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Developmentally-poised chromatin of embryonic stem cells

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1. ABSTRACT

Embryonic stem (ES) cells proliferate indefinitely while maintaining pluripotency. The ability of ES cells to form all cell-types of the embryo can occur because they maintain their genome in an epigenetically-potentiated state that is amenable to a broad series of changes in gene expression. Epigenetic stasis and change occur at a molecular level largely through mechanisms involving chromatin and its modification. This review outlines current knowledge of chromatin homeostasis in undifferentiated ES cells, and the remodeling of chromatin during the course of ES cell differentiation. Furthermore, recent evidence shows that the chromatin of many genes in ES cells is configured in developmentally-potentiated states that index them for later transcriptional outcomes. ES cell chromatin also has dynamic physical and kinetic properties that are probably necessary for rapid and pervasive remodeling upon differentiation. Finally, knowledge of nuclear reprogramming activities in oocytes and ES cells are considered, since these activities may also function in the maintenance of pluripotent ES cell chromatin and are also likely involved in subsequent differentiation.

2. INTRODUCTION

ES cells are unique among cultured mammalian cells in that they are able to undergo unlimited self-renewal without loss of pluripotency. ES cells were first isolated by outgrowth of the inner cell mass (ICM) of mouse embryos under conditions originally optimized for the establishment of teratocarcinoma cells (1). These cells have the ability to proliferate in a perpetually undifferentiated state *in vitro*, while maintaining the potential to contribute to all germ layers (ectoderm, endoderm, and mesoderm) when transplanted into suitable blastocyst-stage embryos, when used to form teratomas *in vivo*, or when coaxed to differentiate *in vitro*. The broad-spectrum ability of ESCs to contribute to chimeric animals remains the gold standard for developmental pluripotency. ES cells were subsequently isolated from human ICM, and their pluripotency was demonstrated by their ability to contribute to all three germ layers of teratomas when injected into immunotolerant host mice. Furthermore, human ES cells were shown to express surface markers characteristic of nonhuman primate ES cells and human embryonal carcinoma (EC) cells (2). ES cells have

garnered a high degree of interest since they offer the possibility to serve as sources of transplantable materials to alleviate a variety of human disorders characterized by cell loss or damage, which are at present poorly alleviated by current medical practices. As an experimental system, ES cells offer a tractable platform for the investigation of key aspects of mammalian developmental biology since these cells can be subjected to experimental approaches that are difficult to apply to intact embryos.

ES cells are remarkable in their ability to proliferate indefinitely while maintaining their pluripotent state. ICM cells (from which ES cells are derived) form the embryo proper, but the ICM exists as a transitory developmental stage in which cells reside for only a few hours. The transitory nature of the ICM makes it clear that ES cells are not equivalent to ICM cells. Furthermore, ES cells undergo a recently appreciated, but poorly understood culture adaptation process during their derivation, which is characterized by changes in the duration of cell cycle phases and other alterations (3). However, like the ICM, ES cells can contribute to all structures in the embryo proper upon reintroduction into embryos, and an increasing number of reports describe the successful recapitulation of developmental processes through ES cell differentiation *in vitro*. This remarkable range of cellular lineages derived from ES cells is all accomplished within the framework of a single fixed genome. Therefore, lineage allocation can best be viewed as the restriction of gene expression over developmental stages to patterns compatible with the establishment and maintenance of proper cellular identity. Precisely how are sets of genes selected for expression from within an otherwise static genome? Increasingly, evidence points to a major role for epigenetics in the establishment and maintenance of patterns of gene expression during embryogenesis.

Epigenetics can be broadly defined as phenomena that influence or predispose states of gene expression independently of DNA sequence. The molecular basis for epigenesis has been sought intensively, and it is now apparent that chromatin is at the center of epigenetic function in eukaryotic cells. The fundamental unit of chromatin, the nucleosome, consists of an octamer of histones comprised of 2 H2As, 2 H2Bs, 2 H3s, and 2 H4s around which 146 base pairs of DNA are wound (4). N-terminal tails of histones are especially rich in lysine and arginine residues, and protrude from the planes of roughly disk-shaped nucleosomes. These tails are subject to a diverse array of post-translational modifications (PTMs) including acetylation, mono-, di-, and trimethylation, phosphorylation and ubiquitination. In addition, a few post-translational modifications are known to occur at accessible surfaces of globular domains of histones, and near histone C-termini. Recently, a system of nomenclature has been proposed that describes specific histone modifications without ambiguity (5). (This nomenclature is used in this review to denote histone PTMs when the type and number of modifications at specific amino acid residues are known. For instance, histone H3 containing a trimethyl modification at lysine 27 is denoted as H3K27me3, and histone H3 containing an acetyl group at lysine 9 is denoted as H3K9ac). Also of note are variant histones, which can replace canonical histones to form specialized nucleosomes (6). The large number of independent histone PTMs, and the added complexity brought about by the existence of several mammalian histone variants means that an extraordinarily large number of modification combinations are theoretically possible for nucleosomes. However, the molecular diversity of nucleosomes in various mammalian cell-types is currently unknown. Current hypotheses hold that the collective status of modified histones can be read out in terms of the transcriptional or genetic regulatory status of the DNA that is associated with (or near) modified nucleosomes (7-10).

