Review

Unraveling Epigenetic Regulation in Embryonic Stem Cells

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Embryonic stem (ES) cells can replicate indefinitely while retaining the capacity to differentiate into function-ally distinct cell types. ES cells proliferate and differentiate without detectable genetic changes, indicating that these processes are controlled by epigenetic factors. Here we describe what is known about the epige-netics of ES cells and speculate that a dynamic balance among at least three epigenetic elements (chromatin structure, DNA methylation, and microRNAs), in conjunction with transcription factors, contributes to the maintenance of pluripotence. Understanding the interactions among these factors will be critical to the development of improved strategies to reprogram differentiated cells or direct differentiation of pluripotent cells.

Embryonic stem (ES) cells, derived from the inner cell mass of blastocysts, have until recently been unique among all cultured cell types, possessing the capability to replicate indefinitely while retaining the ability to differentiate into a host of functionally dis-tinct cell types. Mouse ES (mES) cells are so developmentally po-tent that an entire mouse can be generated from these cultured cells; human ES (hES) cells also exhibit pluripotency when differ-entiated in culture or transplanted into animals. Recent reports of the conversion of differentiated mouse and human cells into ES cell-like induced pluripotent stem cells (iPS cells) are dramatic demonstrations that cellular reprogramming is a reality. However, as detailed molecular characterization of iPS cells is still in an early stage, we will focus on ES cells in this review.

Global gene expression analysis has shown that differentiation of ES cells is accompanied by dramatic changes in the numbers and types of transcripts expressed (Brandenberger et al., 2004; Ivanova et al., 2002; Liu et al., 2006; Ramalho-Santos et al., 2002). While individual studies differ in the details, there is gen-eral agreement that undifferentiated ES cell populations are un-usually transcriptionally active; although many of the genes are generally expressed at low levels. This implies that commitment to differentiation involves downregulation of general transcrip-tional activity, which would enhance the impact of the genes whose transcription is not suppressed. The pluripotence of ES cells and their differentiation into multiple cell lineages makes them an attractive model in which to study the effects of epige-netic factors (Azura et al., 2006; Reik, 2007; Spivakov and Fisher, 2007).

The term "epigenetics" was coined by Waddington before the physical nature of genes was known, to explain the means by which complex multicellular organisms are formed by differenti-ation of totipotent cells in the embryo (Waddington, 1942). Over time, the definition narrowed, and several years ago, epigenetics was defined as "the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence" (Wu and Morris, 2001). Some have argued that this definition is too restrictive, and should include changes that occur in postmitotic cells and gene rearrangements (such as those that occur in antibody genes) (Holliday, 2002).

The best-known epigenetic factors affect transcription of genes: DNA methylation, transcription factors, and changes in chromatin structure through modifications of DNA-binding pro-teins such as histones. These mechanisms are presumably de-pendent on the type and activity of enzymes and binding proteins in the nucleus and/or cytoplasm. Another high-level regulator, mi-croRNA, is less well understood but is drawing increasing interest in studies of epigenetic effectors.

Epigenetic modifications may or may not be retained by prog-eny cells after a cell divides. It is attractive to imagine that each time an ES cell divides, a balance of epigenetic factors deter-mines whether the daughter cells remain pluripotent or commit to a more limited fate. Understanding the role of epigenetic reg-ulation in ES cells may allow us to direct the differentiation of these cells into specific cell types of interest for research or clin-ical applications. It has been demonstrated that experimental manipulations of known epigenetic patterns can be used to con-vert differentiated cells into pluripotent cells (Okita et al., 2007; Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007; Yu et al., 2007).

In this review, we focus on the two types of epigenetic pro-cesses in ES cells that we best understand—DNA methylation and chromatin modification—and briefly touch on miRNA ex-pression. We will also discuss the interactions between these epigenetic processes and transcription factors, where such in-formation is available. We emphasize information about hES cells rather than mES cells when this evidence is available. We propose a model in which multiple epigenetic elements influence ES cell fate and suggest that a balance of these factors may de-termine whether these cells differentiate or remain pluripotent, and their direction of differentiation.

