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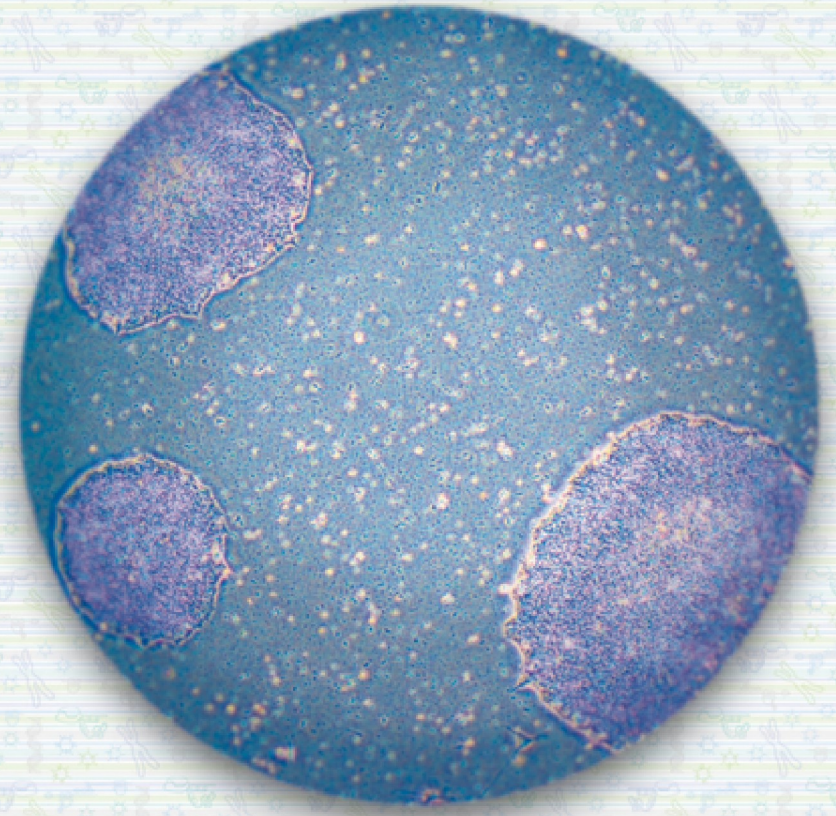
Robert A. Meyers

Stem Cells

From Biology to Therapy

Volume 1



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Cover

Image of human iPSCs in feeder-free culture conditions. For more details see figure 1 in chapter 2 "Induced Pluripotent Stem Cells" authored by Kazutoshi Takahashi and Shinya Yamanaka.

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Contents

Preface

Five Nobel Laureates are associated with this book: the *Encyclopedia of Molecular Biology and Molecular Medicine* Board members, Sir Martin Evans, who won a Nobel in Physiology or Medicine in 2007 for isolating embryonic stem cells and then growing them in culture; as well as David Baltimore, Gunter Blobel, and Phil Sharp; and now contributing author Shinya Yamanaka, whose 2012 Nobel Prize for Physiology or Medicine was awarded for reprogramming mature cells to become pluripotent stem cells. Professor Yamanaka's chapter on Induced Pluripotent Stem Cells, written for our book, forms a central component, tying together all aspects of stem cells biology and applications.

In his chapter, Professor Yamanaka points out the central issues associated with clinical application of stem cells. ''Because pluripotent stem cells can theoretically differentiate into all cell types in the body, applications for cell therapy are expected. However, it is unclear when ES and/or iPS cells would be effective for cell therapy. The most common issue preventing the clinical use of ES and iPS cells is the risk of teratoma formation after transplantation. Residual undifferentiated cells in differentiated cell cultures used for a transplant can cause a teratoma, and should be removed before use. Both effective methods for the removal of undifferentiated cell contamination, such as the use of flow cytometry, and more efficient procedures for differentiation are being developed''. Beyond these, there are additional important potential hurdles to clinical applications, including: the need for xeno-free stem cell lines, epigenetic memory and aberrant genetic errors which may be higher for iPS cells as compared with ES cells. All of these factors are covered in detail in our chapters.

The 26 detailed chapters, prepared by leaders in the field, cover the basic biology of stem cells, laboratory methods, stem cells and disease and stem cell therapy approaches and translation to the clinic for treatment of many diseases including Parkinson's disease, spinal cord trauma, diseases of blood cells, and many types of cancer as well as regeneration of cardiac and other muscle tissue. The chapter on ''Translating Stem Cells to the Clinic: from modeling disease to cellular products'' by Juan Carlos Izpisua Belmonteand his team at the Salk Institute presents the state and future of stem cell clinical applications including 1) ''disease in a dish'' laboratory substrates providing patient-specific iPS cells which can be employed for disease modeling and drug development; 2) the possibility to generate every desired cell type *in vitro* for restoration of any injury from lost tissue by cell replacement and gene-editing technologies that

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efficiently target both and 3) pluripotent cells as well as adult stem cells giving rise to the possibility for gene-correction followed by autologous transplantation which could be employed for the actual cure of monogenic inherited diseases in patients.

Our team hopes that you, the reader, will benefit from our hard work, finding the content useful in your research and educational. We wish to thank our Managing Editor, Sarah Mellor as well as our Executive Editor, Gregor Cicchetti for both their advice and hard work in the course of this project.

Larkspur, California, March 2013

Robert A. Meyers
Editor-in-Chief
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Part I
Basic Biology

Stem Cells: From Biology to Therapy, Advances in Molecular Biology and Medicine, First Edition. Edited by Robert A. Meyers.

1
Epigenetic Regulation in Pluripotent Stem Cells*

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Keywords

Embryonic stem cells (ESCs)
Pluripotent cells derived and cultured from the inner cell mass of blastocysts or from blastomeres of early embryos. These cells are able to proliferate and self-renew indefinitely, and to maintain undifferentiated states under correct culture conditions, while retaining the potential to differentiate into all types of cell in the body.

Induced pluripotent stem cells (iPSCs)
By ectopic expression of a few transcription factors (e.g., Oct4, Sox2, Klf4, and c-Myc), differentiated cells are reprogrammed and give rise to ESC-like cells. The latter are also pluripotent and able to self-renew; hence, they are termed iPS cells (iPSCs).

Totipotency
Cells sufficient to form an entire organism by themselves. Examples are zygotes and few cells in early-cleavage embryos in mammals.

Pluripotency
The developmental potential of a cell to differentiate into all types of cell in the body. The most stringent test for developmental pluripotency is the generation of offspring completely from ESCs/iPSCs by tetraploid embryo complementation, or by four- to eight-cell embryo injection. A less stringent test is the production of germline-competent chimeras by either diploid blastocyst or four- to eight-cell embryo-injection methods.

Reprogramming
An increase in the developmental potency from a differentiated to an undifferentiated stage; also referred to as dedifferentiation in some instances.

Epigenetics
Changes in gene function that are mitotically and/or mitotically inheritable, and that do not entail a change in DNA sequences. Epigenetic information includes changes in gene expression by DNA methylation, microRNAs, histone modifications, histone variants, nucleosome positioning, and higher-order chromatin structure.

DNA methylation
The addition of methyl groups to DNA, mostly at CpG sites, to convert cytosine to 5-methylcytosine. DNA methylation usually represses gene expression.

Histone
Proteins enriched in positively charged amino acid residuals, found in eukaryotic cell nuclei. These proteins package and order the DNA into structural units called nucleosomes.

Nucleosome
The basic unit of chromatin. In a nucleosome, a DNA fragment of 147 bp is wrapped around spools of histone proteins.

Histone modification
Modification in the entire sequence of histones, particularly at the unstructured N-termini (''histone tails''), including acetylation, methylation, ubiquitylation, phosphorylation, and SUMOylation. Histone acetylation or the inhibition of histone deacetylation is generally linked to transcriptional activation.

Imprinting
The allele-specific expression of a small subset of mammalian genes in a parent-of-origin manner (either the paternal or maternal is monoallelically expressed). The establishment of genomic imprinting is controlled mostly by DNA methylation, and also by histone modifications, noncoding RNAs, and specialized chromatin structures. Aberrant imprinting disrupts fetal development, and is associated with genetic diseases, some cancers, and a number of neurological disorders.

X chromosome inactivation
In each mammalian female cell, one of the two X chromosomes is transcriptionally inactivated to compensate any X-linked gene dosage effect between male (XY) and female (XX).

Telomere
Repeated DNA sequences $(TTAGGG)_n$ and associated protein complexes that cap the end of chromosomes to maintain genomic stability. Telomere shortening is associated with cell senescence and organism aging, and also cancer.

Telomerase
An enzyme that specifically adds telomeric repeats *de novo* during each cell division, and is composed of two major components: a telomerase RNA template component (Terc); and Tert, a reverse transcriptase as a catalytic unit. ESCs acquire high telomerase activity to maintain telomere length.

Epigenetic stability is tightly controlled in embryonic stem cells (ESCs) for self-renewal and pluripotency, but is changed during the differentiation of ESCs to various cell lineages. The derivation and culture of ESCs also induce epigenetic alterations, which could have long-term effects on gene expression and the developmental and differentiation potential of ESCs. Developmental and cancer-related genes, and also imprinted genes, are particularly susceptible

to changes in epigenetic remodeling, particularly DNA methylation, microRNA (miRNA), and histone modification. In recognition of the tremendous potential of ESC/induced pluripotent stem cells (iPSCs) in regenerative medicine, the epigenetic instability must be closely monitored when considering human ESCs/iPSCs for therapeutic and technological applications.