The transcriptional status of the entire genome is intimately influenced by the configuration of modified chromatin on a gene-by-gene basis. Numerous recent studies involving chromatin immunoprecipitation (ChIP) followed by analysis on microarrays ("ChIP on chip") have shown that the genome is indexed in terms of its chromatin modification status. Furthermore, this "genomic indexing" through the formation of specialized chromatin differs from cell-type to cell-type and is dynamically regulated during the course of development and during ES cell differentiation. In addition, it now appears that portions of the genome contain precocious chromatin states that mark genes for later expression once an appropriate developmental context is arrived upon. Advances in our understanding of chromatin regulation in ES cells will yield the practical benefit of increasing our expertise in bringing about rationally-guided differentiation outcomes with ES cells. ES cell differentiation probably makes heavy use of pathways and mechanisms that normally operate during embryogenesis. Hence, the scientific community should gain new insights into epigenetic mechanisms that govern development *in vivo* by taking what is learned from ES cells and ascertaining if these same pathways function in embryos. This review explores these exciting new concepts in developmental biology and in particular the case of epigenetic function in ES cells.

3. SELF-RENEWAL AND EPIGENETIC HOMEOSTASIS IN ES CELLS

ES cells are able to undergo unlimited self-renewal while maintaining a degree of pluripotency sufficient to contribute to all cell-types of the embryo proper. Though unlimited self-renewal and pluripotency are conceptually distinct, their biological underpinnings exhibit overlap. For instance, it has long been recognized that the regulated expression of a set of key genes including *Oct4*, *Sox2*, and *Nanog*, are essential for the ability of stem cells to undergo self-renewal in a pluripotent state. Down-regulation of the expression of these genes is also requisite for the execution of developmental programs and associated lineage restriction. For instance, elimination of *Oct4* expression shifts ICM cells to trophoblast lineages (11), whereas modest over-expression of *Oct4* causes differentiation into primitive endoderm and mesoderm (12). Therefore, *Oct4* expression is required for maintenance of pluripotency in mouse ES cells, but silencing of *Oct4* is also required for proper differentiation leading to

production of the embryo proper. Recently, the Oct4 gene was shown to be regulated by Sall4, a factor that binds a distal enhancer of the Oct4 gene. Deletion of Sall4 results in the loss of ES cell pluripotency, and modest reduction of its expression shifts ES cells to a trophoblast identity (13). Sall4 interacts physically with Nanog and regulates both Nanog and Sall4 enhancers (14). Results such as these suggest that ES cell self-renewal may be best viewed as simultaneous proliferation and inhibition of differentiation, which is mediated in large measure by the expression of a distinctive set of transcription factors that are required for the maintenance of pluripotency.

Pluripotency-related genes such as Oct4, Sox2 and Nanog are transcriptionally silenced upon ES cell differentiation. In a study utilizing mouse embryos, ES cells, and embryonal carcinoma (EC) cells, developmentally-regulated silencing of Oct4 was shown to occur by a multi-step process involving H3K9 methylation by the G9a set domain histone methyltransferase, followed by HP1 binding, and subsequent methylation of CpG dinucleotides in the Oct4 promoter (15). Germ cell nuclear factor (GCNF) is an orphan nuclear receptor that has recently been shown to be involved in the repression of Oct4, Nanog, Sox2, and others during early embryonic development through a mechanism involving the recruitment of methyl CpG binding domain proteins Mbd3 and Mbd2 to Oct4 promoter regions (16-18). Interestingly, mouse ES cells that lack Mbd3 (which is a component of the nucleosome remodeling and histone deacetylase complex, NuRD) are relieved of their culture requirement for leukemia inhibitory factor (LIF), but exhibit defects in lineage allocation during embryogenesis and embryoid body (EB) formation in vitro (19, 20). Maintenance of Oct4 silencing in TS cells is by epigenetic means since it can be de-repressed by addition of 5-aza-2'-deoxycytosine (a cytosine demethylating agent) and trichostatin A (a histone deacetylase inhibitor) in TS cells (21). The chromatin of Nanog is also managed epigenetically, in part through a differentially-methylated region (DMR) that is hypomethylated specifically in undifferentiated ES cells, in association with high levels of H3 and H4 acetylation (22). Collectively, these results indicate that key pluripotency transcription factors including Oct4, Sox2, Nanog, are silenced by mechanisms involving the assembly of heterochromatin as ES cells differentiate.

