Stem Cell Epigenetics: Insights from Studies on Embryonic, Induced Pluripotent, and Germline Stem Cells

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Abstract Embryonic stem cells and induced pluripotent stem cells (iPSCs) exhibit similar and unique epigenetic features that endow them with pluripotency. Pluripotent cells have highly plastic genomes that display open chromatin that is abundantly marked by active histone modifications, making it poised for differentiation cues. This is in contrast to lineage-committed cells, which have condensed heterochromatin and large blocks of repressive chromatin domains. A recent study of spermatogonial stem cells showed dynamic epigenetic changes occur during differentiation, suggesting that, in both embryonic- and adult-type stem cells, global epigenetic regulation serves as a barrier between stem and differentiated cells. Reprogramming requires that this epigenetic barrier be overcome for faithful gene expression patterns to be established. Evidence suggests that incomplete epigenetic reprogramming is common in iPSCs, highlighting potentially serious problems for clinical applications. Here, we review insights and major progress obtained from in vitro stem cell studies, as well as in vivo characterization of stem cells in the germline.

Keywords Chromatin · Embryonic stem cells · Epigenetics · Germline · Induced pluripotent stem cells · Spermatogonia

Introduction

Embryonic stem cells (ESCs) isolated from the blastocyst inner cell mass (ICM) have long provided an invaluable tool for understanding how stem cell identity changes during early mammalian development. When cultured under the appropriate conditions, these pluripotent ESCs can be induced to differentiate, recapitulating developmental lineage commitment in vitro and allowing the molecular mechanisms driving these changes to be efficiently studied. Although the ESC system has brought us key insights into cell differentiation, a major challenge in this area remains: what regulatory mechanisms of gene expression drive differentiation and reprogramming? This is not a trivial question because during differentiation, chromatin structural changes are complex, triggered by multiple epigenetic elements, such as DNA methylation, histone modifications and non-coding RNAs, and each of these processes, in turn, can affect gene expression patterns. For instance, while histone H3K4me3 marks promoters of actively transcribed genes, histone H3K27me3 is found at silent promoters. Gene repression has also generally been thought to be a characteristic of cytosine DNA methylation at promoter CpG dinucleotides, although recent evidence indicates more complex scenarios (to be discussed later). Springboarding from this information, high-throughput sequencing technology characterized the epigenetic features of ESCs, and the global epigenetic landscapes of pluripotent and differentiated cells [1]. Such characterization indicated regulatory regions that undergo DNA methylation changes and histone modifications during differentiation, and provided insight into the general roles of each epigenetic mark in the genome.

Recently, Takahashi et al. [2, 3] developed a method for inducing mouse and human differentiated cells to generate

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pluripotent stem cells (iPSCs), a remarkable achievement that greatly expands the study of stem cells and opens new potential for regenerative medicine and biomedical research. Here, the key discovery was that by introducing only four transcription factors—Oct4, Sox2, Klf4 and c-Myc (collectively called OSKM)—differentiated cells can be induced to generate iPSCs. Building from this milestone discovery, a major focus of scientists has been to characterize iPSCs and understand how they are similar to ESCs. Multiple iPSC lines have been characterized by transcriptomic and epigenomic approaches, initially revealing that iPSCs and ESCs share many general characteristics. More recently, however, increasing evidence indicates iPSC lines are epigenetically variable, suggesting that during iPSC generation the reprogramming process is often incomplete and unstable.

Apart from progress with iPSCs, information on the epigenetics of other stem cells remains limited. Among the various stem cells identified in neonatal and adult tissues, testis spermatogonial stem cells are a well-studied example that continue to provide a desirable model to study epigenetic features. Spermatogonial stem cells offer key advantages: multiple marker molecules for the stem cells and their differentiating progeny are known, and spermatogonial stem cells can be grown in culture. Our recent work has revealed dynamic epigenetic changes that occur when mouse spermatogonial stem cells differentiate towards progenitor cells [4•]. Moreover, our work raises the possibility that different types of stem cells might share similar epigenetic features, and epigenetic barriers might exist between stem and differentiated cells, irrespective of their origin.

