Published in final edited form as:

Curr Opin Genet Dev. 2010 October ; 20(5): 492–499. doi:10.1016/j.gde.2010.06.002.

Parallel gateways to pluripotency: open chromatin in stem cells and development

Fong Ming Koh, Michael Sachs, Marcela Guzman-Ayala, and Miguel Ramalho-Santos

Departments of Ob/Gyn and Pathology, Eli and Edythe Broad Center of Regeneration Medicine and Stem Cell Research, Center for Reproductive Sciences and Diabetes Center, University of California, San Francisco, 513 Parnassus Avenue, San Francisco, California 94143-0525, USA

Abstract

Open chromatin is a hallmark of pluripotent stem cells, but the underlying molecular mechanisms are only beginning to be unraveled. In this review we highlight recent studies that employ embryonic stem cells and induced pluripotent stem cells to investigate the regulation of open chromatin and its role in the maintenance and acquisition of pluripotency *in vitro*. We suggest that findings from *in vitro* studies using pluripotent stem cells are predictive of *in vivo* processes of epigenetic regulation of pluripotency, specifically in the development of the zygote and primordial germ cells. The combination of *in vitro* and *in vivo* approaches is expected to provide a comprehensive understanding of the epigenetic regulation of pluripotency and reprogramming.

Introduction

Pluripotent stem cells have a limitless capacity for self-renewal and the unique potential to differentiate into all cell types. With the advent of techniques to reprogram somatic cells into pluripotent stem cells, there is an increased interest in understanding the mechanisms that underlie the maintenance and acquisition of pluripotency. Such understanding may provide important new insights into the regulation of embryonic development, and contribute to the generation of patient-specific pluripotent stem cells for disease modeling and cell replacement therapies.

While transcriptional differences between somatic cells and pluripotent stem cells are well established, there is increasing evidence supporting the critical role that chromatin accessibility plays in pluripotent stem cells. In this review, we highlight recent advancements in our understanding of how open chromatin regulates the maintenance and acquisition of pluripotency. We first describe epigenetic remodelers that regulate open chromatin *in vitro* in pluripotent embryonic stem (ES) cells and reprogrammed induced pluripotent stem (iPS) cells. The large number of ES and iPS cells that can be grown *in vitro* has facilitated the dissection of epigenetic regulation of pluripotency in these cells. We then discuss the potential significance of these recent findings *in vivo*. We propose that epigenetic mechanisms used to maintain and acquire pluripotency *in vitro* operate *in vivo* in the acquisition of totipotency in the nascent zygote and maintenance of pluripotency in germ cells. The integration of studies

Corresponding author: Miguel Ramalho-Santos (mrsantos@diabetes.ucsf.edu).

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in vitro and *in vivo* should thus significantly augment our global understanding of the epigenetic regulation of pluripotency and embryonic development.

ES cell cultures may reflect distinct *in vivo* epigenetic states

ES cells are pluripotent stem cells derived from the inner cell mass of the blastocyst, prior to implantation, and they serve as an excellent *in vitro* model for probing the molecular mechanisms that govern cell fate decisions during early development. Recent data indicate that ES cells are not a homogeneous cell population as previously thought, but rather oscillate between different cell states that may have parallels *in vivo* [1-5]. Mouse ES cell cultures contain significant heterogeneity: the core pluripotency gene Nanog [1] and stem-cell markers Rex1 [2], Pecam1 [3], SSEA1 [3,4] and Stella [5] have all been shown to exhibit a heterogeneous expression pattern, where ES cells are in flux between high and low expression of these genes. The variable phenotype correlates with *in vivo* expression patterns and appears to represent two distinct yet reversible embryonic stages: one that reflects an inner cell masslike state, and another that is closer to an epiblast-like state [2,4,5].