Genes that are required for differentiation can render ES cells incapable of differentiation when mutated. Interestingly, many genes in this class have also been identified, and they turn out to have biochemical activities involved in chromatin homeostasis. For instance, murine ES cells lacking DNA cytosine methyltransferases proliferate readily with typical ES cell morphology, yet fail to differentiate (23-25). These findings raise the interesting possibility that DNA methylation activity is necessary for the silencing of key genes, which prevent differentiation, or kill cells, if not silenced. Conditional inactivation of the gene encoding Dicer in murine ES cells results in defective maturation of microRNAs in ES cells and compromised differentiation (26, 27). Interestingly, Dicer-deficient ES cells produce transcripts from non-coding centromeric repeats with associated reductions in centromeric cytosine methylation and reductions in H3K9me2 and H3K9me3 (26, 27). In addition, dsRNAs emanating from tandem repeats are elevated in ES cells lacking Suv39h proteins, which function as H3K9-specific histone methyltransferases (28). Parp1 encodes poly ADP-ribose polymerase, which ADP-ribosylates chromatin and other proteins. ES cells lacking Parp1 form syncytotrophoblastic giant cells in teratoma assays in mice (29). Parp1-deficient ES cells also form trophoblast cells during differentiation in vitro (30) and activate trophectodermal genes such as H19 after removal of LIF (31). More subtle effects on differentiation have also been described. High-mobility group A (HMGA) proteins directly associate with chromosomes, and ES cells lacking HMGA1 exhibit a decreased ability to differentiate into T-cell precursors, yet can still form B-cells (32). Mutations in genes that encode enzymes with chromatin-modulatory activities are expected to be pleiotropic since the transcription of a large set of target genes are also affected. Therefore, inactivation of chromatin-modulatory genes in ES cells could lead to apoptosis or other lethal events that kill mutant ES cells upon differentiation. This being the case, the results collectively show that proper chromatin homeostasis and micro-RNA expression are critical for the maintenance of ES cells in an undifferentiated state. Additional experiments are needed to tease apart the relative involvement of chromatin homeostasis and downstream effects in ES cells.

4. CHROMATIN REMODELING ASSOCIATED WITH DIFFERENTIATION

A large number of studies document that chromatin remodeling occurs in conjunction with ES cell differentiation. One of the earliest and most exhaustively investigated areas of differentiation-induced chromatin remodeling is that associated with X chromosome inactivation. X chromosomes reside in a pre-inactivation state in undifferentiated ES cells. Upon induction of differentiation, the non-coding Xist RNA (whose gene resides on the X chromosome) is stabilized in association with the inactivating X chromosome as Tsix, an antisense transcript, is silenced. Subsequently, X-linked chromatin becomes hypoacetylated, histones become methylated at H3K27 and other sites, and the histone variant macroH2A becomes incorporated into silenced chromatin. Finally, cytosine methylation occurs near the promoters of silenced X-linked genes. Interestingly, X chromosome inactivation can be induced by conditional expression of Xist RNA in undifferentiated ES cells (33). However, X chromosome inactivation becomes irreversible and independent of Xist in differentiated ES cells. In addition, macroH2A is not recruited to sites of forced Xist expression in undifferentiated ES cells (34). These interesting results show that the chromatin of ES cells differs from that of differentiated derivatives, and that RNA-induced chromatin assembly is exquisitely regulated during the course of differentiation. In short, X chromosome inactivation in differentiating female ES cells is an exquisite and synchronous step-wise heterochromatin assembly process, which has been the subject of a number of excellent recent reviews (35-38).