Here, we will review the rapidly growing area of ESC and iPSC epigenetics. The epigenetic features of stem cells from the mouse germline will be summarized and linked to the features of other types of stem cells. We will also discuss potential problems that might arise if iPSCs are prone to aberrant epigenetic reprogramming and how this might impact clinical applications.

Chromatin States of ESCs and iPSCs

During mammalian development, two phases of dynamic epigenetic reprogramming occur on a global scale [5]. The initial wave commences soon after fertilization, with extensive erasure of DNA methylation and repressive histone modification marks, dramatic changes that unleash transcriptional activation of the embryonic genome. In developing post-implantation embryos, a second wave of reprogramming occurs in primordial germ cells (PGCs); again global DNA methylation is erased and histone modification patterns reprogrammed. Since ESCs are derived from the ICM of blastocysts, they reflect the

pluripotent events that occur after the first wave of reprogramming. Interestingly, iPSCs have undergone OSKM-induced reprogramming in vitro and, consistent with this, they show epigenetic and transcriptomic profiles similar to those in ESCs [2, 6–10, 11•, 12–17].

The genomes of ESCs assume an open chromatin state that is highly accessible to transcription factors. In contrast, differentiated cells have condensed genomes with abundant heterochromatin foci [18–20] (Fig. 1). As expected, changes in chromatin conformation are crucial during formation of iPSCs. During iPSC derivation, the chromatin structure relaxes, generating an ES-like open state. Open chromatin is locally induced by chromatin remodeling factors, such as the SWI–SNF complex and Chd1; and knockdown of Chd1 blocks, and overexpression of SWI–SNF complex improves iPSC formation [21, 22]. In addition, proper reprogramming requires long-range chromatin interactions at genomic regions surrounding pluripotency factors, such as *Oct4* and *Nanog* [23–25].

Chromatin conformation is tightly linked to histone modification patterns. In the case of histone H3K9, methylation allows the histone tails to recruit heterochromatin protein HP1, a necessary component of condensed heterochromatin [26]. Pluripotent ESCs are enriched for active histone marks, including H3K4me3, but as ESCs differentiate, the regions marked by repressive H3K9me3 and H3K27me3 extend and spread [15, 19, 27–29]. H3K9 methylation is a major barrier to reprogramming, so reducing levels of H3K9 methyltransferases or overexpressing H3K9 demethylases improves the efficiency of generating iPSCs [30•, 31•, 32]. In line with such overall epigenetic landscapes, the transcriptionally hyperactive ESC genome undergoes large-scale silencing during differentiation [27]. In addition, the chromatin in ESCs and iPSCs is in a state that is highly plastic and responsive, capable of prompt differentiation in response to extrinsic signals. This plasticity of ESCs and iPSCs is particularly evident at key developmental genes that harbor “bivalent” histone modifications, in which the promoters are marked by both active H3K4me3 and repressive H3K27me3 modifications [3, 33]. Bivalent modifications are considered hallmarks of genes poised to respond to differentiation signals: bivalently marked genes are repressed in pluripotent cells but upon differentiation lose one of the opposing histone modifications, depending on the lineage they become committed to. Thus, bivalency is a unique epigenetic feature of pluripotent cells and an important regulator of differentiation.

DNA Methylation Landscapes in Pluripotent Versus Differentiated Cells

Like histone modifications, DNA methylation status is well characterized in ESCs and iPSCs. DNA methylation has

Fig. 1 Generation of different stem cell lines from embryos, germ cells and somatic cells. Overall epigenomic properties of each cell type are indicated. Embryonic stem cells (ESCs), embryonic germ cells (EGCs), multipotent germline stem cells (mGSCs) and induced pluripotent stem cells (iPSCs) are derived from different origins but likely to share common epigenomic features such as active/open chromatin. Primordial germ cells (PGCs) and the pool of spermatogonial stem cells are lineage-committed but have active/open chromatin, and pluripotent/multipotent cells can be generated without genetic manipulations. In contrast, differentiated somatic cells have more repressive/condensed chromatin domains and efficient reprogramming requires OSKM (Oct4, Sox2, Klf4 and c-Myc) overexpression and Mbd3/NuRD depletion (see text for details)