Strikingly, populations enriched for pluripotency markers SSEA1 or Stella are able to restore the original ratio of mixed populations [3,5]. Stella expression levels correlate with the presence of activating histone marks H3K9ac and H3K4me3 at the Stella gene locus. Interestingly, the Stella+ sub-population is lost when ES cells are cultured in the absence of embryonic fibroblast feeder cells, and addition of the histone deacetylase inhibitor trichostatin A, which promotes active transcription, restores Stella expression in feeder-free conditions [5]. Taken together, the data available suggest that extracellular signaling within ES cell cultures, and potentially *in vivo*, regulates gene expression and differentiation through epigenetic changes. Further evidence comes from a recent study demonstrating that ES cell-like cultures containing a Stella + sub-population can be derived directly from epiblast tissue or epiblast stem cells after prolonged culture with LIF-fetal calf serum on mouse embryonic fibroblast feeders [6]. This transformation is accompanied by DNA demethylation at the Stella and Rex gene loci, further supporting an epigenetic switch between the epiblast and inner cell masslike states.

Direct evidence of epigenetic regulation of cell-fate comes from studies of two closely related DNA-binding transcriptional regulators that are involved in higher-order chromatin organization, Satb1 [7] and Satb2 [8]. The two Satb proteins appear to regulate ES cell self-renewal in an antagonistic manner: while both factors can bind to the Nanog promoter, Satb1 acts to repress Nanog transcription, and Satb2 appears to activate it. Therefore, the balance of Satb1 and Satb2 may underlie the heterogeneity of Nanog expression in ES cells [9]. However, it remains to be seen if extracellular signals lie upstream of these factors. It will be interesting in future studies to determine how LIF and other extracellular signals interact with epigenetic regulators to control pluripotency in ES cells and during development.

New epigenetic regulators of pluripotency

Pluripotent stem cells maintain a globally open chromatin state [10,11], possibly so that genes are readily available for activation during tissue specification [10]. ES cells have low levels of dense, compacted chromatin (heterochromatin) and the ES cell genome is transcriptionally hyperactive, with widespread transcription in both coding and non-coding regions, including sporadic low-level expression of tissue specific genes [10,12]. In addition, a recent study showed that the distribution of repressive marks H3K9me3 and H3K27me3 are significantly expanded in somatic cells relative to pluripotent stem cells [13]. In agreement with these observations, chromatin remodeling factors are over-represented in the ES cell transcriptome, and RNAi knockdown of several chromatin remodelers like Chd1 and Brg1 has been shown to severely impact ES cell proliferation and differentiation potential [10,14].

Chd1 is a chromatin remodeler associated with active transcription that contains a helicase domain, a DNA-binding domain and a pair of chromodomains that binds selectively to the euchromatin mark H3K4me2/3 [14-16] (reviewed in [17]). Chd1 is required to maintain the open chromatin state of pluripotent mouse ES cells (Fig. 1). Chd1-deficient ES cells show an increased number of heterochromatin foci and a pluripotency defect characterized by a high propensity for neural differentiation and an absence of primitive endoderm [14]. The molecular mechanism by which Chd1 regulates open chromatin of ES cells remains unknown. Chromatin immunoprecipitation using promoter tiling arrays shows that Chd1 binding overlaps with markers of transcription, including RNAPolII and H3K4me3 [14]. Interestingly, this distribution at gene promoters is similar to that of histone variant H3.3 [18]. H3.3 is correlated with sites of active transcription in many species [19-21] and appears to maintain open chromatin by inhibiting histone H1 binding to the nucleosome [22] (reviewed in [23]). The incorporation of H3.3 in ES cells is complex and includes the promoters of active and repressed genes, gene bodies only in active genes, transcriptional factor binding sites and telomeres [18]. Evidence from *Drosophila* suggests that Chd1 is required for H3.3 incorporation into chromatin (see below) [24]. It will therefore be of interest to characterize the genomic distribution of Chd1 binding in ES cells beyond gene promoters, determine which aspects of H3.3 incorporation, if any, are dependent on Chd1, and test whether H3.3 mediate the pluripotency defects in Chd1-deficient ES cells.

The BAF (Brg/Brahma-associated factors) complex is a chromatin remodeler of the SWI/SNF family that has been shown to regulate pluripotency [25]. ES cells express a distinctive BAF complex (esBAF) defined by the presence of Brg1 (Brahma-related gene 1), BAF155, and BAF60A, and the absence of Brahma, BAF170, and BAF60C. Using genome-wide ChIP-seq technology, Brg1 was shown to co-localize extensively with the pluripotency transcription factors Oct4, Sox2, and Nanog, thereby suggesting a pluripotency-specific role for esBAF [26]. In addition, Brg1 does not share many targets with the polycomb repressive complex PRC2, suggesting that esBAF is an activator of transcription.