Other dynamic changes of chromatin occur on autosomes of differentiating ES cells, including changes in the methylcytosine content as a function of cellular differentiation state. For instance, an analysis using restriction landmark genome sequencing (RLGS) revealed that a wide range of DMRs become differentially methylated in somatic tissues as compared to ES, embryonic germ (EG), and trophoblast stem (TS) cells (39). Chromatin, and chromatin-associated factors, also change dynamically during ES cell differentiation. In Drosophila, polycomb group complex (PcG) proteins such as Enhancer of Zeste, E (Z) collaborate to produce histone H3 methyltransferase activity and associated silencing of Hox gene expression in embryos (40, 41). A similar situation exists in mammals, where PcG proteins are assembled into polycomb repressive complexes (PRC), which collaborate to silence Hox genes. The recent availability of genomic microarrays that contain tiled sequences of both protein coding and non-coding regions has made possible the comparison of differing chromatin states on a genome-wide basis. In these studies, ChIP-chip analyses have been used to characterize differing configurations of the genome in terms of the distribution of nucleosomal PTMs and associated factors such as PcG proteins. These studies are conceptually similar to earlier studies that have mapped the distribution of CpG methylation across the genome. The EED-EZH2 complex is the human version of the Drosophila ESC-E (Z) complex. EED-EZH2 has been purified from human cells and shown to methylate histone H3 at K27 when expressed in Drosophila (40). The H3K27 methyltransferase Ezh2, a component of PRCs, becomes increasingly less abundant as ES cells differentiate, a process that proceeds with concomitant changes in the content of PRC-associated Eed protein isoforms (42). In a recent study, PRC1 and PRC2 (two distinct PcG-containing repressive complexes) locations were mapped across the mouse genome by a ChIP on chip approach. The results show that promoters of a large number of developmentally-regulated genes are associated with PRCs in undifferentiated murine ES cells. Furthermore, repression of several PcG associated genes was compromised in ES cells lacking Eed, a component of the PRC2 complex (43). These results indicate that the transcriptional silence of a substantial set of developmentally-regulated genes is maintained by PRCs in undifferentiated ESCs. In another study, the genome-wide distribution of PRC1 and PRC2 was mapped in human fibroblasts. This study found that a number of genes implicated in embryonic development and cell fate decisions are associated with PRC1/2 (44). This finding suggests that PRCs may also function to maintain silencing of developmental genes in terminally-differentiated human cells. Further experiments are clearly needed to clarify the developmental timing of PRC action. An attractive hypothesis is that transient de-repression of developmental genes occurs during appropriate embryonic stages, and that silencing is re-imposed in terminally-differentiated cells. Alternatively, PcG proteins may repress substantially different sets of genes in ES cells and fibroblasts.

5. DEVELOPMENTAL PRIMING OF ES CELL CHROMATIN

Studies that merely document differences between the chromatin of ES cells and differentiated cells cannot determine if remodeled chromatin is a consequence or a requirement of developmental processes. Recent findings regarding cell-type specific chromatin states of the Hox genes, and associated methyl-histone and PcG protein content, hinted that chromatin can be precociously "primed" to specify future states of gene expression as lineage allocation proceeds. Exposure of ES cells to retinoic acid (RA) differentiation induces expression of genes of the HoxB cluster. Interestingly, both Hoxb1 and Hoxb9 genes acquire activating histone PTMs (H3K9ac, and H3K4me2) and decondensed chromatin states early during RA-induced differentiation, even though Hoxb9 is expressed in this system much later than Hoxb1 (45). These results indicate that the late expressed Hoxb9 gene is preconfigured by precocious chromatin remodeling events that predispose this gene to transcription at a later developmental stage. In undifferentiated human ES cells, SUZ12 (a subunit of PRC2) and H3K27me3-containing nucleosomes are assembled into chromatin of highly-conserved non-coding DNA and a large number of genes with known developmental functions in human ES cells (46). Genes residing in ES cells may therefore be maintained in a silenced state by the H3K27me3 mark. In a related study, silenced developmentally-regulated genes are marked with regions of methyl-H3K4 embedded within larger stretches of H3K27me3 chromatin in ES cells (47). These "bivalent" chromatin marks are surprising since they consist of nucleosomes containing PTMs that are generally thought to have opposing influences on transcription. In an ES cell context, bivalent domains index genes for silenced (but somewhat leaky) expression. In mouse ES cells, about half of the genes with bivalent chromatin domains are bound by Oct4, Sox2, or Nanog. Upon differentiation along a neuronal lineage, genes with bivalent chromatin domains are resolved in two possible ways: (1) Genes that are expressed upon neuronal differentiation become associated predominantly with methyl-H3K4. (2) Genes that are not expressed upon neuronal lineage become associated predominantly with methyl-H3K27 (Figure 1).

Epigenetic regulation of gene expression by histone methylation also makes use of histone demethylating enzymes. It is becoming increasingly clear that the steady-state levels of mono-, di-, and trimethyl-lysine modifications upon histones are determined by a balance of opposing histone methyltransferase and histone demethylase activities. A recent report describes that RBB2, a JARID1 family member with histone lysine demethylase activity, is displaced from HoxA genes during RA-induced ES cell differentiation, resulting in a net increase in methyl-H3K4 which is associated with transcriptional activation (48). In ES cells, genes marked for later activation by co-modification with H3K27me3 and methyl-H3K4 tend to replicate early in S-phase as compared to differentiated cells (49).