DNA Methylation in ES Cells

The best-known epigenetic process is DNA methylation, the addition or removal of a methyl group symmetrically on CpG dinucleotides. In almost all mammalian cells, 70%–80% of the CpG dinucleotides in the genome are methylated, predominantly in areas of repetitive sequences (Bird, 2002). The idea that DNA methylation controls gene expression comes from the intriguing observation that certain genomic regions appear to be protected from methylation, specifically CpG-rich islands located in the 5′ end of 60% of human genes, usually associated with the promoter and first exon (Bird, 2002). These CpG islands generally remain unmethylated in all tissues at all stages of development (Li, 2002), but a small number become methylated during development, an event that is accompanied by silencing of the associated promoter. These differentially methylated regions (DMRs) are associated with imprinted genes and CpG islands of genes on the inactive X chromosome. In the past 10–15 years, many DNA methylation detection methods have been developed. These include bisulfite sequencing, methylation-specific PCR (MSP), restriction landmark genomic scanning (RLGS), and differential methylation hybridization (DMH) (reviewed in Murrell et al., 2005). Most recently, microarray-based technologies, in combination with either bisulfite conversion or methylated DNA immunoprecipitation (MeDIP), have enabled genome-wide DNA methylation profiling (Weber et al., 2005).

Establishment of DNA Methylation Patterns

Programmed changes in patterns of the cytosine methylation during embryogenesis suggest that methylation may play an important role in cell fate specification (Reik et al., 2001). Mouse studies indicate that after fertilization the male pronucleus is rapidly and actively demethylated, whereas the maternal genome starts to become demethylated after several cleavage divisions (Mayer et al., 2000) and by implantation stage the embryo's DNA is globally demethylated (Morgan et al., 2005). The general process of demethylation does not, however, include the imprinted genes, which maintain their methylation status throughout the cleavage divisions.

The origin of DNA methylation patterning is largely unknown. DNA methyltransferases (Dnmts) are a family of proteins involved in the establishment and maintenance of DNA methylation and are separated into two functional classes—the de novo and the maintenance methyltransferases. Dnmt3a and Dnmt3b are de novo methyltransferases responsible for remethylation in postimplantation mouse embryos and in germ cells (Okano et al., 1999). Inactivation of both Dnmt3a and Dnmt3b in mouse ES cells results in progressive loss of methylation in various repetitive sequences and single-copy genes. In embryonic stem cells, Dnmt3a and Dnmt3b are stably associated with each other (Li et al., 2007b). In differentiating embryonic carcinoma or embryonic stem cells and mouse postimplantation embryos, the two enzymes directly interact and function synergistically to methylate the promoters of the *Pou5F1/Oct4* and *Nanog* genes. Inadequate methylation caused by ablating Dnmt3a and Dnmt3b is associated with dysregulated expression of Oct4 and Nanog during the differentiation of pluripotent cells and mouse embryonic development (Li et al., 2007b). Interestingly, introduction of the Dnmt3a, Dnmt3a2, and Dnmt3b1 isoforms back into highly demethylated mutant ES cells restores genomic methylation patterns; these isoforms appear to have both common and dis-

tinct DNA targets, but none of them appear to be able to restore the methylation patterns of maternally imprinted genes (Chen et al., 2003). Recent studies suggest that, in addition to Dnmts, the epigenetic regulator Hells (Lsh, lymphoid-specific helicase) is directly involved in the control of de novo methylation of DNA (Zhu et al., 2006).

Dnmt1 is a maintenance methyltransferase that prefers hemimethylated templates and is recruited to actively replicating DNA through an association with proliferating cell nuclear antigen (PCNA), the replication fork clamp. Once recruited, Dnmt1 propagates the methylation state by copying the methylation pattern of the template strand onto the nascent strand (Yoder et al., 1997). Three isoforms of *Dnmt1* transcripts, *Dnmt1s*, *Dnmt1o*, and *Dnmt1p*, are produced by alternative usage of multiple first exons. Dnmt1s is expressed in somatic cells, Dnmt1p is found only in spermatocytes at the pachytene stage of meiosis, whereas Dnmt1o is specific to oocytes and preimplantation embryos. Dnmt1-deficient mice lack 30%–80% of their DNA methylation and are early embryonic lethal (Li et al., 1992). mES cells deficient in Dnmt1 are viable but undergo cell death when induced to differentiate (Panning and Jaenisch, 1996), while conditional knockout murine fibroblasts die within a few cell divisions after deletion of the *Dnmt1* gene (Jackson-Grusby et al., 2001). Although early studies in human cells suggested that DNMT1 plays a more minor role in differentiated human cells (Rhee et al., 2000), a recent study supports a similarly critical role for DNMT1 in the human system (Chen et al., 2007b; Egger et al., 2006). These studies suggest that global methylation may be dispensable for the undifferentiated state but is critical for differentiation.

