-Mulia and Gilbert, 2016). Therefore, we can speculate that, as there is no major difference in the overall 3D genomic organization between 2i- and serum-grown ESCs, we can expect no major difference in the global replication timing patterns.

While a global direct comparison of replication timing between 2i- and serum-grown ESCs has yet to be reported, several studies examined replication timing of monoallelically expressed genes in 2i- and serum-grown ESCs. Monoallelically expressed genes (or chromosome in the case of X inactivation) replicate asynchronously in S phase with one, usually the active, allele replicating earlier. This mechanism serves as an epigenetic mark for distinguishing between the two alleles. In a recent study carried out on ESCs derived from hybrid crosses of *M. musculus musculus* × *M. musculus castaneus*, a relatively high degree of asynchrony (~17% of the genes) was reported in ESCs (Rivera-Mulia et al., 2018). Both serum- and 2i-grown ESCs showed this high degree of asynchrony, which was lost during differentiation. By contrast, in a study conducted in ESCs of inbred mice strains, the examined alleles replicated synchronously in ground-state ESCs but asynchronously in serum, switching between late and early replication timing (Figure 5C) (Masika et al., 2017). This serum-related asynchrony is DNA methylation dependent since it disappears in ESCs lacking all DNMTs (DNMT-TKO) (Masika et al., 2017). Together, these data may suggest that the oscillations in CpG methylation in serum-grown ESCs might affect the establishment of the allelic discrimination and thus be responsible for generating allelic choice diversity in the organism. The differences observed in replication timing in ESCs derived from inbred versus non-inbred mice may suggest that *cis*-acting sequences regulate synchronous replication in ground-state conditions. What these sequences are and whether replication timing changes globally between 2i- and serum-grown ESCs await additional research.

Chromatin Proteins Are Hyperdynamic in Pluripotent Cells

Using fluorescence recovery after photobleaching (FRAP), the association between chromatin and structural chromatin proteins including HP1 proteins (Christogianni et al., 2017; Dialynas et al., 2007; Stixová et al., 2011), HMG proteins (Deng et al., 2013; Melcer et al., 2012), and core and linker histones (Bhattacharya et al., 2009; Melcer et al., 2012; Meshorer et al., 2006; Morozumi et al., 2016) was shown to be hyperdynamic in serum-grown pluripotent cells. Congruently, reprogramming somatic cells to pluripotency reverts the more restricted protein-chromatin association to the pluripotent hyperdynamic state (Hezroni et al.,

2011). This dynamic association is restricted upon differentiation and was suggested to be important for the pluripotent state (Mattout and Meshorer, 2010; Melcer and Meshorer, 2010; Suvà et al., 2013). Interestingly, only pluripotent and multipotent cells, although to a lesser extent, displayed this hyperdynamic behavior compared with differentiated cells, and differentiation *per se* did not alter the dynamic state of chromatin proteins (Meshorer et al., 2006). The only histone protein that did not show hyperdynamic behavior in ESCs is the core histone variant H3.3 (Meshorer et al., 2006). Intriguingly, however, subsequent genome-wide turnover studies using sequencing approaches at a single nucleosome resolution revealed a single hyperdynamic H3.3 nucleosome marking promoters of undifferentiated 2i-grown ESCs (Schlesinger et al., 2017). This resembles the behavior of the core histone variant MacroH2A2, which was also studied using a similar approach, in serum-grown ESCs: MacroH2A2 was stably associated with large genomic blocks, which further extended upon ESC differentiation, but in promoter regions, it displayed a high turnover rate in undifferentiated ESCs (Yildirim et al., 2014). These results suggest that the global hyperdynamic behavior observed for several structural components of chromatin is not a general feature of all chromatin proteins in pluripotent cells and that at least some variants display promoter-specific dynamic turnover in ESCs (Table 1).

But why are chromatin proteins more dynamic in pluripotent cells? One potential explanation is the relative paucity of constitutive heterochromatin, especially H3K9me3 domains. Since the levels of HP1 proteins, for example, are not lower in ESCs compared with differentiated cells (Mattout et al., 2015), a larger unbound fraction of HP1 to H3K9me3 is expected, leading to the observed hyperdynamic behavior. As H3K9me3 domains are formed during differentiation, more and more HP1 molecules become associated with them, and their diffusion mobility is restricted. In the case of linker and core histones, one intriguing possibility is a change in phase separation (Erdel and Rippe, 2018), caused, indirectly, by the formation of heterochromatin (Larson et al., 2017; Strom et al., 2017).