1 Introduction

Embryonic stem cells (ESCs) are derived from the inner cell mass (ICM) of blastocysts [1–3]. Under the correct conditions, ESCs are able to proliferate indefinitely. A group of genes is required for ESC self-renewal. These pluripotency-associated genes, which are highly expressed in ESCs, are mostly downregulated upon ESC differentiation [4–6]. Among these genes, three transcription factors – Oct4, Sox2, and Nanog – play pivotal roles in the maintenance of pluripotency [7–10]. These three transcription factors regulate themselves and crossregulate each other, thus, forming a core regulatory circuitry for pluripotency [11]. Moreover, Oct4, Sox2, and Nanog activate many pluripotency-associated genes, while suppressing the expression of genes that encode developmental regulators [11–14].

Importantly, ESCs also have the potential to differentiate into all types of cell in the organism. Typically, ESCs form embryoid bodies (EBs) *in vitro*, which resemble early embryogenesis [1, 2]. Following the subcutaneous injection of ESCs into immunodeficient mice, the cells develop into a benign tumor (a teratoma), which consists of cells from three germ layers [2]. When injected into blastocysts, ESCs contribute to embryonic development and give rise to chimeric animals; subsequently, through germline transmission in chimera, the genetic information from the ESCs can be passed to the progeny [15]. Most importantly, live pups composed totally of ESCs can be derived by the tetraploid complementation or four- to eight-cell embryo injection [16–18].

These unique properties of ESCs – notably, self-renewal and differentiation potential – are referred to as *pluripotency*. The self-renewal of ESCs can provide an unlimited supply of cells, whereas the differentiation potential of ESCs allows any desired type of cell to be derived. Consequently, ESCs hold great promise for the future development of regenerative medicine.

Epigenetic events are defined as changes in gene function that are mitotically and/or miotically inheritable and that do not entail a change in DNA sequences. Epigenetic information includes DNA methylation, histone modifications, histone variants, nucleosome positioning, and higher-order chromatin structure. The activities of many enzymes, including DNA methyltransferases (DNMTs), histone demethylases, histone methyltransferases (HMTs), histone deacetylases (HDACs), histone acetyltransferases (HATs), and chromatin-remodeling enzymes, are involved in the regulation of epigenetics [19]. Moreover, as ESCs and differentiated cells share the same genetic materials, the pluripotency of ESCs is mainly attributed to the unique epigenetic regulation within ESCs.

2 DNA Methylation

DNA methylation, which serves as a key epigenetic event in the regulation of gene expression, is in dynamic mode during development. Typically, the paternal genome is actively demethylated in the male pronucleus shortly after fertilization, and this is followed by a passive DNA demethylation of the maternal genome [20]. Global *de novo* methylation increases rapidly in the blastocysts, the earliest stage of differentiation into trophectoderm cells, and also in the ICM, from which the ESCs are isolated. The reprogramming of promoter methylation represents one of the key determinants of the epigenetic regulation of pluripotency genes [21]. The methylation of DNA occurs on the cytosine in most cytosine-guanine dinucleotide (CpG) islands in mammalian genomes, and is carried out by various DNMTs. For example, DNMT1 prefers hemimethylated CpGs as a substrate, and maintains the pre-existing DNA methylation pattern during DNA replication. In contrast, DNMT3a and DNMT3b, which are known as *de novo methyltransferases*, prefer unmethylated CpGs as substrate and are responsible for the *de novo* methylation of DNA. The hypermethylation of DNA usually results in repression of gene transcription. Many CpG islands through the genome are hypomethylated and are actively transcribed in undifferentiated ESCs, but subsequently become methylated and silenced during differentiation. Those genes that are repressed in ESCs but required for later differentiation are marked by bivalent H3K4me3 and H3K27me3 domains, that render them poised for activation [22, 23]. Approximately one-third of genes that are not marked by histone H3 lysine 4 trimethylation (H3K4me3) or H3K27me3, but are mostly repressed in ESCs, are marked by DNA methylation, complementary to histone modifications [21, 22, 24]. The DNA methylation patterns are better correlated with histone methylation patterns than with the underlying genome sequence context. DNA methylation and histone modification pathways may be interdependent, with any crosstalk being mediated by biochemical interactions between the SET domain histone methyltransferases and the DNMTs [25]. Moreover, the polycomb group (PcG) protein Enhancer of Zeste homolog 2 (EZH2) is a histone methyltransferase that is associated with transcriptional repression, interacts (within the context of the Polycomb repressive complexes (PRC) 2 and 3) with DNMTs, and also exerts a direct control over DNA methylation [26].

Undifferentiated ESCs express high levels of the *de novo* DNA methyltransferases DNMT3a and DNMT3b, which may repress differentiation-related genes, thereby maintaining the ESCs in undifferentiated states. Both DNMT3a and DNMT3b are directly regulated by the core pluripotency transcription factors Oct4, Sox2, Nanog, and Tcf3. In addition, they are also indirectly regulated by the miR-290 cluster that represses retinoblastoma-like 2 (Rbl2) [27], which in turn downregulates DNMT3a and DNMT3b [28]. The inactivation of both DNMT3a and DNMT3b in mouse ESCs was shown to cause a progressive loss of methylation in various repetitive sequences and single-copy genes. Typically, DNMT3a and 3b are both stably associated with each other in ESCs [29], with the two enzymes interacting to methylate the promoters of Oct4 and Nanog genes in differentiating ESCs. The methylation of key regulatory genes Oct4 and Nanog

plays an important role in the differentiation of ESCs [30]. Generally, DNMT3a and 3b are required for remethylation in post-implantation mouse embryos and in germ cells [31]. ESCs which are deficient in DNMT1 are viable, but undergo cell death when induced to differentiate [32], whereas fibroblasts die within a few cell divisions after the conditional deletion of DNMT1 [33]. DNA methylation is also involved in chromatin structure regulation [34] and, in ESCs, also requires the lysine methyltransferase G9a [35]. Whilst, together, the activities of DNMTs and DNA methylation are not essential for the self-renewal of ESCs, they are rather critical in order for the pluripotent cells to differentiate into various types of specialized cell [36, 37] (Table 1).

A comprehensive map of DNA methylation in 11 201 proximal promoters in mouse embryonic stem cells (mESCs), using methyl-DNA immunoprecipitation (MeDIP) in combination with microarrays, showed that approximately 40% of the interrogated promoter regions are methylated, 32% are unmethylated, and 28% are indeterminate [59]. The methylated promoter regions are located primarily outside of the CpG islands, of which only about 3% are methylated to some degree in mESCs [59]. DNA methylation maps, created by high-throughput reduced representation bisulfite sequencing and single-molecule-based sequencing, have shown that the methylation of CpGs undergoes extensive changes during cell differentiation, particularly in regulatory regions outside of core promoters. Any ''weak'' CpG islands that are associated with a specific set of developmentally regulated genes undergo aberrant hypermethylation during extended proliferation *in vitro* [24]. Furthermore, genome-wide, single-base-resolution maps of methylated cytosines in a mammalian genome, from both human embryonic stem cells (hESC) and fetal fibroblasts have shown widespread differences in the composition and patterning of cytosine methylation between the two genomes [60]. Almost one-quarter of all methylations identified in ESCs were in a non-CG context, which suggested that ESCs might also use different methylation mechanisms to affect gene regulation. Non-CG methylation has been shown to disappear upon the induced differentiation of ESCs, but to be restored in induced pluripotent stem cells (iPSCs) [60].

Whilst ESC lines may differ in their DNA methylation profiles, methylation changes have been shown to accumulate during prolonged culture [61]. Such epigenetic changes are thought to reduce the developmental potential of high-passage ESC lines [62, 63]. Many female ESC lines rapidly lose their global DNA methylation following their derivation, and are associated with the activation of both X chromosomes [62, 64]. However, female ESCs are difficult to maintain in culture, and often tend to lose one of their two X chromosomes and thus to exhibit genetic instability [64]. Methylation changes are observed at the imprinting control regions (ICRs), including those at the growth-related imprinted *Igf2* and *Igf2r* loci [62, 63]. In addition, a prolonged culture period and varying culture conditions can affect the methylation patterns of undifferentiated hESCs [65, 66]. The DNA methylation profile clearly distinguishes hESCs from all other cell types, including somatic stem cells. Yet, different hESC lines exhibit different changes randomly with time in culture, and the degree of overall change in methylation is related to the number of passages [67]. It should also be noted that some

Tab. 1 Epigenetic modifying enzymes involved in the maintenance of cell pluripotency.

Epigenetic modification		*Enzyme*		*Knockout/knockdown phenotype in ESCs*	*Knockout phenotype in mouse*	*Reference(s)*
DNA methylation		DNMT1		Normal proliferation, but defects in differentiation	Embryos die at E9.5	[38, 39]
		DNMT3a/3b		Normal proliferation, but defects in differentiation	Embryos die at E11.5	[31, 40]
		DNMT1/3a/3b		Normal proliferation, but defects in differentiation	NA	[41]
Histone modifications	H3K27 methylation	PRC2	Eed	Reduction in mono-, di-, and trimethylation of H3K27. Upregulation of PcG target genes. Strong tendency to differentiate	Gastrulation failure. Defect in embryonic mesoderm development. Embryos die at ~E8.5	[42–44]
			Suz12	Global loss of H3K27me2 and H3K27me3. Impaired differentiation, failing to repress ESC markers and to activate differentiation-specific genes	Early developmental defect. Embryos die at ~E7.5–E8.5	[45]
			Ezh2	Reduction in H3K27me2 and H3K27me3, only negligible effect on H3K27me1. Impaired differentiation, yet less severe than Eed null ESCs	Lethal around gastrulation. Embryos die at ~E7.5–E8.5	[46]
			Jarid2	Unaffected global H3K27me3. Impaired ESC differentiation	Defect in neurulation. Embryos die before E15.5	[47–49]

(*continued overleaf*)

Tab. 1 (*continued*)

Epigenetic modification	*Enzyme*		*Knockout/knockdown phenotype in ESCs*	*Knockout phenotype in mouse*	*Reference(s)*
Histone acetylation H3K9 methylation	SetDB1[a]		Differentiation toward trophectoderm lineage	Peri-implantation lethal. Embryos die at ~E3.5–E5.5	[50–52]
	Jmjd1a[a]		Increased level of H3K9me2. ESC differentiation	Viable mice. Defect in spermatogenesis and become obese in adult mice	[53–55]
	Jmjd2c[a]		Increased level of H3K9me3. ESC differentiation	NA	[53]
	Tip60-p400	Tip60[a]	Flattened colony morphology. AP activity, EB formation, and teratoma formation are compromised. Upregulation of many developmental genes	Embryo dies before implantation	[56, 57]
		Trrap[a]	Flattened colony morphology	Peri-implantation lethality	[56, 58]

[a]The phenotypes described here are knockdown ESCs.
NA, data not available.

genes which frequently gain aberrant DNA methylation are related to tumorigenesis [68].