Characterization of DNA Methylation in ES Cells

How DNA methylation specifically contributes to pluripotence, commitment, and phenotypic maturation of specific differentiated cells is not well understood. Table 1 summarizes the studies of global DNA methylation in ES cells to date. Using RLGS in differentiating mouse stem cells, sperm, and somatic tissues, Shiota et al. (2002) reported that the methylation pattern in these cells was specific and varied in a precise manner according to cell lineage and tissue type, and during cell differentiation (Shiota et al., 2002). More recently, Fouse et al. (2008) (this issue of *Cell Stem Cell*) provided a comprehensive map of DNA methylation in 11,201 proximal promoters in mES cells using MeDIP in combination with microarrays. This study indicated that in mES cells, ∼40% of the interrogated promoter regions are methylated, 32% are unmethylated, and 28% are indeterminate (Fouse et al., 2008). By analyzing the CpG density and distribution in methylated and unmethylated promoter regions, the authors uncovered that the methylated promoter regions are primarily outside of CpG islands and only ∼3% of the CpG islands are methylated to some degree in mES cells.

There are conflicting reports about whether prolonged culture of undifferentiated hES cells affects their methylation patterns (Allegrucci et al., 2007; Bibikova et al., 2006; Maitra et al., 2005; Rugg-Gunn et al., 2005). One study (Rugg-Gunn et al., 2005) examined the allele-specific expression of six imprinted genes and the methylation profiles of three imprinting control regions to assess the epigenetic status of hES cells cultured for 40–155 passages. Three of the analyzed imprinted genes are paternally expressed (*IGF2*, *IPW*, and *KCNQ1OT1*), and the other three are maternally expressed (*H19*, *SLC22A18*, and *NESP55*). While

Table 1. DNA Methylation Profiling in ES Cells

Organism	Cell Types	Number of CpG Sites Monitored	Method	Reference
Mouse	Stem cells, sperm, and somatic tissues	1500 genomic loci	RLGS	Shiota et al., 2002
Mouse	ES cell	11,201 proximal promoters	MeDIP and promoter arrays	Fouse et al., 2008
Human	ES cell	three imprinting control regions	Bisulfite sequencing	Rugg-Gunn et al., 2005
Human	ES cell	14 genes	MSP	Maitra et al., 2005
Human	ES cell, somatic stem cells, cancer cell lines, and lymphoblastoid cell lines	1536 CpG sites from 371 genes	Bead array	Bibikova et al., 2006
Human	ES cell, NPC	4608 CpG islands	DMH	Shen et al., 2006
Human	ES cell	>2000 genomic loci	RLGS	Allegrucci et al., 2007
Human	ES cell, embryoid bodies	four to five genes	MSP	Lagarkova et al., 2006; Yeo et al., 2007

there was no evidence of abnormal methylation of these genes, one cell line became biallelic for *H19*. While these data argue for a substantial degree of stability in the DNA methylation pattern of hES cells, another study (Maitra et al., 2005) compared methylation patterns of a panel of 14 genes known to be differentially methylated in cancers in early- and late- passage hES cell lines (ranging from 22 to 175 passages). In this study, a majority of late-passage hES cell lines had significantly increased methylation of a putative tumor suppressor gene *RASSF1*, and less frequent changes for two other genes (*DCR1* and *PTPN6*). The authors suggested that methylation changes accumulate in culture over time and argued that late-passage hES cells may be unsuitable for cell therapy applications. However, a small number of evaluated genes in the Rugg-Gunn et al. (2005) and Maitra et al. (2005) studies does not allow drawing conclusions about global methylation profile changes in hES cells.