Polycomb repressive complexes (PRCs) coordinate the transcriptional repression of lineage-specific developmental genes in ES cells in multiple ways, including mediating H3K27 di-/tri-methylations and H2A ubiquitination [27-29]. Disruption of either PRC1 or PRC2 results in early embryonic lethality *in vivo* [30-32]. This observation is mirrored *in vitro* by the propensity of PRC1- or PRC2-deficient ES cells to differentiate [27,33]. Cell survival is greatly reduced upon initiation of differentiation in PRC-deficient ES cells, possibly due to activation of endogenous retroviruses [33]. Novel components of the PRC2 complex have recently been shown to be enriched in undifferentiated ES cells: Jarid2 was identified as a regulatory component that modulates PRC2 localization and activity [34,35], and Pcl2 was described as another component required for proper regulation of both pluripotency and lineage-specific genes in ES cells [36].

Finally, DNA methylation is another epigenetic mechanism by which ES cells may regulate gene expression. Recent studies challenge the classical view that ES cells have reduced global DNA methylation, but rather reveal that they use ES cell-specific non-CpG methylation in addition to the canonical CpG methylation [37,38]. While DNA methylation is generally associated with transcriptional silencing, the functional significance of this alternative type of DNA methylation in ES cells remains to be determined. It should also be noted that a marker of active transcription, H3K36me3, is highly correlated with the presence of DNA methylation within gene bodies, suggesting a role for DNA methylation beyond transcriptional repression [13,39]. Future investigations will be necessary to explore the functional significance of this alternative regulatory node.

In sum, recent findings indicate that the open, accessible chromatin state of ES cells is actively maintained by chromatin remodelers such as Chd1, and that PRCs and DNA methylation are involved in repression of developmental genes until differentiation is triggered. Therefore, a complex, dynamic balance is at play in the epigenetic regulation of ES cell pluripotency. How this balance may be reestablished during reprogramming of somatic cells to pluripotency is the focus of the next section.

Reacquiring pluripotency *in vitro*: epigenetics is key

Cellular reprogramming by the ectopic expression of defined transcription factors offers a reliable, albeit inefficient, method of obtaining iPS cells from somatic cells [40]. Although detailed analyses comparing mouse and human ES and iPS cells have uncovered subtle differences in gene expression patterns [41], iPS cells have consistently been found to reactivate pluripotency-related genes as well as re-establish an ES cell-like open chromatin state via global DNA demethylation and H3K4 and H3K27 methylation changes [41-43]. Therefore, it is expected that global chromatin opening and DNA demethylation will be important components of reprogramming (Fig. 1).

In order to gain insight into the epigenetic changes that may underlie reprogramming, several groups have compared stable partially reprogrammed cell lines, which have not turned on the endogenous Oct4 gene, to true iPS cells that have activated Oct4 [42,44,45]. These partially reprogrammed cells exhibit DNA hypermethylation and a failure to remethylate H3K4 relative to ES and true iPS cells [42], supporting the notion that proper epigenetic remodeling may be required for successful reprogramming to pluripotency. Direct manipulation of the chromatin of these partially reprogrammed cells and of somatic cells during reprogramming have proved to be effective at enhancing reprogramming efficiency. DNA methyltransferase inhibitor 5-azacytidine (5-azaC) induces partially reprogrammed cells to undergo a rapid and stable transition to a fully reprogrammed state [42], while 5-azaC and histone deacetylase inhibitors like valproic acid increase the efficiency of iPS cell generation up to 40-fold [46]. These observations parallel earlier ones made using somatic cell nuclear transfer (SCNT), which established DNA demethylation of somatic cell nuclei as a necessary step to successful reprogramming in mouse and *Xenopus* [47,48].

In addition, targeted downregulation by RNAi has enabled the identification of specific molecular complexes involved in the reprogramming process. Downregulation of Chd1, a regulator of open chromatin and pluripotency of ES cells (see above), strongly inhibits generation of iPS cells [14]. It will be of interest to determine whether H3.3 incorporation or other functions associated with Chd1 underlie its role in reprogramming. Recently, activation-induced cytidine deaminase (AID), which is involved in DNA demethylation [49], has been implicated in reprogramming. Knockdown of AID during nuclear reprogramming by somatic cell fusion with ES cells leads to a failure to reactivate Oct4 and Nanog, possibly due to defective DNA demethylation at their promoters [50]. Future work using RNAi screens will likely elucidate other molecular complexes involved in reprogramming towards pluripotency *in vitro*.