long been considered a repressive mark but more recently, genome-wide studies indicate methylation plays more complex roles and is not always repressive [34, 35]. In general, regions rich in CpG (CpG islands) that overlap gene promoters are unmethylated and expression is repressed instead by H3K27me3 marks [34, 36, 37]. Nevertheless, many promoters in CpG islands undergo methylation changes during differentiation, like promoters of pluripotency genes, which are devoid of methylation in undifferentiated ESCs and iPSCs, but become methylated upon differentiation [2, 3, 7, 38]. Indeed, for ESCs to differentiate, DNA methylation is required: differentiation is blocked by global hypomethylation induced by depletion of either maintenance methyltransferase (Dnmt1) or de novo methyltransferase enzymes (Dnmt3a and Dnmt3b) [39]. Interestingly, Dnmt-depleted cells retain stem cell characteristics and remain viable, indicating that DNA methylation is dispensable for the maintenance of stemness.

Paradoxically, a global map of ESC methylation made available by high-throughput sequencing showed that, unlike hypomethylated ICM cells from which ESCs are derived, the ESC genome is, overall, highly methylated,

resembling that of somatic cells [40–42]. These ESCs had been cultured under standard conditions [i.e., containing fetal calf serum (FCS) and leukemia inhibitory factor (LIF)], priming them for differentiation by fibroblast growth factor (FGF)/extracellular signal-regulated kinase (ERK) signaling [43], which it was assumed, promoted ESCs to acquire methylation. Blocking this pathway by culturing ESCs in the presence of two selective small molecule inhibitors (2i), thereby inducing ground-state pluripotency [44], generated a globally hypomethylated genome with lower expression levels of Dnmt3a, 3b and their regulator Dnmt3L. These results suggested that 2i culture conditions push the ESC epigenome into a state similar to that of ICM or PGCs [45, 46, 47, 48, 49]. Altogether, evidence indicates that global hypomethylation appears to be one of the epigenetic features of the ground-state pluripotent stem cells. Whether other epigenomic landscapes, including histone modifications, are further reprogrammed in 2i culture remains an open question.

For differentiated cells to become reprogrammed, DNA methylation appears to be a crucial barrier that must be removed. In mammals, DNA methylation at the 5 position

of cytosine (5mC) is a key epigenetic modification. Global hypomethylation is promoted by the ten-eleven translocation (Tet) family of 5mC-hydroxylases (Tet1–3), which convert 5mC to 5-hydroxymethylcytosine (5hmC) [50]. When OSKM is overexpressed, Tet2 expression rises and decreases global levels of 5mC, an event that triggers hydroxylation of pluripotency genes such as *Nanog* and *Esrrb* [51]. Accordingly, Tet2 knockdown suppresses induction of 5hmC and abolishes iPSC colony formation. Under the 2i culture conditions, Tet1/2 physically interacts with Prdm14, a key factor for germ cells to acquire pluripotency, synergistically downregulating Dnmt3a/b, and evoking the hypomethylated state [52]. Interestingly, recent studies revealed that vitamin C is also a key molecule that facilitates DNA demethylation and increases reprogramming efficiency [48, 53–58]. Vitamin C does not affect Tet gene expression but rather it is likely a co-factor for Tet enzymes that enhances hydroxylation [48, 57]. When ESCs are cultured in 2i, vitamin C appears to induce Tet1/2-mediated hydroxylation and thereby DNA demethylation, giving rise to an ICM-like epigenetic state [48].