In contrast, DNA demethylation is important for the full reprogramming of somatic cells into iPSCs, by an enforced expression of defined sets of transcription factors in somatic cells. Stable partially reprogrammed cell lines show the reactivation of a distinctive subset of stem cell-related genes, an incomplete repression of lineage-specifying transcription factors, and DNA hypermethylation at pluripotency-related loci [69]. Thus, DNA demethylation might represent an inefficient step in the transition to pluripotency. Down-regulation of lineage-specifying transcription factors can facilitate reprogramming, and treatment with DNMT inhibitors can improve the overall efficiency of the reprogramming process [69]. Activation-induced cytidine deaminase (AID; also referred to as *AICDA*) is also required for promoter active demethylation and the induction of OCT4 and NANOG gene expression to initiate nuclear reprogramming towards pluripotency in human somatic cells [70]. Small molecules that modulate DNA and histone methylation have also been shown useful for facilitating the epigenetic modification and reprogramming of somatic cells to iPSCs [71, 72].

3 Histone Modifications and Histone Variants

In eukaryotic cells, DNA is organized into chromatin, the basic unit of which is the *nucleosome*. In a nucleosome, a DNA segment of approximately 147 bp is wrapped around a histone octamer, which is itself composed of two copies each of four histones (H2A, H2B, H3, and H4) [73]. The histones in nucleosomes are subjected to many types of modification, including methylation, acetylation, ubiquitination, phosphorylation, and SUMOylation. Many of these histone modifications reside on the amino- and carboxy-terminal histone tails, including the methylation of Lys4, Lys9, and Lys27 in histone H3 (H3K4, H3K9, and H3K27), the acetylation of H3K9 and H3K14, the acetylation of H4K5, H4K8, H4K13 and H4K16, and the ubiquitination of H2BK123 (in yeast), and H2BK120 (in mammals) [19]. Histone modifications can be classified broadly into two types – repressing and activating. H3K4me3 and histone acetylation are frequently associated with active transcription, while H3K9me3 and H3K27me3 belong to the repressive histone marks. The language of histone modification is not always ''black and white'', for example, an active histone modification H3K4me3 does not always mark actively transcribed genes, and in many cases genes marked with H3K4me3 are neither expressed, nor stably bound, by RNA polymerase II (RNA Pol II) [74]. H3K9me3, a repressive histone modification, is found at the coding regions of active genes [75]. Moreover, at some specific genomic loci, both active and inactive histone modifications are present simultaneously. Such a combination of H3K4me3 (active modification) and H3K9me3 (repressive modification) is detected within open reading frames (ORFs), which indicate a dynamic transcriptional activity [76]. By contrast the so-called ''bivalent domain,'' which harbors both H3K4me3 (active) and H3K27me3 (repressive) modifications, maintains genes at a poised stage ready for transcription [23].

More recently, several studies have been conducted to characterize the

genome-wide profiles of histone modifications in ESCs [23, 75–78]. In general, these profiles have revealed the relationships between various histone modifications and gene expression; for example, H3K4me3 and H3K27me3 can effectively discriminate between genes that are expressed, poised for expression, or stably repressed [77].

These genome-wide profiles of histone modifications have also revealed some novel regulation mechanisms for transcription. Although most promoters in hESCs have nucleosomes marked with H3K4me3 [75, 76, 78], only a small subset of these genes will express full-length transcripts. Genes with H3K4me3, but not producing detectable full-length transcripts, actually experience a transcriptional initiation, as evidenced by the presence of H3K9,14 acetylation, and RNA Pol II at their promoters. Yet, the fact that no elongation marker H3K36me3 is detected at these genes suggests that they are regulated at post-initiation steps. The means by which transcription is suppressed following transcriptional initiation remains elusive; however. it should be noted that this regulation mechanism is not limited to ESCs, as the same phenomenon is also observed in differentiated cells [78].

These genome-wide analyses of histone modifications have revealed a specific modification pattern, consisting of a large region of H3K27me3 harboring a smaller region of H4K4me3 (Fig. 1). As this modification pattern has both repressive and activating histone modifications, it is termed "bivalent domain." In ESCs, genes marked with bivalent domains are normally expressed at low levels, and are enriched in developmental function. Such genes also become either activated or suppressed upon differentiation [23], which leads to the intriguing hypothesis that bivalent domains maintain developmental genes at a status which

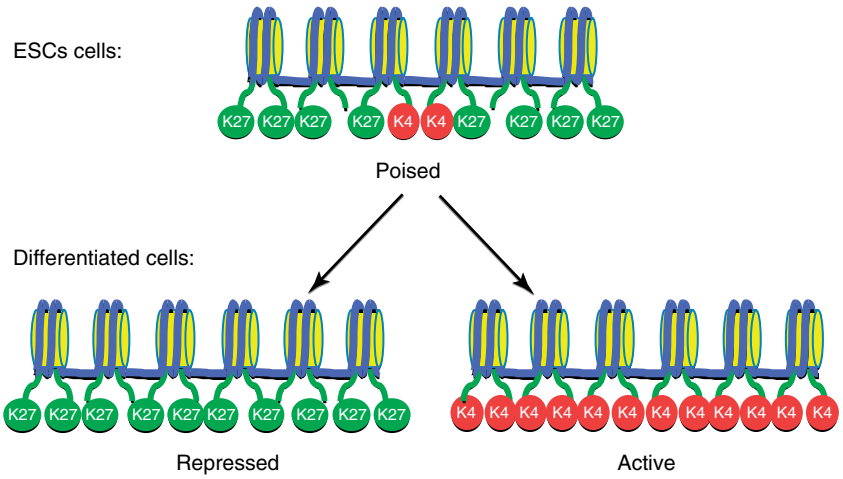

Fig. 1 Bivalent domain facilitates rapid gene activation in ESCs. Bivalent domains are chromatin regions marked with both active H3K4me3 and repressive H3K27me3. Genes associated with bivalent domains are not expressed or expressed at low levels in ESCs. Upon differentiation, bivalent domains become either H3K27me3 or H3K4me3, resulting in gene repression or gene activation, respectively.

is transcriptionally inactive, but capable of being activated; clearly, bivalent domains may play a critical role in the maintenance of pluripotency. Although the inactivation of developmental genes allows ESCs to be self-renewed, maintenance of the "activatability" of these genes is essential to maintain the differentiation potential of ESCs. Again, bivalent domains are not restricted to ESCs. Some pluripotency-associated genes – notably *SOX2*, *OCT4*, and *NANOG* – which are marked with H3K4me3 alone in ESCs, become associated with bivalent domains during differentiation. Moreover, in a human lung fibroblast cell line, IMR90, bivalent domains were also detected at some ES-specific and lineage-specific genes [65]. It appeared that these ES-specific and lineage-specific genes were not ready to be activated in IMR90 cells. Therefore, in addition to the bivalent domain, there might be other mechanism(s) available to suppress these genes in differentiated cells. Alternative, bivalent domains might function in unison with other mechanism(s) to maintain developmental genes poised for transcriptional activation in ESCs.

The importance of histone modifications in the maintenance of pluripotency has been further elucidated by studies of histone-modifying enzymes [42, 45–48, 50, 53, 56–58, 79–81]. The PcG proteins have essential roles in early embryogenesis, thus implying their functions in ESC pluripotency. PcG proteins function in two distinct Polycomb repressive complexes, PRC1 and PRC2, with the PRC2-mediated methylation of H3K27 having been implicated in the maintenance of pluripotency. The core of PRC2 is composed of three PcG proteins, Ezh2, Suz12, and Eed. Mouse ESCs lacking the individual PRC2 core subunit can be established from respective homozygous knockout blastocysts. Although these null ESCs retain a normal self-renewal capacity, they display defects in differentiation [45, 46, 81]. For example, $Eed^{-/-}$ ESCs lack di- and trimethylation on H3K27, show significantly reduced H3K27me1, and also upregulate PcG target genes [42, 43]; consequently, $Eed^{-/-}$ ESCs have a strong propensity to differentiate [42]. Similar phenotypes have been observed in $Suz12^{-/-}$ ESCs [45], whereas the knockout of *Ezh2* results in reductions of H3K27me2 and H3K27me3, but has a negligible effect on H3K27me1. A less-severe differentiation defect is also observed in $Ezh2^{-/-}$ ESCs than in $Eed^{-/-}$ ESCs. The residual HMT activity in $Ezh2^{-/-}$ ESCs is provided by Ezh1, since cells lacking *Ezh2* and depleted of *Ezh1* resemble $Eed^{-/-}$ ESCs [46]. Mapping the genome-wide binding sites of PCR2 has shown that PRC2 occupies many of the genes that encode developmental regulators in ESCs. These genes are associated with H3K27me3-modified nucleosomes, which suggests their transcriptional inactive state, and they are preferentially activated during ESC differentiation [42, 82]. In addition, PRC1 co-occupies many PRC2 target genes, which implies that PRC1 might also be involved in suppressing developmental regulators [42]. Moreover, both $Eed^{-/-}$ and $Ezh2^{-/-}$ ESCs fail to completely silence a set of ES-specific genes following a six-day differentiation; this suggests that PRC2 is also required for the suppression of pluripotency-associated genes during differentiation [46]. Taken together, PRC2 is capable of maintaining ESC pluripotency by suppressing the expression of developmental regulators in ESCs, and also contributes to ESC differentiation by suppressing the expression of pluripotency genes upon differentiation.