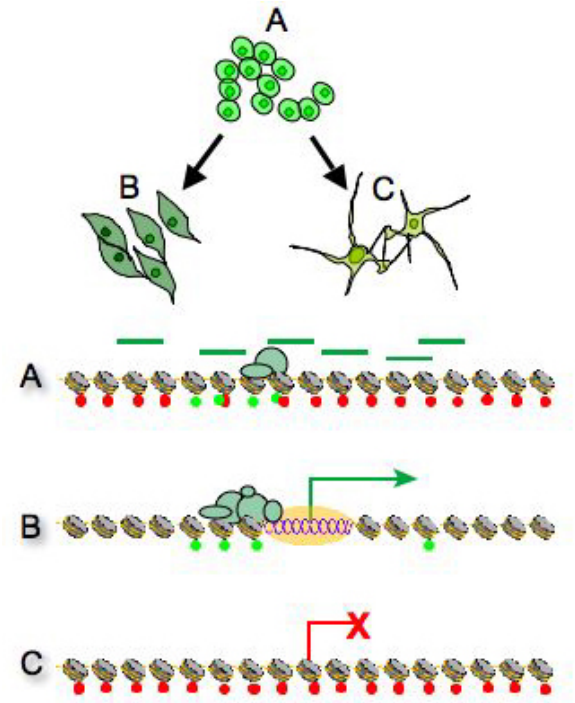

Figure 1. Model for developmentally-primed chromatin. ES cells (A) can be differentiated into two cell-types (B and C). A developmentally-regulated gene (chromatin diagrams) is primed for expression in differentiated cells. In ES cells (A) the gene is essentially silent except for low-level non-specific transcription. Nucleosomes associated with the gene are modified with repressive histone PTMs (red circles) such as H3K27me3 via the action of PcG proteins. The promoter region contains a local concentration of activating PTMs (green circles) such as H3K4me3 or H3K4ac, and an incomplete transcription initiation complex. In type B cells, the gene is expressed and only activating histone PTMs remain. In type C cells, the gene is stably silenced, and repressive histones PTMs lock the gene in a silenced mode.

Developmentally-primed chromatin in ES cells has been studied near other genes and gene clusters. For instance, the LCR and linked globin genes of the murine β-globin locus contains chromatin that is differentially acetylated in various tissues at differing developmental stages. The β-globin LCR also contains DNase hypersensitive sites in mouse ES cells, suggesting the existence of an open chromatin structure in pluripotent cells (50). In another report, an LCR-like non-coding regulatory region in proximity to the *λ5* and *VpreB1* genes is precociously marked by methyl-H3K4 and H3K4ac PTMs in ES cells, a chromatin signature dubbed an early transcription competence mark (ETCM) by these authors (51). In comparisons of pro-B and pre-B cells, the methyl-H3K4 and acetyl-H3 signature spreads into adjacent regions that include the *λ5* and *VpreB1* genes, which become highly transcribed in pre-B cells. Interestingly, the β-globin ETCM is also the site of recruitment of components of the transcription factor IID (TFIID) complex in ES cells, as well as RNA polymerase II, even though the linked *λ5* and *VpreB1* genes are not expressed. However, in ES cells subjected to a general differentiation protocol (which did not produce lymphoid cells), the ETCM chromatin signature was lost. The ETCM recruits E2A and PU.1 transcription factors in pro-B cells, and the occupancy of these factors spread to the *λ5* and *VpreB1* promoter regions in pre-B cells, where these genes become highly transcribed. In pre-B cells, ChIP analyses showed abundant occupancy of TFIID and RNA polymerase II at the expressed *λ5* and *VpreB1* genes, as well as the ETCM. Collectively, these findings suggest that genes are indexed in a permissive chromatin state in pluripotent cells that poises them for later expression once proper cellular lineage allocation is achieved. Furthermore, these data strongly suggest that "primed" chromatin might function by providing a permissive molecular microenvironment that allows the assembly of partial transcriptional activating complexes in juxtaposition to promoters.