Recent work in iPSCs reported that, if cells are depleted of Mbd3, a core component of the Mbd3/NuRD (nucleosome remodeling and acetylation) complex, OSKM-induced reprogramming efficiency reaches nearly 100 % [59•]. Without OSKM overexpression, Mbd3 depletion was not sufficient to induce iPSC formation. Interestingly, in the background of OKSM expression, Mbd3 localizes to target genes for pluripotency factors (i.e., *Klf4*, *Oct4*, *Sox2* and *Esrrb*) and Mbd3 deficiency rendered these genes in an open chromatin state, with high levels of H3K4me3 and H3K27 acetylation and a reduced level of H3K27me3. Hypothetically, this remarkable improvement in generating pluripotent conditions may be the result of an association between Mbd3 and Tet1 and 5hmC-enriched promoter/enhancer regions [60, 61]. This idea is unproven, however, and binding of Mbd3/NuRD could be independent of 5hmC and 5mC status [62]. Whatever the case, the MBD3/NuRD complex clearly blocks the exit from pluripotency and so interferes with reprogramming [63]. Overall, abundant evidence suggests that epigenetic reprogramming of differentiated cells to ground-state pluripotency is apparently the key to generating iPSCs efficiently, and this could be substantially affected by a strikingly small number of culture components and cellular proteins.

Pluripotent and Multipotent Stem Cells from Lineage-Committed Cells

Before Yamanaka's group published the milestone report that they established iPSCs [2], somatic tissues had never

been coaxed to produce pluripotent stem cells that can differentiate into germ line cells. Likewise, freshly isolated PGCs (diploid, embryonic, germ cell precursors) injected into blastocysts could not be made to contribute to chimeras or become embryoid bodies in culture, despite that they endogenously express *Oct4*, *Sox2* and *Nanog* [64, 65]. Indeed, PGCs in and of themselves are not pluripotent. Moreover, PGC survival was problematic [66–68], hindering long-term maintenance of PGC properties in culture. An important breakthrough was achieved by culturing PGCs on embryonic fibroblast feeder cells with three cytokines (FGF2, LIF and SCF), which enabled long-term culture and resulted in conversion of unipotent PGCs into pluripotent embryonic germ cell (EGC) lines [69, 70] (Fig. 1).

EGCs can be established from PGCs at the pre-migratory stage, the migrating stage, and after the entry into the genital ridge, but from these phases onward, global DNA demethylation, erasure of methylation imprints, and reactivation of the X chromosome proceed [71]. Therefore, DNA methylation profiling shows that, depending on the developmental stage from which PGCs are derived, the DNA methylation patterns of EGCs differ [72]. In EGCs derived from pre-migratory (E8.5) embryos, the level of DNA methylation is similar to ESCs, but upon transfer into 2i culture, the extent of methylation drops [47•]. EGC pluripotency was tested by in vitro differentiation as well as in chimera formation assays, showing EGCs can contribute to all three germ layers including germ cells [68, 69, 73]. Thus, it is conceivable that the PGC epigenome is plastic and under the proper culture conditions it can convert from the metastable epigenetic state to the level of ground-state pluripotency. Like pluripotent ESCs, PGCs at the reprogrammed stage do not require DNA methylation, suggesting the transcriptional regulatory machinery is probably compensated by other epigenetic systems. This could explain why only the germ cell lineage can be transformed to pluripotent stem cells without any genetic and epigenetic manipulations.

In 2004, Shinohara's group made a breakthrough report that they had established multipotent germ stem cells (mGSCs) from neonatal gonocyte/spermatogonia [74] (Fig. 1). Since then other groups have reported similar multipotent stem cells could be derived from postnatal male germ cells (hereinafter called ES-like cells in this review), although origins of these cell types and the culture protocols used are surprisingly different from one another: Guan et al. [75] established ES-like cells from Stra8-GFP-tagged spermatogonia from 4-week to 6-week-old mice in a short-term culture condition with GDNF; Seandel et al. [76] used 3-week to 8-month-old GPR125+ spermatogonial progenitor cells with added GDNF, and co-cultured with mixed primary testicular somatic cells. The above groups, however, shared the fact that they applied two-step culture protocols: in step one, spermatogonia-like cells were maintained in

culture, while in step two, culture conditions were changed to establish ES-like cells. Nevertheless, the initial culture conditions differed. Guan et al., initially cultured cells for only two weeks, while Shinohara and Seandel maintained spermatogonia in vitro for at least two to three months. It is not yet clear how epigenetically similar these ES-like lines are, but further integrated comparisons of their phenotypic, genetic and epigenetic properties should be forthcoming.