In addition to the core of PRC2, a founding member of the Jumonji family, Jarid2, is associated with PRC2 complex. Jarid2 and PRC2 co-occupy the same genomic regions and, indeed, the occupancy of Jarid2 and PRC2 at target genes is mutually dependent [47, 48, 80]. Interestingly, Jarid2 modulates the HMT activity of PRC2, and fine-tunes the H3K27me3 *in vivo*. Similar to null mutations of the core subunits of PRC2, knockout of *Jarid2* does not affect ESC self-renewal, but impairs ESC differentiation [48, 80].

Histone H3K9 also plays a role in ESC pluripotency. In a high-throughput short hairpin RNA (shRNA) screen for novel chromatin regulators that influence the ESC state, a group of H3K9 methyltransferases was identified as essential chromatin regulators for the maintenance of pluripotency. In particular, the loss of SetDB1 (also named ESET), which is an H3K9 HMT, had the most profound effect on the ESC state [79]. A *SetDB1*-null mutation was shown to lead to peri-implantation lethality between 3.5 and 5.5 days post coitus (dpc), and no ESC lines were obtained from the *SetDB1*-null blastocysts [50]. *SetDB1* knockdown was shown to reduce both SetDB1 and Oct4 expression levels, whereas the expression levels of differentiation markers were enhanced [51, 52, 79]. Taken together, these data suggest a role for SetDB1 in the maintenance of ESC pluripotency. The results of recent studies have shown that the knockdown of SetDB1 results in the differentiation of ESCs into a trophectoderm lineage. In this case, SetDB1 and Oct4 interact with each other, and co-occupy the *Cdx2* promoter to inactivate transcription. Hence, SetDB1 is required for the maintenance of ESC pluripotency by suppressing trophectoderm differentiation [51, 52]. Chromatin immunoprecipitation, coupled with massively parallel DNA sequencing (ChIP-Seq), has revealed that SetDB1 binds to both the active and repressed genes. The repressed genes, which were bound by SetDB1, were significantly enriched for developmental regulators, whereas the active genes were enriched for gene expression and metabolism. About one-third of the genes occupied and repressed by SetDB1 were also targets of PRC2. Consequently, the ChIP-Seq result suggests a broader function of SetDB1 in maintaining ESC pluripotency, by suppressing the genes that encode the developmental regulators [79].

H3K9 methylation is regulated by both HMTs and histone demethylases. H3K9 demethylases also play important roles in the maintenance of pluripotency. Two JmjC domain-containing histone demethylases, Jmjd1a and Jmjd2c, are involved in the regulation of ESC self-renewal, such that the depletion of either Jmjd1a or Jmjd2c causes ESC differentiation. Jmjd1a demethylates H3K9me2 at the promoter regions of pluripotency genes, such as *Tcl1*, *Tcfcp2l1*, and *Zfp57*, and activates the expression of these genes. Jmjd2c promote ESC self-renewal by positively regulating a key pluripotency factor Nanog. Jmjd2c removes the H3K9me3 marks at the *Nanog* promoter, and consequently prevents binding of the transcriptional repressors heterochromatin protein 1 (HP1) and KAP1 [53].

The Tip60-p400 HAT and nucleosome remodeling complex is essential for ESC maintenance. The deletion of *Tip60* or *Trrap*, which are two components of the Tip60-p400 complex, results in preimplantation embryonic lethality [57, 58]. The colonies of ESCs depleted Tip60-p400

complex exhibit a flattened and elongated morphology, which is different from that of typical ESC colonies. Moreover, depletion of the Tip60-p400 complex compromises three features of ESCs, namely alkaline phosphatase (AP) activity, EB formation, and teratoma formation. Knockdown of the Tip60-p400 complex also leads to an upregulation of many developmental genes, despite the expression levels of the ESC markers not being significantly affected. Interestingly, the Tip60-P400 knockdown expression profile overlaps with that of Nanog, while the latter promotes Tip60-p400 binding to its target sites. Since Tip60-p400 binding also requires H3K4me3 at the binding sites, it has been suggested that Tip60-p400 regulates gene expression in ESCs through integrating the signals from Nanog and H3K4me3 [56].

In summary, many histone-modifying enzymes are essential to maintain the pluripotency of ESCs, by catalyzing histone modification reactions to repress or activate target gene expression, and to maintain the unique transcriptional profile in ESCs. For example, PRC2 and SetBD1 methylate H3K27 and H3K9, respectively, thereby repressing many developmental genes. In contrast, Jmjd1a and Jmjd2c remove the methylation from H3K9, and positively regulate pluripotency-associated genes, such as Nanog. The ablation of these enzymatic activities leads to changes in the epigenetic profile, in association with a compromised ESC pluripotency (Table 1).

It is not only the canonical histones (H2A, H2B, H3, and H4) but also noncanonical histone variants that contribute to the formation of nucleosomes. The histone variants, which add another layer of complexity to the regulation of nucleosome dynamics and chromatin structure, may be classified as two types: universal and lineage-specific variants. The universal variants, such as centromeric histone variant H3 (CenH3), H3.3, H2A.Z, and H2A.X, are found in almost all eukaryotes, whereas lineage-specific variants, with their unique biological functions, are only found in certain organisms. For example, in animal sperm the DNA is tightly packaged with histone variants, protamines, and protamine-like proteins. Another example is the mammal-specific H2A Barr body-deficient (H2A.Bbd) which lacks a complete docking domain at the C terminus. Typically, H2A.Bbd appears to contribute to active chromatin, being absent on inactive X chromosomes in fibroblasts and coinciding with acetylated H4. Moreover, H2A.Bbd-GFP (green fluorescent protein) undergoes a quicker exchange in the nucleosome than does H2A-GFP [83].

These noncanonical histone variants are involved in a wide range of biological processes, including DNA repair, meiotic recombination, chromosome segregation, transcription initiation and termination, sex chromosome condensation, and sperm chromatin packaging. The histone variants also contribute to the maintenance of pluripotency in ESCs. For example, H2AZ has been shown recently to be essential for ESC differentiation, since H2AZ-depleted ESCs could not support normal development *in vivo* by tetraploid complementation and chimeric analysis. Upon the withdrawal of leukemia inhibitory factor (LIF) under non-adherent conditions, the H2AZ-depleted ESCs were seen to differentiate into EBs. However, the H2AZ-depleted EBs proved to be more disorganized than egg cylinder-stage embryos, and failed to form typical structures representing differentiated cell types. The differentiation deficiency of

the H2AZ-depleted ESCs was similar to that of *Suz12* (a component of the PRC2 core)-null ESCs. Consistent with these observations, genome-wide binding profile analysis revealed that H2AZ would mainly occupy the promoter regions, while H2AZ and Suz12 shared a highly similar set of genes in ESCs. Most importantly, the occupancy of H2AZ and Suz12 at promoters was shown to be mutually dependent in ESCs [84]. Taken together, H2AZ might cooperate with PRC2 to regulate the expression of key developmental regulators in ESCs, and also during ES differentiation.

4 Higher-Order Structure of Chromatin

Nucleosome organization is the first-order structure of chromatin, resulting in a ''beads-on-a-string'' fiber structure on the basis of which chromatin is further folded to form higher-order structures. This leads to the formation of two distinct types of chromatin, namely euchromatin and heterochromatin. Although the way in which chromatin is further packed is a controversial topic, the higher-order organization of chromatin represents an important mechanism with regards to gene regulation.

The chromatin in ESCs is maintained in a unique state compared to other differentiated cells:

1) The staining of HP1α, H3K9me3, or DNA reflects a large, poorly defined heterochromatin region in undifferentiated ESCs. In neural progenitor cells (NPCs) which have been differentiated from ESCs, the heterochromatin is organized into small, discrete foci with well-defined borders. And the number of heterochromatin foci per nucleus increasing as the cells differentiate.
2) By using the technique of fluorescence recovery after photobleaching (FRAP), the exchange dynamics of architectural chromatin proteins, including HP1, H2B, H3, and the linker histone H1, have been shown to be faster in undifferentiated ESCs than in NPCs. These rapid exchange dynamics of architectural chromatin proteins in ESCs might be due to an increased loosely bound or soluble pool of these molecules. Consistently, biochemical studies have also shown that both endogenous H1 and HP1 are released more easily from ESC chromatin than from NPC chromatin.
3) By regulating the hyperdynamic plasticity of chromatin proteins it is possible to affect the differentiation of the ESCs. The deletion of HirA (a nucleosome assembly factor) leads to a dramatic increase in the rapid exchange of the unbound and loosely bound fractions of both H3 and H3.3. As a result of these enhanced exchange dynamics, $HirA^{-/-}$ ESCs show an accelerated differentiation. Conversely, when the exchange dynamics is reduced by the expression of H1cc (an H1 mutant with an increased binding affinity to chromatin), the ESCs do not differentiate normally into neuroblasts [85].

Taken together, these data suggest that ESCs maintain their chromatin in an open state, and the hyperdynamic binding of chromatin proteins promotes an early differentiation of ESCs.

Chd1, a chromatin-remodeling enzyme, has been shown to be an essential regulator of open chromatin in ESCs. Chd1 extensively colocalizes with Pol II and

H3K4me3, which suggests that Chd1 associates globally with euchromatin in ESCs. When Chd1 is knocked down in ESCs, the number of heterochromatin foci is increased, even in the undifferentiated ESCs expressing Oct4. Moreover, the depletion of Chd1 compromises the rapid exchange of H1 in heterochromatin, indicating that the chromatin is condensed. As a consequence, *Chd1* RNA interference (RNAi) results in a decreased expansion of ESCs. The differentiation potential of ESCs is also compromised by *Chd1* RNAi. The loss of primitive endoderm and cardiac mesoderm differentiation, as well as an enhanced neural differentiation, is observed in EBs from *Chd1* RNAi ESCs [86]. These data suggest that Chd1 is required for the maintenance of open chromatin in ESCs, while open chromatin is essential to maintain ESC pluripotency.