In our own study, we compared the DNA methylation profiles in 14 independently isolated hES cell lines at various times in culture and asked whether they differed from other types of cells, including other types of stem cells (Bibikova et al., 2006). We used a microarray-based method to analyze the methylation status of 1536 CpG sites selected from the 5′-regulatory regions of 371 genes. We found that the DNA methylation profile clearly distinguished the hES cells from all of the other cell types, including somatic stem cells; this suggests that embryonic stem cells and somatic stem cells may be under different methylation regulations. Among the genes with elevated methylation levels in hES cells are genes encoding proteins involved in nuclear and extracellular signaling (e.g., *THBS2*, *IL13*, *IL16*, *TNF*, *MSF*, and *PI3*), stress response and apoptosis (*ASC* and *CASP8*), and cell-cycle control (*CDKN1B* and *RASSF1*), and genes from the HLA locus (HLA-DQA1 and HLA-DPA1). Two growth factor receptor genes, *FGFR3* and *TGFBR1*, had a very low methylation level in hES cells as compared to that in differentiated cells and somatic stem cells. TGF-β signaling has been shown to play a crucial role in the maintenance of the undifferentiated state of hES cells (James et al., 2005), while basic fibroblast growth factor (FGF2) has been shown to promote long-term undifferentiated proliferation of hES cells (Xu et al., 2005). A set of 25 CpG sites from 23 genes contributed most to distinguish hES cells from normal differentiated cells (Bibikova et al., 2006), although their cellular functions related to the pluripotent property of hES cells remain

unclear. We also observed that different hES cell lines showed different changes with time in culture, and that the degree of overall change in methylation was roughly proportional to the number of passages separating compared preparations. However, we did not find a specific set of genes in the group studied that changed predictably.

A recent report also noted that no single set of genes changed predictably with passage number in multiple hES cell lines (Allegrucci et al., 2007). Using RLGS, this group analyzed DNA methylation of >2000 genomic loci in six independently derived hES cell lines and observed changes in DNA methylation over time in culture, especially when cells were transferred to serum-free conditions. Although the affected loci did not seem to be linked to any particular pathway of differentiation, many had been previously associated with an adult tumor phenotype.

DNA Methylation during ES Cell Differentiation

A concern about the use of hES cells in replacement therapies is that differentiation of the cells may result in hypermethylation of CpG islands, which may in turn progress into malignancy. The limited information available seems to indicate that this is not the case. Using a DMH method, Shen et al. (2006) profiled DNA methylation changes in a library of 4608 CpG islands during differentiation of hES cells along a neuronal lineage. The results indicated that only a small fraction (1.4%) of the CpG islands studied underwent de novo DNA methylation during differentiation, and this subset of CpG islands was distinctively different from those reported to be methylated in cancer cells (Shen et al., 2006).

Other recent reports support the idea that methylation of key regulatory genes may play an important role in differentiation of ES cells. Lagarkova and colleagues (Lagarkova et al., 2006) studied the expression of pluripotence-associated genes *POU5F1/OCT4*, *NANOG*, *DPPA3*, and *DPPA5* during spontaneous differentiation of hES cells. Methylation profiles of the promoters or putative regulatory regions of these genes demonstrated that expression of the transcription factors *POU5F1/OCT4* and *NANOG*, but not *DPPA3* and *DPPA5*, was correlated with their methylation status. Another study (Yeo et al., 2007) confirmed that following exposure to differentiation stimuli *POU5F1/OCT4* and *NANOG* gene loci were rapidly modified by DNA methylation.

These studies indicate that DNA methylation is active in ES cells, and that methylation and demethylation may control

transcription of key developmentally regulated genes in these cells. But gauging the importance of this epigenetic mechanism to the stability of ES cells and to their differentiation will require a comprehensive analysis linking methylation, gene expression, and functional analysis of ES cells and their derivatives. In the first report integrating DNA methylation, gene expression, and chromatin modification data in mES cells, a recent study of control and demethylated mutant mES cells found that the expression levels of 390 annotated genes (∼1.7%) are significantly increased in the demethylated cells (Fouse et al., 2008). Gene ontology analysis of this group of genes showed an overrepresentation of tissue-specific genes, which was consistent with the mapping of DNA methylation patterns in mES cells. Compared to the deregulation of up to 10% of genes in demethylated fibroblasts (Jackson-Grusby et al., 2001), the smaller proportion (1.7%) of upregulated genes seen in the demethylated mES cells implies that mechanisms other than DNA methylation may be important in maintaining the undifferentiated state in mES cells. This notion, that repression of differentiation-associated genes in mES cells may be mediated by several different mechanisms, is supported by the observation that 87% of the promoters that lack both the H3K4 and H3K27 trimethylation marks are methylated in mES cells (Fouse et al., 2008). It would also be interesting to compare global DNA methylation profiles in human ES cells and investigate their changes in the course of differentiation. This information will be extremely useful as a basis for novel strategies for modifying the differentiation of hES cells to produce functional cells for therapy, and perhaps to minimize their tumorigenic potential.