During germ cell development in vivo, evidence suggests dynamic epigenetic changes may impact the acquisition of pluripotency. At the migration stage, PGCs stop expressing Gfp, a component of the histone H3K9 methyltransferase complex, resulting in loss of H3K9me1/2; nevertheless, these cells acquire high levels of H3K27me3 [77, 78]. PGCs have a very low level of DNA methylation at E9.5, which is further reduced by E12.5 [79–81]; and when PGCs commit to a lineage, Dnmt3b is no longer expressed, although Dnmt3a is maintained at a low level throughout embryogenesis [77]. Neonatal and adult mouse spermatogonia also exhibit a dynamic epigenetic status [4]. Postnatally, male germ cell cytokinesis is incomplete, leaving divided cells interconnected from the spermatogonial stage onward and allowing cell division history to be easily inferred by counting the number of cells in a chain. In adult testes, the first single spermatogonial cell—called the As cell—is believed to be a stem cell. We recently found that adult undifferentiated spermatogonia consisting not only of As cells but also 2-chained to 8-chained cells (that is until they start expressing a progenitor cell marker, c-Kit), share a similar epigenetic status: they contain euchromatin-rich nuclei; they express low levels of de novo Dnmt3 proteins; immunohistochemistry (IHC) detects only weak signals of H3K9me2; and IHC and ELISA analyses detect only low levels of 5mC. All of these features change dynamically once the cells enter the c-Kit-expressing (differentiating) state. This raises the possibility that the 2-chained to 8-chained cells are epigenetically similar to As cells, maintaining an intrinsic stem cell activity that allows them to comprise a “stem cell pool”. Furthermore, in mice, disrupting the DNA methylation maintenance machinery (by Np95 deficiency) did not affect the cells in the stem cell pool, suggesting that these cells can tolerate low levels of DNA methylation [4]. Thus, an epigenetic barrier may exist between stem and differentiating cells, implying that various stem cell systems, including ESCs and iPSCs, share a general feature of exit from stemness that may require epigenetic switching (Fig. 1).

Aberrant Epigenetic Patterns of iPSCs and Potential Problems for Clinical Use

The emergence of techniques to generate iPSCs raises the anticipation that these cells might be useful for regenerative

medicine, such as cell transplantation therapy. Clearly, iPSCs hold great therapeutic potential; however, further studies are necessary before they will offer practical use at the bedside. Any real breakthrough for regenerative medicine will depend on the fidelity of reprogramming of the iPSCs to the pluripotent state and on their maintenance of normal genetic and epigenetic status. For iPSCs and ESCs, the genomic regions prone to amplification, deletion, or point mutation seem to be enriched for genes involved in cell-cycle regulation and cancer [82–84], and this phenomenon may be linked to an ineluctable feature of iPSCs that is required at the establishment and expansion phases. In addition to these genetic abnormalities (which include variation in chromosomal copy number and a high frequency of mutation), epigenetic instability has been reported, such as aberrant DNA methylation and histone tail modifications that lead to abnormal gene expression. In particular, disappointing reports describe aberrant CpG methylation of genes and imprinted loci [11•, 85–88]. These abnormalities can occur at both the reprogramming and expansion steps (during adaptation to long term culture), or be inherited from original somatic cells. Whole-genome profiles of DNA methylation at single-base resolution in iPSCs were compared to similar datasets of ESCs and somatic cells [86, 87] showing that iPSCs and ESCs display similar global methylome patterns. CpG islands in iPSCs were mostly reprogrammed to an ESC-like stage in that hypermethylated CpG islands were not resistant to reprogramming. Nevertheless, a comprehensive analysis of iPSCs and ESCs by Lister et al. [11•] identified >1,000 differentially CpG-methylated regions (CG-DMRs) that likely arose either because the original differentiated cells were incompletely reprogrammed and so retained some original methylation patterns (somatic memory), or because some DMRs are not derived from the original somatic cells and ESCs, but rather are iPSC-specific. Comparison of iPSC methylation patterns with those of the respective original somatic cells from which they were derived revealed that 44–49 % of DMRs were unlike with respect to ESCs, reflecting the original methylation pattern. The aberrant methylation at CpG sites appeared to be transmitted to differentiated cells, resulting in transcriptional changes distinct from differentiated ESCs [87]. In addition to DMRs of CpG methylation, regions of differentially methylated non-CpG sites (non-CG DMRs) were identified, which tended to be very large, half of them being greater than 1 Mb in length and the longest being 4.8 Mb [11•].