Another factor involved in the regulation of open chromatin is the SWI/SNF chromatin-remodeling complex (also known as the *Brg*/brahma-associated factors; BAFs). The BAF complexes utilize energy derived from ATP hydrolysis to alter the DNA–nucleosome contact and to modulate chromatin structure; thus, they have a critical role in gene regulation. The BAF complexes consist of 11 core subunits, several of which are encoded by gene families. The combinatorial assembly of alternative family members diversifies the BAF complexes with different functional specificities. The ESCs have been shown to possess a distinct subunit composition of BAF complexes which differs from those in fibroblasts, brain, and some mammalian cell lines [87, 88]. The distinctive BAF complexes in ESCs (esBAF) are defined by the presence of Brg, BAF155, and BAF60A, and the absence of Brm, BAF170, and BAF60C [88]. The esBAF complexes are critical for pluripotency. Null mutations of *Brg*, *BAF155*, and *BAF47* all cause peri-implantation death. Neither the ICM nor trophectoderm of these mutant blastocysts can give rise to outgrowth *in vitro* [89–91]. The inactivation or downregulation of subunits in the BAF complexes, such as BAF250, Brg, BAF47, BAF155, and BAF57, compromises ESC self-renewal and differentiation [56, 88, 92–94]. In undifferentiated ESCs, the esBAF complexes colocalize extensively with the key pluripotency factors Oct4, Nanog, and Sox2. In addition, the esBAF complexes occupy a large number of Smad1 and Stat3 target genes. Both, Smad1 and Stat3 are transcription factors downstream of the bone morphogenetic protein (BMP) and LIF signaling pathways, respectively. Thus, esBAF complexes participate in ESC maintenance by cooperating with not only key pluripotency factors but also with transcription factors involved in the signaling pathways [95]. During differentiation, esBAF complexes are required for the repression of Nanog and other self-renewal genes. Most importantly, BAF155 is necessary for heterochromatin formation during the retinoic acid-induced differentiation of ESCs [92].

The important role of open chromatin in pluripotency is further elucidated by the derivation of iPSCs. The latter are established from differentiated cells by the ectopic expression of certain transcription factors, such as Oct4, Sox2, Klf4, and cMyc. The knockdown of *Chd1* compromises the efficiency of iPSC derivation [86], and two components of esBAF complex, Brg1, and BAF155, synergistically promote the reprogramming efficiency [96].

5
X-Chromosome Inactivation

In each mammalian female cell, one of the two X chromosomes is transcriptionally inactivated to compensate for the X-linked gene dosage effect between male (XY) and female (XX). This phenomenon, which is referred to as X-chromosome inactivation (XCI) [97], is a critical epigenetic event in the establishment of pluripotency and in differentiation. Two types of XCI have been identified during embryogenesis, namely imprinted XCI and random XCI (Fig. 2). Imprinted XCI initiates at the two-cell stage, and preferentially silences the paternal X chromosome [98, 99]. Subsequently, at the morula stage, the paternal X chromosomes in all blastomeres are inactivated. As the embryos further develop into blastocysts, however, the inactivated paternal X chromosome is reactivated in the epiblast, while imprinted XCI is maintained in the trophectoderm and the primitive endoderm [98, 100]. Following implantation, the epiblast cells undergo another round of XCI, in which one of the two X chromosomes is randomly silenced, regardless of their parental origin [101]. Hence, this round of XCI is known as *random XCI*.

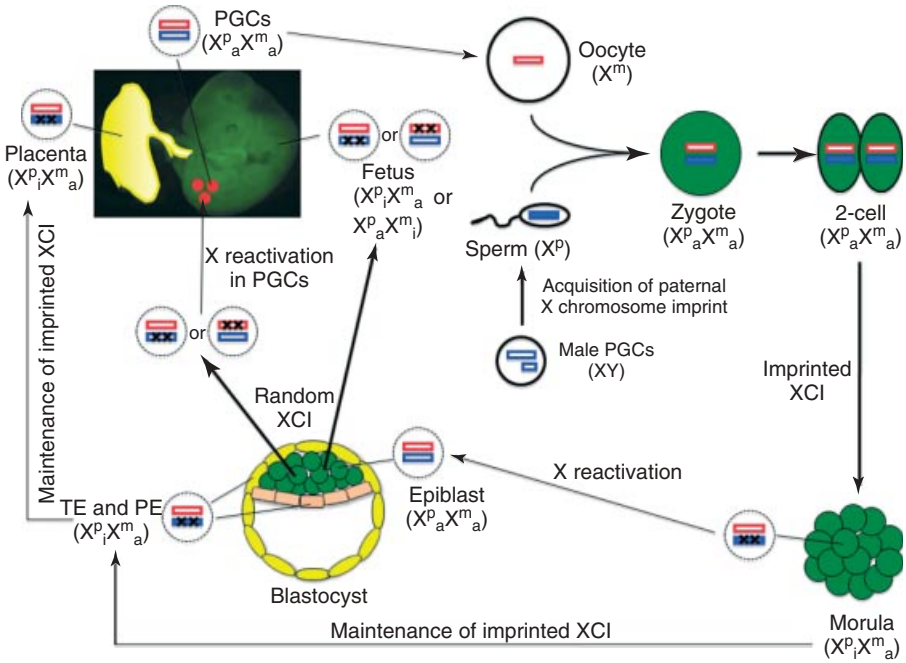

Fig. 2 X-chromosome inactivation (XCI) and reactivation cycle in mouse development. Several key events in XCI and inactivation, including imprinted XCI from two-cell to morula stage, X-reactivation in epiblast, and random XCI as epiblast further develops, are illustrated in the diagram. The paternal and maternal X chromosomes are shown in blue and red rectangles, respectively. The blue shading on the paternal X chromosomes symbolizes paternal imprints. Rectangles marked with two black ''X'' are inactive X chromosomes. TE, trophectoderm; PE, primitive endoderm; PGCs, primordial germ cells; X^p, paternal X; X^m, maternal X; X_i, inactive X; X_a, active X.

Two large noncoding RNAs – Xist and Tsix – play pivotal roles in XCI [102–104]. The genes encoding Xist and Tsix are located at the same genomic locus, and are transcribed in opposite directions. Xist and Tsix antagonize the expression of each other [103], with Xist expression initiating silencing of the X chromosome from which Xist is transcribed. XCI involves multiple steps of various chromosome-wide epigenetic modifications, including H3K4 hypomethylation, H3K9 hypoacetylation, Eed/Enx1 accumulation, H3K27 methylation, macroH2A association, and H3K9 methylation [98].

In female mouse ESCs, the two X chromosomes are both active, and resemble the epiblast stage after X chromosome reactivation and before random XCI. During differentiation, the mouse ESCs undergo random XCI; however, in female hESC lines the transcriptional status of the X chromosomes may be variable, with some cells having two active X chromosomes and others one active and one inactive X chromosome [105, 106]. The various degrees of XCI in hESCs might be established during the process of derivation and/or propagation. hESCs depend on activin (INHBA)/nodal (NODAL) and fibroblast growth factor (FGF), whereas mouse ESCs rely on LIF and BMP. When cultured in the hESC medium, epiblast cells in the post-implantation embryo give rise to mouse epiblast stem cells (EpiSCs), which resemble hESCs [107, 108]. Taken together, these data suggest that hESCs are likely derivatives of post-XCI epiblast cells, and are different from ICM-derived mouse ESCs. The post-XCI epiblast origin might cause the various statuses of XCI in hESCs. Moreover, hESCs with two active X chromosomes could be established under physiological oxygen concentrations. Chronic exposure to atmospheric oxygen induces irreversible XCI in these hESC lines, while only minor changes in the transcriptome are detected [109]. Hence, the long-term culture of hESCs in 20% oxygen might also contribute to the various statuses of XCI in these cells.

6 Regulation of ESC Pluripotency and Differentiation by miRNAs

MicroRNAs (miRNAs) are short single-stranded RNAs (18–25 nucleotides), which do not translate into protein but rather regulate gene expression by interacting with specific mRNAs, which results in mRNA degradation, deadenylation, or translational inhibition [65, 110]. The miRNAs play essential roles in regulating ESC self-renewal, pluripotency and differentiation, and also in the regulation of early mammalian development [28, 111–113].

Oct4, Nanog, Klf4, and Sox2 are core transcription factors required for the maintenance of ESC identity and pluripotency. These transcription factors regulate the epigenetic network to support ESC pluripotency by affecting chromatin structure, DNA methylation, miRNA, and XCI [13, 114–116]. The miRNA cluster miR302-367 is differentially expressed in ESCs, with the ESC-specific expression of the cluster being fully conferred by its core promoter transcriptional activity and the cluster activity decaying upon differentiation of the ESCs [117]. Both, Oct4 and Sox2 have been shown to bind to a conserved promoter region of miR-302. The miR-302a may target many cell cycle regulators, and repress the productive translation of cyclin D1, an important G_1 regulator in hESCs [118]. The miRNA clusters miR-290 to miR-295 in chromosome 7 are

specific to ESCs [112]. miR-294, miR-295, and miR-292-3p are enriched in undifferentiated ESCs, and reduced following ESC differentiation [119]. In contrast, miR-134, miR-296, and miR-470 are upregulated upon the retinoic acid-induced differentiation of mouse ESCs and target the coding sequence of mRNA of Nanog, Oct4, and Sox2 [120]. This further supports the idea that miRNAs play an important role in ESC pluripotency and differentiation. The miR-290 cluster may fine-tune ESC maintenance and differentiation by regulating *de novo* DNA methylation via Rbl2, which in turn inhibits DNMT3a and DNMT3b expression [27, 28]. Notably, the core pluripotency factors Oct4, Sox2, Nanog, and Tcf3 bind to miRNA genes, and most likely regulate the expression of these miRNAs in ESCs [115]. Similarly, OCT4, SOX2, and NANOG are associated with the miRNA genes, miR-137 and miR-301, in hESCs [11, 82].