While studies of the epigenetic regulation of iPS cell generation are expected to have broad application in regenerative medicine, they may in addition reveal potential molecular parallels between experimental reprogramming *in vitro* in iPS cells and physiological reprogramming *in vivo* [51]. In the next sections we describe some of these potential parallels, focusing on recent insights into epigenetic reprogramming in the zygote and mid-gestation germ cells (Fig. 1).

Epigenetic reprogramming towards totipotency in the zygote

The zygote marks the starting point of development, and represents the reacquisition of totipotency from fusion of two highly differentiated gametes. The two parental genomes in the zygote have highly asymmetric chromatin organization [52,53]. Most histones are stripped from the paternal genome during spermatogenesis and replaced with highly basic protamines that allow for a very tight compaction of DNA [54-56]. A restricted set of nucleosomes is retained in about 4% of the genome, preferentially at developmental genes [57]. These histones appear to be enriched in modifications like H3K27me3 and H3K4me3 in patterns that overlap significantly with those in ES cells. It thus appears that sperm DNA, while densely packed with protamines, may also transmit epigenetic marks important for early development [57].

Shortly after fertilization, H3.3 is preferentially incorporated into the male pronucleus [53, 58], presumably by maternal stores of chromatin factors that complement the transcriptionally silent zygote [59-61]. Although these factors have not been characterized in the mammalian zygote, maternal Chd1 has been shown to be required for incorporation of H3.3 into the male pronucleus in the *Drosophila* embryo [24]. *Drosophila* embryos derived from Chd1-null mothers exhibit a loss of the paternal genome and the resulting haploid embryos arrest before hatching. Functional studies of Chd1 in mouse should reveal whether its role in H3.3 incorporation in the male pronucleus is conserved, and if so, what is the significance of this process for mammalian development (Fig. 1).

Another important epigenetic asymmetry between the parental genomes is that paternal (but not maternal) DNA is actively demethylated immediately following fertilization [62,63]. No DNA demethylase has been described despite extensive efforts [64]. With the aid of a live cell imaging reporter system, a recent RNAi screen implicated the transcription elongator complex in paternal DNA demethylation [65]. The molecular mechanism behind elongator-mediated DNA demethylation needs to be further explored. In addition, it will be important to determine whether this represents a unique DNA demethylation mechanism, or whether AID is involved. Finally, the relationship between H3.3 incorporation and DNA demethylation, both of which occur prior to the first cell division, remains to be explored.

Epigenetic reprogramming in primordial germ cells

Another context where extensive chromatin remodeling occurs *in vivo* that may have parallels with reprogramming *in vitro* is in primordial germ cells (PGCs). PGCs are specified by inductive signals around the time of gastrulation and represent the only lineage from the epiblast that actively represses the somatic cell fate in order to form oocytes and sperm later in development (reviewed in [66]). PGCs are also the only embryonic cells post-gastrulation that can still give rise to pluripotent stem cells when cultured *in vitro* [67]. These observations, coupled with the large number of regulators of ES cell pluripotency that are expressed in PGCs [68], suggest that some of same molecular mechanisms that maintain pluripotency *in vitro* may operate in PGCs.

Several recent studies [69-71] paint an intricate picture of the dynamic epigenetic reprogramming that takes place *in vivo* during PGC maturation. PGCs experience a large-scale loss of DNA methylation [72-74] and many histone marks, including H3K9Ac, H3K9me3 and H3K27me3, around E11.5. Concurrently, linker histone H1 staining is lost and DAPI-stained chromatin becomes noticeably 'loosened'. Subsequently by E12.5, the transient loosening of the chromatin is reversed with the return of brightly stained DAPI foci, histone H1, H3K9me3, H3K27me3 and pericentromeric heterochromatin marks [69].