6. METASTABLE STATE OF ES CELL CHROMATIN

ES cells are perpetually poised in a pluripotent, self-renewing state. Evidence strongly suggests that ES cell chromatin is maintained in a unique metastable state that is amenable to extensive remodeling that is associated with (and in many cases required for) allocation of subsequent cellular lineages. The existence of metastable chromatin in ES cells is ample: Human ES cells express a larger set of genes than differentiated cells, though many of these are expressed at moderate levels, suggesting that ES cells may exhibit a broad range of low-level transcription across much of the genome (52). Metastability of ES cell chromatin has recently been demonstrated through the use of cell biological approaches including fluorescence recovery after photobleaching (FRAP) (53). FRAP provides a cell-biological approach to determine molecular retention and dissociation rates in live cells by photobleaching green fluorescence protein (GFP)-tagged chromatin components and monitoring the time required for recovery of fluorescence. FRAP studies show that rate of exchange of heterochromatin protein 1 (HP1) α, β, and γ are much faster in the heterochromatin of ES cells as compared to their differentiated derivatives (53). In these studies recovery after photobleaching is biphasic in ES cells, with a rapid initial phase of recovery that is unique to ES cells, followed by additional recovery of much longer duration. Recovery of the linker histone $H1^{o}$ was also faster in ES cells than differentiated cells. Exchange rates of core histones H3 and H2B were also faster in ES cells, though much slower than linker H1 histones. These experiments demonstrate that ES cells contain a hyperdynamic pool of chromatin components, which rapidly associate and dissociate from chromosomes as compared to differentiated cells. In the same study, ES cell chromatin was found to be less stable in the presence of salt, providing independent confirmation of the hyperdynamic nature of ES cell chromatin. Furthermore, EB formation is accelerated in ES cells deficient for the nucleosome assembly factor HirA, but EB formation is impeded when ES cells are made to express a form of $H1^{o}$ that is mutated to have a higher affinity for chromatin.

These results imply that altering the association of architectural components with chromatin can induce ES cell differentiation.

Dynamic alterations occur in nuclear organization and chromatin environment when ES cells are coaxed to differentiate. A picture of how nuclear dynamics interplays with regulated gene expression in differentiating ES cells is emerging for *Hox* gene regulation, where gene expression is sequential with regards to developmental anterior-posterior patterning in the embryo and correlated with the linear organization of *Hox* genes within a cluster. One study observed that as *Hoxb* genes become expressed in appropriate tissues of the developing mouse embryo, transcribed *Hox* loci decondense and loop out from their chromosome territories (54). However, decondensation and looping out from chromosome territories are separable events. *Hoxd* alleles can occur in decondensed form in ES cells undergoing differentiation, even though the locus remains embedded within its normal chromosomal territory, while in other cases, loci can loop out of territories without decondensation (55). Looping out and decondensation imply that genes can become accessible to auxiliary regulatory molecules due to regulated accessibility. New approaches such as chromosome conformation capture (3C), a procedure that detects the position of loci of interest within the interphase nucleus, show that the *Hoxb1* gene becomes juxtaposed to distant loci only after ES cell differentiation (56).

Pluripotency genes are also positioned at intriguing intranuclear positions in ES cells. Chromosome 12p contains a cluster of pluripotency genes including NANOG, which is positioned in the transcriptionally permissive nuclear interior in human ES cells (57). This same study shows that the *OCT4* gene is positioned outside of its chromosome territory in ES cells. In another report, the *Mash1* locus (which encodes a factor involved in neural development) is located near the nuclear periphery and replicates late in S-phase. Furthermore, the *Mash1* promoter is enriched in methyl-H3K27 and acetyl-H3K9 in ES cells. Surprisingly, loss of Exh2/Eed histone methylation, other histone methyltransferases, or cytosine DNA methyltransferases failed to perturb *Mash1* localization to the nuclear periphery in ES cells. With neural induction, *Mash1* replication shifted to an earlier time in S-phase and the *Mash1* alleles were positioned to a more central locale within the nucleus. Intranuclear localization and replication timing are phenomena that are results of the molecular microenvironment of epigenetically-regulated alleles. Silent genes are also often found near centromeric heterochromatin, which contains a number of protein factors including HP1 isoforms. TIF1β is a repressor that is important for early embryogenesis. TIF1β exhibits a diffuse nuclear localization in EC cells, but is concentrated in foci corresponding to pericentromeric heterochromatin after RA-induced differentiation (58). Amino acid substitutions in an HP1 interaction motif of TIF1β abolish the association of this repressor with pericentric heterochromatin. Since HP1 is known to bind methyl-H3K9, these results suggest the existence of a molecular mechanism in which a repressor protein (TIF1β) is recruited to genes with methyl-H3K9 content. Clearly, future studies of interactions between chromatin of developmentally-regulated genes and auxiliary, chromatin-binding factors will hasten our understanding of molecular-epigenetic mechanisms that participate in processes such ES cell differentiation and embryogenesis.