The miRNAs are also involved in the differentiation of ESCs, and may function by inhibiting tissue-specific gene expression. The serum response factor (SRF)-dependent muscle-specific microRNAs, miR-1 and miR-133, promote mesoderm formation from ESCs but have opposing functions during further differentiation into cardiac muscle progenitors [121]. The miRNAs may induce differentiation via the downregulation of pluripotency-associated genes. For example, miR-134 promotes ESC differentiation into the ectodermal lineage by the post-transcriptional attenuation of Nanog and LRH1 [122], while miR-145 represses the core pluripotency factors OCT4, SOX2, and KLF4, and facilitates ESC differentiation [123].

The requirement for miRNAs in the maintenance of ESC pluripotency and differentiation capacity was initially demonstrated in genetic manipulation studies. Dicer is an RNase III-family nuclease required for miRNA generation and RNAi, and a deficiency in this enzyme reduces ESC proliferation and differentiation [124, 125]. The loss of DGCR8, an RNA-binding protein that is essential for the biogenesis of miRNAs, leads to the absence of mature miRNAs and defective ESC differentiation. This suggests that miRNAs function in the silencing of ESC self-renewal that normally occurs with the induction of differentiation [126]. Many miRNA primary transcripts, including members of the Let-7 family, are present at high levels but are not processed by Drosha in ESCs [127]. Inhibition of the let-7 family promotes the de-differentiation of somatic cells to iPSCs [128]. As ESCs differentiate, the primary miRNA transcripts are processed to create mature miRNAs which then facilitate differentiation. Lin28, as a negative regulator of miRNA biogenesis, has been found to block miRNA-mediated differentiation in stem cells [129], and to enhance the reprogramming of somatic cells into iPSCs [130]. Furthermore, ESC-specific miRNAs promote the induction of an homogeneous population of iPSC colonies [131].

The comparison of genetically identical mouse ESCs and iPSCs shows that their overall messenger RNA and miRNA expression patterns are indistinguishable, with the exception of a few transcripts encoded within the imprinted Dlk1-Dio3 gene cluster on chromosome 12qF1, which are aberrantly silenced in most of the iPSC clones. The normal expression of the Dlk1-Dio3 cluster contributes to the full development potential (or true developmental pluripotency) of iPSCs, as

evidenced by the generation of entirely iPSC derived animals (''all-iPSC mice'') [132]. A mammalian conserved cluster of miRNAs encoded by this region exhibits significant expression differences between full- and partial-pluripotent stem cells. The degree of activation of the Dlk1-Dio3 region correlates positively with the pluripotency level of the stem cells [133]. Several miRNAs from this cluster potentially target the PRC2 silencing complex, and may form a feed-forward regulatory loop resulting in the expression of all genes and noncoding RNAs encoded by this region in full-pluripotent stem cells [133]. Interestingly, specific large noncoding RNAs also are transcriptionally regulated by key transcription factors such as Sox2, Oct4, and Nanog, and p53, thereby demonstrating a diverse range of roles for lncRNAs in processes from ESC pluripotency to cell proliferation [134].

7 Telomere Function and Genomic Stability in ESCs

Telomeres consist of repeated DNA sequences $(TTAGGG)_n$ and an associated protein known as ''*Shelterin*'' that cap the end of chromosomes to maintain genomic stability [135, 136]. The telomere length usually is maintained by telomerase, which in turn is composed of a telomerase RNA component (Terc) and Tert, a reverse transcriptase that adds telomeric repeats *de novo* during each cell division. The telomeres also are elongated by an alternative lengthening of telomeres (ALT) mechanism in some circumstances, which relies on homologous recombination between telomeric sequences [137, 138]. ESCs acquire a high telomerase activity that helps to maintain the telomere length [3]. Telomerase mTert-deficient ESCs exhibit genomic instability, aneuploidy, and telomeric fusions [139]. In addition, Terc-deficient ESCs lack any detectable telomerase activity, and their growth rate is reduced after more than 300 divisions, becoming almost zero after 450 cell divisions. Following this growth crisis, however, survivor cells with a rapid growth rate emerge, and the survivors are able to maintain functional telomeres in a telomerase-independent fashion [140]. ESCs also may use an ALT mechanism to lengthen the telomeres. Zscan4, which is highly expressed in two-cell embryos [141], was recently shown to regulate telomere elongation by recombination in ESCs [142].

Epigenetic modification by histone and DNA methylation also regulates telomere length and integrity [143, 144]. Mammalian telomeres have heterochromatic features, including trimethylated histone H3 at Lys9 (H3K9me3) and trimethylated histone H4 at Lys20 (H4K20me3). In addition, subtelomeric DNA is hypermethylated. H4K20me3 at telomeres can be catalyzed by Suv4-20h1 and Suv4-20h2 HMTs [143, 145]. The abrogation of master epigenetic regulators, such as HMTs and DNMTs, correlates with a loss of telomere-length control. On the other hand, telomere repeats are important in the establishment of constitutive heterochromatin at mammalian telomeres and subtelomeres, while histone modifications are important in counting telomere repeats [146]. Telomere shortening to a critical length affects the epigenetic status of the telomeres and subtelomeres. Suv39h1 and Suv39h2 govern the methylation of histone H3 Lys9 (H3-Lys9) in heterochromatic regions, while cells that lack the Suv39h1 and Suv39h2 HMTs

show decreased levels of H3K9 trimethylation at telomeres, and are associated with an aberrant telomere elongation [147]. Suv4-20h HMTs are responsible for histone modification at telomeres, and play a role in telomere length control. Cells deficient in Suv4-20h2, or in both Suv4-20h1 and Suv4-20h2, show decreased levels of H4K20me3 at telomeres and subtelomeres in the absence of any changes in H3K9me3, accompanied by telomere elongation. A deficiency in Suv4-20h or Suv39h HMTs increases telomere recombination in the absence of any changes in subtelomeric DNA methylation, which suggests an important role for chromatin architecture and histone lysine methylation in the maintenance of telomere length homeostasis and telomere recombination [145].

DNA methylation may serve as a secondary mechanism to reinforce the telomere position effect (TPE) and repress homologous recombination at telomeres in maintaining telomere integrity [143]. Mouse ESCs deficient in DNMT1, or both DNMT3a and DNMT3b, have dramatically elongated telomeres. Decreases in DNA methylation, both globally and specifically at subtelomeric regions, lead to an increased telomeric recombination and telomere elongation by ALT, even when there is no loss of heterochromatic histone-methylation marks [144]. Moreover, the miRNA cluster miR-290 directly regulates Rbl2-dependent DNMT expression, indirectly affecting telomere-length homeostasis. Reduced miR-290, in the absence of Dicer, leads to increased levels of Rbl2, a transcriptional repressor of DNMT3a,3b. Decreased DNMT expression leads to a hypomethylation of the genome, including the subtelomeric regions, and an increased telomere recombination and aberrantly long telomeres [28]. The miR-290 cluster-dependent regulation of DNA methylation may also have an important impact on the regulation of telomere recombination and telomere length during early embryonic development [148]. Taken together, a knockout deletion of the histone methyltransferase and DNMTs leads to an aberrantly increased telomere length and chromosomal instability. Thus, the repressive histone and DNA methylation are critical for telomere length maintenance and structural integrity.

The somewhat common histone H3.3 is preferentially integrated into transcription sites, and is associated with active and open chromatin [149]. H3.3 regulates telomere chromatin integrity in ESCs, and undergoes dynamic differentiation-dependent remodeling during the process of differentiation [150]. ATRX (alpha thalassemia/mental retardation syndrome, X-linked) is a member of the SWI2/SNF2 family of chromatin-remodeling proteins. Mutations in the *ATRX* gene are associated with X-linked mental retardation (XLMR), often accompanied by alpha thalassemia syndrome. Interestingly, ATRX localizes at the telomeres in interphase mouse ESCs in synchrony with the incorporation of H3.3 during telomere replication at S-phase. ATRX also is associated with the DNMTs 3 to 3L (ADD) domain [151]. The chromobox homolog 5 (CBX5; also known as heterochromatin protein 1α; HP1α) is present at the telomeres in ESCs. It appears that ATRX, when operating in conjunction with H3.3 and CBX5, has a novel function as a key regulator of ESC telomere chromatin [152]. Indeed, ATRX is required for the Hira-independent localization of H3.3 at telomeres, and also for the repression of telomeric RNA [153]. A loss of ATRX in ESCs leads to reduced

cell growth and to a higher rate of spontaneous differentiation. This suggests that ATRX plays a role in controlling ESC proliferation and differentiation [152, 154].

Telomeres are also important for the maintenance of genomic stability in iPSCs, to ensure the long-term survival and function of iPSC derived cells following transplantation therapy. Telomeres are elongated during iPSC formation, and acquire the length and epigenetic marks of ESCs [155, 156]. Immortal cells, such as cancer, male germline and ESCs, can maintain their telomere reserves for prolonged periods through the action of telomerase, the activation of which via an increased expression of Terc and/or Tert most likely plays a critical role in telomere reprogramming and maintenance. The activation of telomerase-independent telomere elongation mechanisms might also occur in some Terc-deficient iPSC clones. TERRA (telomere-repeat-encoding RNA) is transcribed from telomeres, and it has been proposed that TERRA can negatively regulate telomerase activity. TERRA levels are efficiently increased in iPSCs, and an increased expression of TERRA in iPSCs may serve as a counting mechanism of telomere length that would inhibit telomerase activity once the iPSCs had reached the ESC telomere length. As the number of cell divisions drives epigenetic reprogramming to pluripotency [130, 157], sufficient cell divisions also are required for telomere elongation by telomerase during iPSC induction [155, 156]. Likewise, adult somatic cells also can be reprogrammed and elongated following somatic cell nuclear transfer or fusion with ESCs.