On examination of the reorganization process of the heterochromatin landscape during early serum-grown differentiating ESCs, fewer dynamic heterochromatin domains were observed during the exit from pluripotency, resulting in a highly heterogeneous process, which becomes more and more stable over time (Christogianni et al., 2017). A dynamic chromatin state in serum-grown ESCs was further confirmed using correlated spatiotemporal fluctuations (Talwar et al., 2013). Photobleaching-based experiments performed in early developing mouse embryos revealed that hyperdynamic plasticity of core histones *in vivo* precedes the pluripotent state with the highest dynamic association of H3.1 and H3.2 at 2-cell-stage embryos (Bošković et al., 2014). As in ESCs, the core histone variant H3.3 also did not show this typical hyperdynamic association in early embryos (Bošković et al., 2014), suggesting at least partial selectivity or reflecting the different chaperone activities for the two classes of variants. In addition to structural components of chromatin, the nuclear lamina protein Lamin B1 (Bhattacharya et al., 2009) as well as the helicase Topoisomerase I (Topol) (Harikumar et al., 2017) were also shown to be more dynamic in ESCs compared with differentiated cells. Together, these data suggest that several architectural proteins, all of which are chromatin associated,

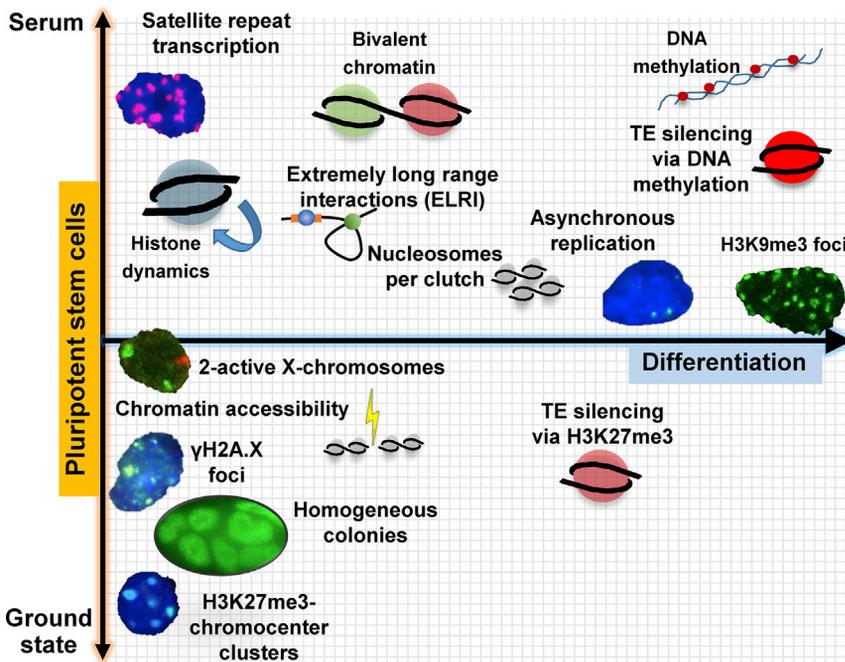


Figure 6. Schematic Summary of Chromatin Features in Pluripotent and Differentiated Cells

x axis denotes differentiation; y axis denotes culture conditions; bottom: 2i, top: serum. Overall, ESCs begin their journey with an “open” chromatin structure, which supports pluripotency; during differentiation, the potential of the cells gradually decreases, while gaining their specific functions.

ESCs (Pagliara et al., 2014). Taken together, these results imply that ESCs transition through a more permissive state when undergoing differentiation. It is likely that several of the recorded features of ESCs described above including chromatin protein hyperdynamics, permissive transcription, decondensed chromatin, active histone modifications, etc. are transiently enriched in this transition state and that serum-grown ESCs show a high degree of variability resulting in promiscuous phenotypes. Regardless, essentially all studies argue that both ground-state and serum-grown mouse ESCs possess some degree of open, accessible, and permissive chromatin.

show hyperdynamic plasticity in undifferentiated serum-grown ESCs, supporting the open chromatin view of ESCs. However, most studies reported to date were performed in serum conditions, and hence, it cannot be ruled out that the hyperdynamic plasticity is, at least partially, due to the confusing signals obtained from growing ESCs in the presence of serum. This is supported by the high variation of chromatin protein plasticity observed in serum conditions (Christogianni et al., 2017) and might suggest that the ground state has a lower epigenetic “Waddington landscape energy,” which is elevated during serum and perhaps during ESC priming and then restricted again during differentiation and specification.