7. MECHANISMS OF EPIGENETIC CHANGE

At a molecular level, the chromatin of ES cells is metastable in comparison to that of differentiated cells. In addition, the chromatin of key developmentally-regulated genes is precociously marked in ES cells in a way that seems to index them for subsequent transcriptional states. Though these observations are suggestive, the involvement of chromatin in mechanisms related to cell lineage decisions and other developmental regulation are only now becoming elucidated. In short, much of the literature to date consists of comparisons of chromatin states before and after differentiation. Such comparisons, though intriguing, do not allow us to ascertain if chromatin remodeling is causally involved in differentiation, or merely a consequence. These comparisons beg a pair of interesting questions: Is it possible that the perpetual pluripotency of ES cells is directly related to their kinetically metastable chromatin? Is the kinetic metastablity of ES cell chromatin an indicator of a highly active and responsive epigenetic system in ES cells, or merely a correlate of a developmentally-primitive chromatin state?

Clearly, the ES cell genome is maintained in a state that is amenable to broad epigenetic change during differentiation. Advances in nuclear reprogramming research demonstrate that broad epigenetic change can occur in the reverse direction, allowing the production of pluripotent cells from differentiated cells. Since differentiation and reprogramming both feature massive epigenetic change, it is likely that they share common molecular mechanisms. For this reason, a consideration of mechanisms of reprogramming is warranted here, since reprogramming may rely upon some of the same epigenetic mechanisms that operate in ES cells (Figure 2).

Reprogramming procedures are artificial manipulations that make use of biochemical activities resident in cells that likely have much to do with epigenetics and chromatin metabolism. Somatic cell nuclear transfer (SCNT) experiments demonstrate that vertebrate oocytes contain biochemical activities that can reprogram somatic nuclei to a state of pluripotency (59, 60). Ooplasm contains biochemical activities that can rapidly reconfigure the chromatin of the paternal genome immediately following fertilization. In addition, numerous studies show that maternal chromatin, too, is subject to wide-ranging chromatin remodeling events during preimplantation development. These same activities probably participate in reprogramming chromatin of the somatic cell genome during the artificial SCNT process. Interestingly, reprogramming activities seem to be sequestered in blastomere nuclei of preimplantation embryos since enucleated blastomeres no longer support reprogramming by SNCT (61, 62).

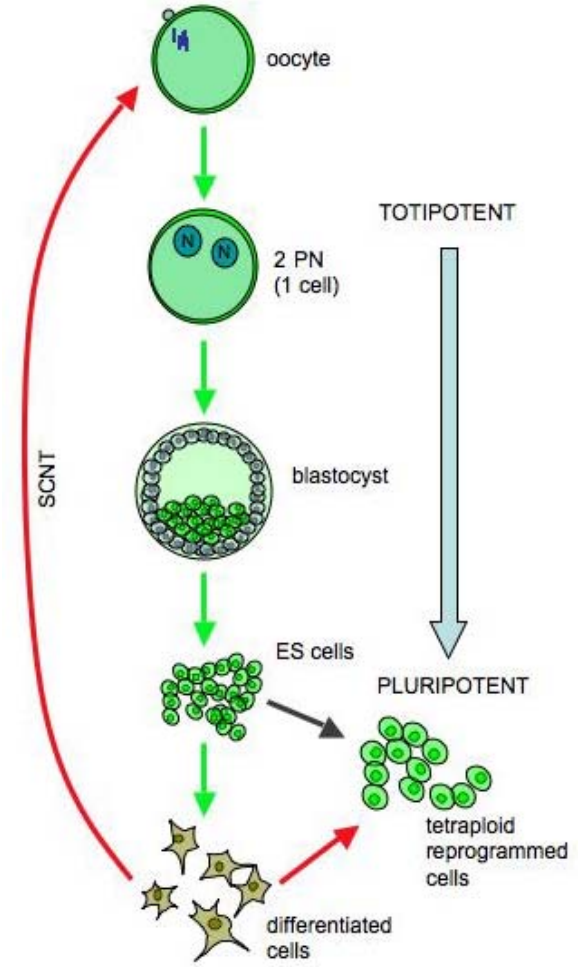

Figure 2. Pluripotency in the context of differentiation and reprogramming. A normal developmental sequence from oocyte, to 2 pronuclei (2 PN) 1 cell embryo, to blastocyst is shown. ES cells are derived from the inner cell mass of the blastocyst, and these can be coaxed to form differentiated cells. Green arrows indicate differentiation steps. Reprogramming pathways (red arrows) include somatic cell nuclear transfer (SCNT) and fusion of differentiated cells with ES cells to form tetraploid reprogrammed cells. Reprogramming is accomplished by biochemical activities present in ooplasm (for SCNT), but reprogramming activities are sequestered in nuclei after nuclear envelopes of male and female pronuclei form in 1 cell embryos. Reprogramming activity remains nuclear until the ICM stage in blastocysts, from which ES cells are derived. Reprogramming activity is also nuclear in ES cells, and these can reprogram somatic cells upon artificial fusion.