Typically, telomeres shorten primarily as a consequence of gradual end replication losses with ongoing cell division. The telomere length of hESCs is heterogeneous, with telomeres ranging from 3.0 kb to over 25 kb [158]. Although some hESCs display karyotypic changes following prolonged periods in culture, the predominant aberrations are aneuploidy, specifically gains of chromosomes 17, 12, and X, with less evidence of nonreciprocal translocations that occur as a consequence of telomere dysfunction. Telomere shortening is implicated in cellular and organism aging. Telomere lengthening and reprogramming are important for iPSC generation and functionality, while the iPS technology possibly provides rejuvenation and a reversal of developmental aging.

8 Imprinting and ESC Stability

Imprints are established during gametogenesis, and play important roles in fetal growth and development [159]. Imprinted genes represent a small subset of mammalian genes that are monoallelically expressed in a parent-of-origin manner (either the paternal or maternal allele). Any aberrant allele-specific expression of imprinted genes will disrupt fetal development, and is associated with genetically related diseases, some cancers, and a number of neurological disorders [20]. The establishment of genomic imprinting is controlled by DNA methylation, histone modifications, noncoding RNAs, and specialized chromatin structures. Allele-specific DNA methylation is thought to be a major factor regulating genomic imprinting. Specific DNA methylation in the differentially methylated regions (DMRs) of parental origin allows a discrimination to be made between paternal and maternal alleles, and leads to the monoallelic expression of imprinted genes [20, 160]. HMTs Suv4-20h also regulates H4K20me3 at ICRs [161].

Culture of ESCs affects their pluripotency, and may give rise to fetal abnormalities [62]. Altered allelic methylation patterns have been detected in two maternally expressed genes (*Igf2r*, *H19*) and two paternally expressed genes (*Igf2*, *U2af1-rs1*), and these are consistently associated with allelic changes in gene expression. All of the methylation changes that have arisen in the ESCs persist on *in vivo* differentiation to fetal stages [62]. Alterations include a loss of methylation with bi-allelic expression of *U2af1-rs1*, a maternal methylation and predominantly maternal expression of *Igf2*, and a bi-allelic methylation and expression of *Igf2r*. hESCs also demonstrate gene-specific differences in the stability of imprinted loci, related to disrupted DNA methylation, warranting comprehensive imprinting analysis in the continued characterization of hESC lines [162]. Moreover, DNA methylation is globally reduced in XX ESC lines, in association with reduced levels of DNMT3a and DNMT3b, while selection against the loss of methylation may provide the basis for X-chromosome instability [64].

Parthenogenetic embryonic stem cells (pESCs; XX) can be derived from parthenogenetic embryos which are unable to develop to term because they lack the paternal expression of imprinted genes and cannot develop a functional placenta to support fetal development [163, 164]. In studies of mouse pESCs conducted over more than two decades, an extensive differentiation potential has been demonstrated both *in vitro* and *in vivo*, although the true pluripotency of these cells was questioned previously, notably when considering a low chimera production and deficiency in germline competence, which is a common standard used to test the genetic integrity and pluripotency of ESCs in rodents. As the mechanisms of oocyte activation by sperm during fertilization have become better understood, artificial methods for the activation of oocytes have been improved to mimic sperm-induced oocyte activation, such that parthenogenetic embryos develop in similar fashion to fertilized embryos during the preimplantation stages [165–169]. With improved methods for oocyte activation, pESC lines of a higher quality have been isolated from mice. Notably, a dramatic epigenetic reprogramming was found to occur during the isolation and culture *in vitro* of pESCs from their progenitor embryos, and this led to an improved pluripotency of pESCs [170–172]. Whereas, parthenogenetic embryos and fetuses fail to express paternally expressed imprinted genes, pESCs express those genes in a pattern which resembles that of ESCs derived from fertilized embryos. An increased expression of *U2af1-rs1* and *Snrpn*, and a decreased expression of *Igf2r*, correlate with the pluripotency of pESCs [171]. Moreover, mouse parthenogenetic pups can be produced directly from pESCs by tetraploid embryo complementation, which contributes to placenta development [173]. The full-term developmental potential of pESCs suggests that they can differentiate into all cell types and functioning organs in the body. In this regard, human pESCs may serve as an additional source of histocompatible tissues for cell transplantation therapy [174–177].

9 Epigenetic Interconversion among Mouse ESCs, EpiSCs, and Human ESCs

Mouse ESCs are obtained from the ICMs of blastocysts prior to implantation in the uterus. The EpiSCs (which

are post-implantation epiblast-derived stem cells) can be derived from the epiblast, a tissue of the post-implantation embryo that generates the embryo proper [107, 108]. The EpiSCs express transcription factors that are known to regulate pluripotency, maintain their genomic integrity, and robustly differentiate into the major somatic cell types as well as primordial germ cells, but exhibit only limited pluripotency *in vivo*, as evidenced by a restricted ability to contribute to chimeric mice. EpiSCs are distinct from mouse ESCs in terms of their epigenetic state and the signals that control their differentiation. Rather, EpiSCs resemble hESCs more closely than mouse ESCs in patterns of gene expression and signaling responses. bFGF/Activin/Nodal signaling controls the expression of the key pluripotency factor Nanog in hESCs and in mouse EpiSCs [3, 157, 178]. Because FGF and activin – the factors used to promote hESC self-renewal – also promote trophoblast stem cell self-renewal, any tendency towards trophoblast differentiation in hESCs or mouse EpiSCs will be accentuated by an expansion of these cells [179]. hESCs differ from mouse ESCs not only morphologically, but also epigenetically in XCI, and the occupancy of pluripotent factors [11, 105, 108]. Both, human and rhesus macaque ESCs resemble the EpiSCs in pluripotent state rather than ICM-derived ESCs [179]. These similarities between hESCs and mouse EpiSCs have led to the suggestion that hESCs are actually equivalent to the early post-implantation epiblast, rather than to its ICM progenitor. Mouse ESCs, EpiSCs, and hESCs represent two different pluripotent states – the naïve (ICM-like or ESC-like) and primed (epiblast-like or EpiSC-like) – that can be converted from one to another under appropriate conditions [157, 180–182] (Fig. 3).

Constitutive expression of Klf4 or c-Myc in EpiSCs can regenerate the naïve ground state of ESCs and EpiSC-derived induced pluripotent stem cells (Epi-iPSCs) produce high-contribution chimeras with germline transmission [157, 183]. Moreover, EpiSCs have an infinite capacity for generating PGCs, under conditions that sustain their pluripotency and self-renewal [184]. These PGCs, when generated *in vitro*, demonstrate appropriate transcriptional and epigenetic reprogramming events, and can be induced to undergo dedifferentiation into pluripotent embryonic germ cells (EGCs), which resemble ESCs, and not the EpiSCs from which they are derived. Intrinsic reprogramming during the specification of PGCs results in an erasure of the epigenetic memory of EpiSCs following reactivation of the X chromosome, DNA demethylation, and the re-expression of key pluripotency genes [184]. Advanced epiblast cells from embryonic day 5.5–7.5 mouse embryos with a uniform expression of N-cadherin and inactive X chromosome, also can be reprogrammed to ESC-like cells (rESCs) in response to LIF–STAT3 signaling following extended culture [185]. EpiSCs and ESCs also can be interchangeable by the DNA methylation of Stella [186].

Although human and mouse ESCs are derived from blastocyst-stage embryos, they have very different biological properties. The pluripotent state of hESCs corresponds to that of mouse-derived EpiSCs. Recently, it was shown possible to convert the identity of conventional hESCs into a more immature state that shares many defining features with pluripotent mouse ESCs, by the ectopic induction of Oct4, Klf4, and Klf2 factors, combined with LIF and inhibitors of glycogen synthase kinase

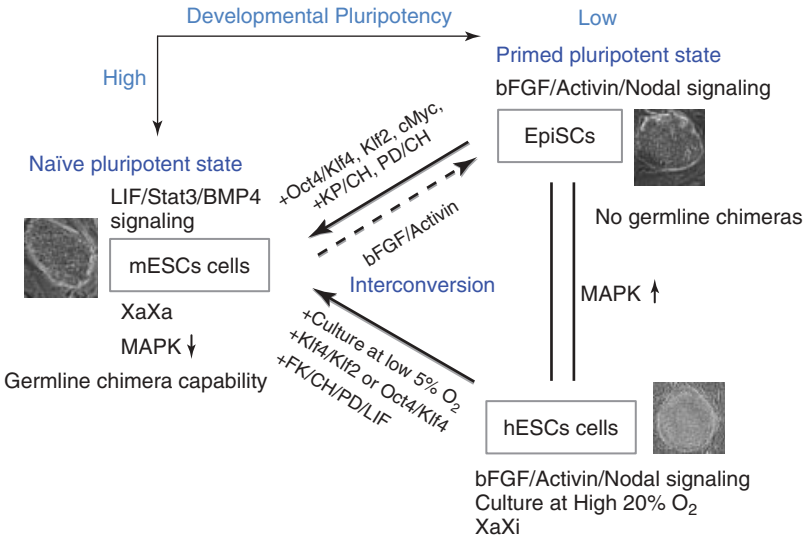

Fig. 3 Two different states of developmental pluripotency can be interconverted by defined signals. Conventional human ESCs (hES cells) resemble mouse-derived epiblast stem cells (EpiSCs), and can be converted to pluripotent states, like mouse ESCs (mES cells) by culture under physiological low-oxygen level, by ectopic induction of Oct4, Klf4, and Klf2 factors combined with LIF and inhibitors of glycogen synthase kinase 3β (GSK3β) and the mitogen-activated protein kinase (ERK1/2) pathway, or by small molecules Forskolin (FK) or Kenpaullone (KP) that can induce Klf4 and Klf2 expression. PD, ERK1/2 inhibitor PD0325901; CH, GSK3β inhibitor CHIR99021.