This view is supported by a study that directly compared, using FRAP experiments, the dynamic plasticity of core histones in Nanog-positive and Nanog-negative serum-grown ESCs (Chalut et al., 2012). Although the authors did not grow the cells in ground-state 2i conditions, they identified naive (Nanog-high) versus primed (Nanog-low) ESCs according to Nanog expression. They demonstrated that the Nanog-high naive ESCs show restricted dynamics and transcriptional activity compared with the primed Nanog-low cells. They further developed an optical stretching method to reveal that naive ESCs have a stiffer nucleus, which is coupled to a globally more condensed chromatin state (Chalut et al., 2012). In a follow-up study, the authors used 2i ground-state conditions and identified three distinct states: 2i-grown naive ESCs expressing both Nanog and Rex1, primed ESCs 48 h after withdrawal of the two inhibitors, and a “transition” state (T) 24 h after 2i removal where the cells still express Rex1 but not Nanog. Remarkably, T-ESCs have auxetic nuclei, meaning that they become stiffer with increasing compression. Moreover, using electron microscopy, the authors show that chromatin is less compact in T-ESCs than in naive

matin. Based on all combined literature, one intriguing possibility is that ground-state ESCs possess a semi-restricted state, which is lifted to a higher energy hyperdynamic state when they are grown in serum or even when primed for differentiation. Once differentiation begins, the various open chromatin plasticity features depicted above become gradually restricted (Figure 6).

Human Embryonic Stem Cells Also Display Open Chromatin Features

While conditions mimicking ground-state pluripotency in mouse were identified in 2008 (Ying et al., 2008) and very recently modified and improved (Choi et al., 2017), the situation with hESCs remains somewhat controversial, having been shown to represent epiblast stage, rather than blastocyst stage, mouse ESCs (Brons et al., 2007; Tesar et al., 2007), at least before naive conditions were developed for hESCs. In one example supporting this view, hESC replication timing profiles were significantly more aligned to mouse EpiSCs than to mouse ESCs (Ryba et al., 2010). Nonetheless, several studies convincingly showed that hESCs possess chromatin and nuclear features that support an open and relaxed state. For example, nuclei of hESCs were shown to have high physical plasticity that stiffens upon differentiation (Pajerowski et al., 2007), enrichment of active histone modifications (Bártová et al., 2008a), and an overall decondensed chromatin (Bártová et al., 2008b), and ultrastructural imaging studies revealed a conspicuous absence of heterochromatin, including peripheral heterochromatin (Underwood et al., 2017). We will therefore only briefly summarize the situation in human pluripotency and select representative examples to illustrate the current state of affairs of chromatin plasticity in hESCs.

Direct measurement of open chromatin content in hESCs was carried out using DHSs and FAIRE. The authors compared several human cell lines, including hESCs, and found that the latter possessed the higher fraction of DNase-I sites, with >3%, compared with an average of <2% in six other human cell lines tested (Song et al., 2011). The same was true for open chromatin, which the authors calculated based on their experiments. In a more recent study, ATAC-seq was used to map open chromatin during hESC differentiation into cardiomyocytes (Liu et al., 2017). In this study, the authors analyzed the relative genomic fraction rather than overall accessibility. They found that the fraction of open sites corresponding to promoter regions increased during differentiation at the expense of intergenic regions. This transition likely reflects the changes in transcription that are associated with differentiation, resulting in many open sites in intergenic regions. Less compact chromatin in hESCs was also observed in a study, which compared hESCs with human CD34+ cord blood cells (Lorzadeh et al., 2016). Unlike the CD34+ cells, which were shown to be enriched with 2-nucleosome fragment sizes, hESCs were highly enriched with single nucleosome fragments, likely reflecting a more open chromatin. Together, these data argue for a less compact chromatin in hESCs compared with differentiated cells.