ES cells, like oocytes, contain reprogramming activities that can reprogram the somatic genome to a state of pluripotency upon fusion with somatic cells (63-65). Interestingly, the reprogramming activity of ES cells is primarily nuclear (66). Reprogramming, be it by SNCT or ES cell fusion, results in the erasure of developmentally-imposed chromatin marks and the restoration of pluripotency. In either case, the entire genome is subjected to the reprogramming process, which must make use of naturally-occurring activities present in oocytes and ES cells. Since these activities clearly involve the reconfiguration of chromatin, it is very tempting to conclude that ES cell nuclei maintain their chromatin in a pluripotent ground state through an active process. Thus, observations having to do with chromatin reprogramming in hybrids of ES cells and somatic cells are of interest. X chromosomes residing in female somatic cells are reprogrammed to a pre-inactivation state after fusion of differentiated cells with ES cells (64, 67, 68). When hybrids were made between ES cells and thymocytes, levels of H3K4me2 and H3K4me3 were reprogrammed to levels similar to unfused ES cells and *Oct4* was activated from the somatic genome, in conjunction with reprogrammed promoter chromatin (69). The nuclear proteome of ES cells contains a number of chromatin remodeling proteins whose levels decrease after differentiation caused by removal of LIF (70). This finding that ES cell nuclei contain high levels of proteins implicated in chromatin modification and remodeling suggests that maintenance of a pluripotent epigenome in ES cell may be an active process.

Reprogramming to a state of pluripotency can also be induced directly by introducing a select set of four transgenes (*Oct4*, *Sox2*, *c-Myc*, and *Klf4*) into fibroblasts (71). Furthermore, cells created by induced pluripotency by these methods exhibit extensive epigenetic reprogramming, and can readily contribute to chimeras (72-74). These exciting results show that expression of a select set of transcription factors can induce chromatin remodeling events culminating in the induction of a robust state of pluripotency.

Reprogramming of somatic nuclei, whether by SCNT or fusion with ES cells, presumably occurs because ooplasm and ES cell nuclei contain a robust set of chromatin modulatory activities (Figure 2). In the case of oocytes, chromatin modulatory activities are present to remodel the incoming male nucleus upon fertilization, and to provide a maternal store of proteins to manage the epigenome during early preimplantation development. Because these activities are in apparent excess, introduction of a somatic nucleus by SCNT places the somatic genome in a biochemical environment that restores its chromatin to a developmental ground state. Similar processes take place when ES cells are fused to somatic cells, since nuclear ES cell reprogramming activities can restore pluripotency to the somatic genome. Thus, reprogramming advances indicate that oocytes and ES cells both maintain their developmental potency through active biochemical processes. Furthermore, since the chromatin of ES cells contains genes whose expression state is pre-specified by bivalent chromatin domains, it seem possible that these marks are established and maintained by active mechanisms as well. Furthermore, since ES cells are highly proliferative, bivalent domains must be preserved with each cell cycle, a fact that also points to the existence of active epigenetic maintenance activities in ES cells.

8. PERSPECTIVE

This review details much evidence regarding the existence of epigenetic functions in ES cells that maintain the pluripotent state. This epigenetic system must change when ES cells differentiate. The existence of primed states of chromatin that specify later states of gene expression point to a role for epigenetics and chromatin remodeling in differentiation as well as the maintenance of pluripotency. These recent ideas now point the way to an interesting future that will lead to a greater understanding of epigenetics in ES cells and their differentiated derivatives.

Key questions remain, which will need to be resolved. For instance, it is unclear precisely how unique chromatin configurations are targeted to specific genes in ES cells. One idea is that highly conserved elements in DNA primary sequence serve to specify an initial chromatin state in ES cells, and a very similar situation might exist in ICM cells of blastocysts. Another question has to do with the relative order of action of chromatin remodeling and transcription factor action. It seems plausible that binding of transcription factors could nucleate chromatin remodeling, but conversely, it is easy to imagine that specialized chromatin might be required for transcription factor binding. This chicken and egg question will be an important and challenging area of study, and the solution to this problem may allow the rational manipulation of gene expression during ES cell differentiation, thus bringing us closer to new insights in developmental biology and advances in regenerative medicine.

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