3β (GSK3β) and the mitogen-activated protein kinase (ERK1/2) pathway [180]. In contrast to conventional hESCs, these epigenetically converted hESCs demonstrate growth properties, a X-chromosome activation state (XaXa), a gene expression profile, and a signaling pathway dependence that are all very similar to those of mouse ESCs.

The presence of two active X chromosomes (XaXa) is a hallmark of the ground state of pluripotency specific to murine ESCs. Transcription factors for pluripotency cooperate to repress Xist and couple X inactivation reprogramming to the control of pluripotency during embryogenesis [116]. Conventional hESCs invariably exhibit signs of XCI, and are considered developmentally more advanced than their murine counterparts [105, 106, 187]. Interestingly, the derivation and culture of hESCs in physiological O_2 ($\sim 5\%, pO_2, 36$ mmHg) prevents precocious XCI, maintains pluripotency, and suppresses the spontaneous differentiation of hESCs, such that XaXa hESCs are acquired under physiological oxygen concentrations. This suggests that a physiological O_2 level would help to maintain hESCs in a more developmentally immature state [109]. Together, EpiSCs and hESCs can be epigenetically reprogrammed to resemble mouse ESCs.

10 Summary

ESCs face a paradoxical situation to maintain their pluripotency. Whilst the

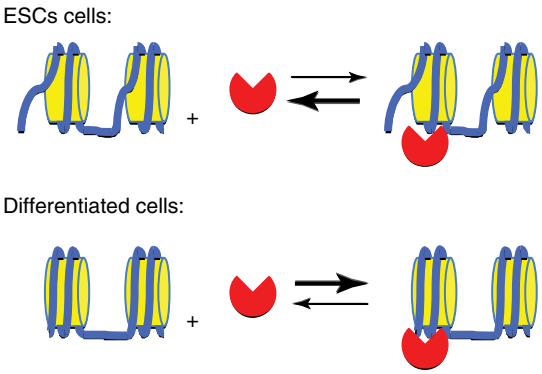

Fig. 4 Open chromatin allows more epigenetic plasticity in ESCs. ESCs have a unique global chromatin conformation; hence, chromatin-associated proteins are exchanged in a faster dynamics in ESCs than in differentiated cells. This figure illustrates a potential mechanism for open chromatin. By changing the histone–DNA interaction, the nucleosomes in ESCs are less compacted than those in differentiated cells. Thus, histones, as well as other chromatin-binding proteins (red), can disassociate more easily from chromatin.

pluripotency genes must be active in undifferentiated ESCs, their expression must be turned off upon differentiation. In contrast, developmental genes remain transcriptionally inactive in ESCs; during differentiation, some of these are activated while others are further silenced. Consequently, a stable epigenetic profile is required for self-renewal, while plasticity in the epigenetic profile is required for a quick adaption into the various transcriptional profiles of differentiated cells. Hence, the epigenetic profile in ESCs should maintain the balance between stability and instability.

Open chromatin is a key factor contributing to the plasticity of the epigenetic profile in ESCs, as it allows a more permissive environment for transcription. Clearly, open chromatin is not only beneficial for the expression of pluripotency genes in ESCs, but it also facilitates the activation of developmental genes during differentiation. In ESCs, suppressors – such as PcG proteins and HP1, as well as suppressive histone modifications – cooperatively inactivate developmental genes. The loose binding of suppressor proteins to chromatin allows the rapid removal of suppressors upon gene activation (Fig. 4). Furthermore, an open chromatin conformation renders DNA and histones more accessible to transcription factors and to histone-modifying enzymes.

Bivalent domain represents another mechanism by which the instability of the epigenetic profile in ESCs can be enhanced. Bivalent domains comprise a large region of repressive mark H3K27me3 and a smaller region of active mark H3K4me3. This coexistence of repressive and active marks maintains genes in a transcriptionally inactive state [23]. As the active marks in bivalent domains might serve as seeds to promote gene activation, it might be easier to activate genes associated with bivalent domains than genes marked only by H3K27me3. Bivalent domains are found not only in ESCs, but also in differentiated cells. For example, in a human lung fibroblast cell line, IMR90, some ES-specific

and lineage-specific genes are marked by bivalent domains [65]. Apparently, the ES-specific and lineage-specific genes associated with these bivalent domains are not ready to be activated; otherwise, it would be not only straightforward but also highly efficient to derive iPSCs or to trans-differentiate them into other lineages from IMR90 cells. Therefore, it can be argued that a bivalent domain in itself is insufficient to keep genes in a transcriptionally poised state, and additional mechanisms should be involved to maintain a poised transcriptional status.

DNA methylation is considered to be a more stable epigenetic modification than histone modifications. If a gene is silenced by DNA methylation, it is relatively difficult to activate its transcription. In order to ensure the activatability of tissue-specific genes, windows of unmethylated CpGs are located in the enhancers of such genes in ESCs (Fig. 5). The maintenance of these unmethylated CpG windows depends on the binding of pioneer transcription factors. Unmethylated CpG windows might serve as a platform to recruit activators and/or coactivators, thus facilitating the rapid activation of genes [188]. Moreover, gene repression in ESCs relies less on DNA methylation than that in differentiated cells. The inactivation of DNMT1 or DNMT3a/DNMT3b leads to a hypomethylation of DNA in ESCs. Both, $DNMT1^{-/-}$ and $DNMT3a^{-/-}DNMT3b^{-/-}$ ESCs are able to self-renew normally, but they have defects in differentiation [32, 36, 40, 189]. Interestingly, the introduction of DNMT3a and DNMT3b back into highly demethylated $DNMT3a^{-/-}DNMT3b^{-/-}$ ESCs restores genomic methylation patterns and, more importantly, rescues the differentiation defect [40]. In contrast, the deletion of DNMT1 in murine fibroblast cells leads to

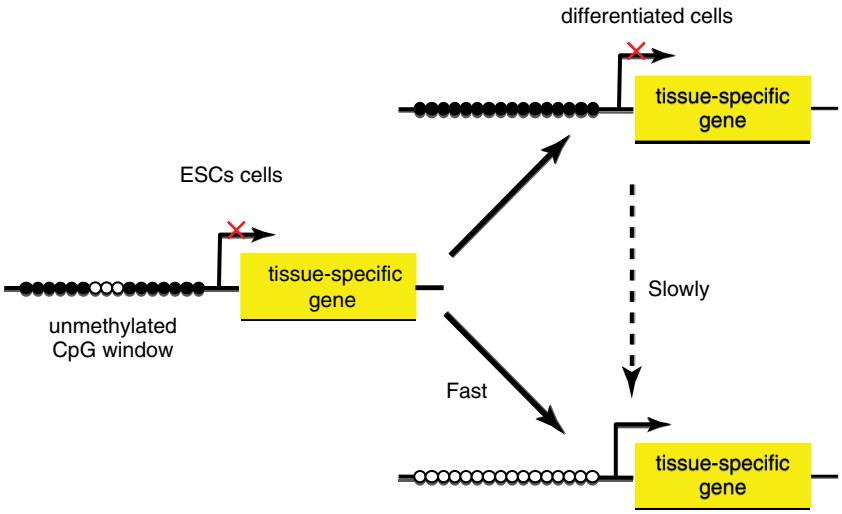

Fig. 5 Unmethylated CpG windows ensure a permissive chromatin environment for gene activation. In ESCs, certain tissue-specific genes are associated with methylated CpG islands, which harbor smaller windows of unmethylated CpG dinucleotides. These genes are not expressed in ESCs; however, during differentiation they become fully methylated and repressed, or fully unmethylated and activated. Unmethylated CpG windows might act as an initiate point for gene activation.

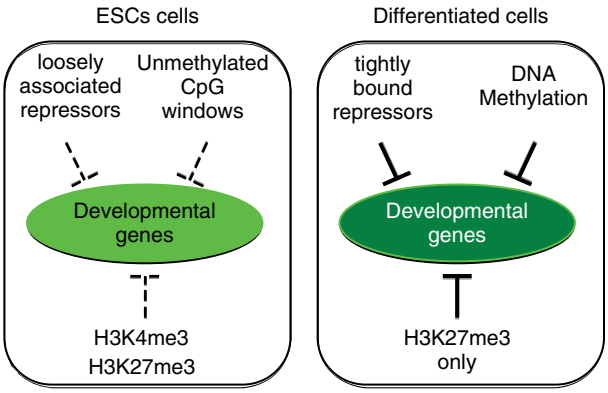

Fig. 6 Multiple mechanisms underlie epigenetic instability in ESCs. Developmental genes are repressed in ESCs, but open chromatin, bivalent domains, unmethylated CpG windows, and dispensable global DNA methylation function together to ensure a permissive chromatin environment for gene activation. In contrast, differentiated cells repressed certain developmental genes with more stable mechanisms, such as compacted chromatin structure, a H3K27me3 only histone mark, and completely methylated CpG islands.

cell death within a few cell divisions [33]. These data suggest that global DNA methylation is dispensable for ESC maintenance; rather, it is critical for differentiated cells. It is likely that DNA methylation is required for the stable silencing of pluripotent genes and some lineage-specific genes in differentiated cells.

Open chromatin, bivalent domains, unmethylated CpG windows, and dispensable global DNA methylation allow a greater plasticity of the epigenetic profile in ESCs (Fig. 6). With these unique epigenetic characteristics, ESCs are able to respond to differentiation signals and adapt rapidly into new transcriptional and epigenetic profiles.

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