A relatively open chromatin structure in hESCs compared with differentiated cells was also found using epigenomic mapping. High-resolution mapping of several histone modifications in hESCs using ChIP-seq analyses revealed that the two most predominantly redistributed modifications were the heterochromatin-associated H3K27me3 and H3K9me3, demonstrating heterochromatin increase during ESC differentiation (Hawkins et al., 2010). Notwithstanding, differentiation into primordial-germ-cell-like cells (PGCLCs) resulted in a global decrease in H3K27me3, although ESCs showed the highest number of H3K27ac-specific regions (Kurimoto et al., 2015). In the most comprehensive epigenomic mapping conducted as yet, chromatin state dynamics were analyzed in some 300 human cell types and tissues (Zhu et al., 2013). This study confirmed that human ESCs, akin to their mouse counterparts, are indeed largely depleted for heterochromatin-associated histone modifications, especially the Polycomb-associated H3K27me3, which spreads during cellular differentiation and specification. Unlike H3K27me3, spreading of the constitutive-heterochromatin-associated H3K9me3 was shown to be largely a consequence of the *in vitro* culture conditions. Statistical modeling incorporating multiple chromatin states revealed that cellular specification was accompanied by a global transition from a uniquely accessible chromatin state to increasingly restrictive configurations. Supporting this view was the reorganization of the histone variant H2A.Z from a widespread distribution in ESCs to confined concentration in promoters and distal elements in differentiated cells (Zhu et al., 2013). Finally, the dynamic association of a heterochromatin protein, i.e., HP1 β , was reported to significantly increase in pluripotent human cells following reprogramming of somatic fibroblasts (Manukyan and Singh, 2014) and was suggested to act as a quantifiable measure for human pluripotency, mimicking the situation in mouse (Meshorer et al., 2006). Taken together, the epigenomic and chromatin state of hESCs, even when grown in the more classical conditions that parallel EpiSCs, is distinct from that of so-

matic cells, supporting, once again, a more open chromatin conformation.

Functional Consequences and Conclusions

Serum-grown mouse ESCs have been used to generate mice by blastocyst injections ever since the 1980s; hence, their pluripotency is not questionable. However, it is possible that only a fraction of the cells are truly pluripotent and that these cells are selected *in vivo* during blastocyst injections, exclusively contributing to the chimeric animals produced. Therefore, pluripotency-promoting conditions, such as increased histone acetylation (Hezroni et al., 2011), naive conditions (Ying et al., 2008), or a2i conditions (Choi et al., 2017; Yagi et al., 2017), would be highly beneficial from a practical point of view. Another important motivation to seek conditions that mimic the ICM of the early embryo (Habibi and Stunnenberg, 2017) is to better model development and to enable proper differentiation *in vitro*. Although 2i conditions were used in multiple studies to generate animals, the recent finding that prolonged culturing in these conditions hamper embryonic development (Choi et al., 2017; Yagi et al., 2017) casts doubt on the suitability of these conditions for properly mimicking the ICM. Having said that, the ICM stage is characterized by low-level DNA methylation, while both serum-grown and a2i-grown ESCs are hypermethylated. This suggests that DNA methylation in ESCs may have little functional consequences, likely reflecting the relatively high levels of the *de novo* DNMTs present in these cells (Figure 4) and that during differentiation, the proper methylation patterns are established and erased, as dictated by the developmental route. Supporting this notion is the fact that ESCs devoid of all three DNMTs (Tsumura et al., 2006) or all three TET proteins (Dawlaty et al., 2014) maintain their growth and self-renewal. Thus, *in vitro* conditions that truly capture the epigenetic state of the ICM without compromising developmental potential are yet to be identified. To sum up, mESCs grown in either 2i (“ground-state”) or serum (“confused”) are both naive pluripotent ESCs, while classically cultured hESCs likely represent EpiSCs. As such, they are less naive in their pluripotent state than mouse ESCs, but all of these different pluripotent states display features of chromatin “hyper-plasticity.” Assays that measure open chromatin accurately will enable, for example, the identification of properly reprogrammed iPSCs. Tracking the chromatin landscape from pluripotency through priming to differentiated cells will further enable understanding of the molecular events that shape our genome and likely reflect the chromatin restriction, which occurs during development. A better understanding of the unique gene silencing mechanisms used by pluripotent cells, in the generally “open” environment, will enable examining and assessing the safety of the cells in use. Taking into account everything we have learned, pluripotent stem cells, whether ground state, serum-grown, or even primed, show a higher degree of chromatin decompaction and “openness” than somatic cells do (Figure 6). However, not all open chromatin features correlate directly with pluripotency, and some, such as promiscuous transcription, seem to be related to the multiple signals emerging from growing the cells in serum. Once conditions to grow naive hESCs become standardized, it will be possible to comprehensively

re-examine all epigenomic and chromatin-related features and, using the mouse model for *in vivo* studies, correlate them directly with pluripotency.

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