DNA methylation is a major barrier to iPSC reprogramming and, therefore, hydroxymethylation followed by demethylation is required for optimal induction of iPSCs. It is notable that bisulfite sequencing cannot distinguish 5mC and 5hmC. This is an issue that must be taken into consideration when interpreting previously reported bisulfite

sequencing data. Unconverted cytosines represent the sum of 5mC and 5hmC. Wang et al. [89••] found 5hmC levels increase significantly when iPSCs are reprogrammed, mainly because Tet1 is activated. Furthermore, they identified twenty regions in iPSCs that are hotspots of large-scale aberrant reprogramming; these extend from 100 kb to 1.3 Mb in telomeric-proximal regions, particularly at chromosomes 2, 7, 8, 12 and 20, and most of them exhibit incomplete hydroxymethylation at CpG sites. In iPSCs, these hotspots of aberrant 5hmC largely coincide with previously reported aberrantly methylated non-CG DMRs but not with the CG-DMRs. Therefore, 5hmC modification mediated by Tet1 could result in the epigenetic variation of iPSCs and iPSC-ESC. Furthermore, when comparing iPSCs and ESCs, Guenther et al. [9] identified 31 differentially modified bivalent regions, while Hawkins et al. [15] found clearer differences in the H3K9me3 repressive modifications than in H3K27me3 modifications. Overall, variation in DNA methylation is generally common in iPSCs, and it is key that when generating iPSCs, depleting Mbd3 increases reprogramming efficiency to almost 100 % [59••]. This approach will likely improve the quality of reprogrammed cells and reduce the frequency of obtaining aberrantly reprogrammed iPSCs. Moreover, epigenetic status could vary under different cell culture conditions, such as the 2i condition and various oxygen concentrations [90]. Therefore, large-scale studies with a unified protocol will be an important and necessary advance in evaluating the epigenetic status of iPSCs.

Conclusion

In this review, we have focused on some of the representative epigenetic events in stem cells. Figure 1 summarizes the generation of pluripotent stem cells and their typical epigenetic properties. It must be noted that a variety of additional histone modifications, non-coding RNAs and ATP-dependent chromatin remodeling factors also collaborate to render chromatin into the proper conformation. Limited space demands, however, that we leave the detailed discussion of such epigenetic events to others. Nevertheless, the rapidly growing literature describing iPSC studies illuminates the reprogramming mechanisms that might be applied to fully committed and differentiated cells although these mechanisms are obviously artificial and different from what occurs in fertilized eggs. This work shows iPSCs are not always faithfully reprogrammed and are distinguishable from pluripotent-stage embryos or even embryo-derived ESCs revealing serious obstacles to clinical application of iPSCs. The ineluctable steps of iPSC generation, i.e., reprogramming and subsequent expansion of iPSCs in culture, can lead to the accumulation of diverse

abnormalities at the chromosomal, sub-chromosomal and single base levels. Therefore, although we still do not understand the epigenetic modifications required for “good-quality” iPSCs, high-throughput functional genomics should provide important insight into the quality of iPSCs. Still, these technical issues might be further complicated by the high cost associated with stem-cell-based therapies, which could limit the scope for patients and burden health care systems. In the meantime, patient-derived iPSCs are already playing an important role in drug screening and analysis of molecular mechanisms of rare diseases. For the future, identification of “good-quality iPSCs” for cell therapy application is an urgent task.

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Compliance with Ethics Guidelines

Conflict of Interest Shin-ichi Tomizawa, Takayuki Shirakawa, and Kazuyuki Ohbo declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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