It is possible that the rapid chromatin opening in E11.5 PGCs results from a large-scale incorporation of H3.3. In support of this hypothesis, the histone chaperone HIRA, which is

essential for delivering H3.3 to active and repressed genes [18,75], is enriched in the nucleus of E11.5 PGCs [18,69]. Chd1, which directly facilitates H3.3 deposition [24], is highly expressed in E11.5 PGCs [68]. Finally, H3.3 is essential for chromatin remodeling in the *Drosophila* germline, with both male and female flies mutant for H3.3 being sterile [76,77]. Altogether, it is tempting to speculate that maintenance of germline pluripotency, from flies to mice, requires the deposition of H3.3 by Chd1. It will therefore be of great interest to determine the function of Chd1, HIRA and H3.3 during mammalian PGC development (Fig. 1).

The functional significance of this large-scale chromatin remodeling in PGCs remains unclear, but it may limit the transmission of epigenetic information across generations. It may not be a coincidence that, as in the case of the zygote, extensive chromatin remodeling in PGCs coincides with large-scale DNA demethylation [73]. Careful observations suggest that DNA demethylation may precede chromatin remodeling in PGCs [69], but this remains to be demonstrated functionally. Intriguingly, AID was recently shown to be essential for efficient DNA demethylation in PGCs [74], highlighting another potential parallel between reprogramming in PGCs and *in vitro*.

Perspectives and future directions

Increasing evidence supports the notion that an open, decondensed chromatin state plays a vital role in the regulation of pluripotency in stem cells *in vitro* and during critical events of mammalian embryogenesis, including zygote and PGC development. The ease with which ES and iPS cells can be obtained in large numbers allows the application of unbiased genome-wide approaches such as ChIP-Seq and RNAi screens. The integration of data from these approaches should shed light on the epigenetic architecture of the pluripotent stem cell state, and how it is reconfigured during differentiation. The process of reprogramming to iPS cells remains largely a black box, and future studies are likely to reveal the epigenetic steps undertaken by somatic cells on the way to the open pluripotent chromatin state. The limited numbers of zygotes and PGCs and their rapidly changing transcriptional and epigenetic states during embryonic development pose significant technical hurdles to genome-wide analyses. Nevertheless, recent advancements have made genome-wide studies of chromatin states feasible for limited numbers of primary cells, and we expect that novel insights will be gained from applying these methods to pluripotent cells *in vivo*. In addition, candidate gene approaches that determine the *in vivo* roles of regulators of the open chromatin state of pluripotent stem cells, such as Chd1, may reveal common themes in the regulation of open chromatin. Research in the years ahead will likely reveal fascinating insights into the epigenetic regulation of pluripotency *in vitro* and its significance *in vivo*.

Acknowledgments

We thank Marco Conti, Robert Blelloch, Diana Laird and members of the Santos lab for helpful discussions and critical reading of the manuscript. We apologize to authors whose work we were unable to describe due to space constraints. F.M.K. is supported by the Agency for Science, Technology and Research (Singapore). M.G.-A. is the recipient of a Center for Reproductive Sciences Institutional Research Training Grant (T32) from NIH. Research in the Santos lab is supported by the NIH Director's New Innovator Award, California Institute for Regenerative Medicine, Juvenile Diabetes Research Foundation and the Helmsley Trust.

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Figure 1. Potential parallels in epigenetic regulation of pluripotency in stem cells *in vitro* and the germline *in vivo*

ES and iPS cells are useful *in vitro* models to study the regulation of open chromatin and associated epigenetic marks in pluripotent cells. Several epigenetic regulators have been shown to be important for the establishment and maintenance of the open chromatin state of pluripotent stem cells, although the picture is far from complete. Much less is known about the epigenetic regulators that operate *in vivo* in epigenetic reprogramming in the early zygote and PGCs. Note that the column “zygote” refers to chromatin decondensation of the male pronucleus after fertilization. Recent evidence suggests that Chd1 and AID are essential chromatin regulators of pluripotency both *in vitro* and *in vivo*. Future studies may reveal the potential molecular parallels, as well as the differences, in epigenetic regulation between the various cell types depicted. Shaded columns represent cell types and states associated with open chromatin. For simplicity, several chromatin factors known to have roles in each specific cell type are not shown. See text for details.

Footnotes:

^a iPS cells have a significant expansion of H3K9me3 into genes compared to ES cells.
N.D. Not determined.