Chromatin Modification in ES Cells

In mammalian cells, DNA is never alone. Chromatin is a complex structure comprised of DNA and associated proteins, and transcriptional activity is intertwined with modifications to the chromatin structure. Thus, a complete understanding of the transcriptional regulatory networks in ES cells must be accompanied by analysis of the assembly, disassembly, and covalent modifications of chromatin and DNA (Li et al., 2007a). A nucleosome, the basic subunit of chromatin, consists of an octamer of histone proteins including H2A, H2B, H3, and H4. Protein machinery, such as the SWI/SNF complex, can move nucleosomes along the DNA in an ATP-dependent mechanism. In addition, transcriptional activity is correlated with dissociation or reassembly of nucleosomes on specific sequences. Moreover, the histone proteins are subject to at least 100 distinct covalent modifications, catalyzed by a growing list of enzymatic activities that are recruited to the chromatin by sequence-specific DNA-binding proteins, or as components of the general transcription machinery (Berger, 2007). The collection of histone modification states is considered to be a key component of the epigenome (Bernstein et al., 2007).

The last 2 years have witnessed rapid progress in the development of technologies for mapping histone modifications in mammalian genomes. High-resolution, genome-wide maps of key histone modification marks have been obtained in ES cells and cell lineages at various stages of differentiation. These maps have provided a comprehensive view of the chromatin architecture in the mammalian genome, revealed the dynamics of chromatin structure during lineage specification, and uncovered functional relationships between chromatin structure and transcriptional activity.

Analysis by Chromatin Immunoprecipitation-Based Methodology

To map histone modifications in mammalian cells, including ES cells, two methods have been developed, both involving chromatin immunoprecipitation (ChIP) (Kim and Ren, 2006). For ChIP, cells are treated with formaldehyde to crosslink the DNA to the proteins bound to them in vivo. The chromosomes are then extracted from the cells and broken into small (500–1000 bp) fragments. Subsequently, antibodies that recognize specific DNA-binding proteins, modified histones, or DNA modifications are used to isolate the specific chromatin fragments bound to the proteins. Finally, the DNA is separated from the rest of the mixture by protease digestion after reversal of crosslinking.

Following ChIP, one method, known as ChIP-chip (or ChIP-on-chip), uses genomic tiling microarrays to identify the locations of a particular antibody target (e.g., a histone modification) along the genome. Tiling microarrays contain short DNA oligonucleotides or PCR fragments that represent a nearly continuous genome. The immunoprecipitated DNA fragments, after amplification and labeling with fluorescent dyes, are hybridized to these microarrays, and the regions bound by a particular antibody target are identified by the probes that show a significant enrichment compared to control DNA. In the last few years, the ChIP-chip method has provided high-resolution and genome-wide views of histone modifications. However, it is limited by the resolution and coverage of the tiling arrays, which typically neglect repetitive sequences because they crosshybridize with multiple genomic sequences. Next-generation microarrays are being designed to interrogate many of the repeat-rich regions using carefully selected oligonucleotide probes within such sequences.

A recently developed alternative method for chromatin analysis, known as "ChIP-Seq," relies on new massively parallel sequencing technologies. ChIP-Seq identifies the regions of DNA associated with specific DNA-binding proteins, modified histones, or DNA modifications via direct sequencing of the DNA immunoprecipitated by the ChIP procedure (Barski et al., 2007; Johnson et al., 2007; Loh et al., 2006; Mikkelsen et al., 2007). A new generation of sequencing technologies is capable of generating tens of millions of short sequencing reads from nanograms of DNA in a single run (Bentley, 2006). While the length of the sequence reads are generally short (between 25 and 35), they can serve as molecular tags to uniquely locate the genomic positions of the immunoprecipitated DNA fragments, and provide a digital profile of the relative enrichment of different genomic regions in the DNA pool. To interpret the data, the short DNA sequence reads are aligned to the complete genomic sequence, and statistical algorithms are employed to identify regions where these sequence tags are significantly enriched compared to control samples. The statistically enriched genomic regions are thus identified as potential sequences associated with the antibody targets.

Characteristics of Embryonic Stem Cell Chromatin

Using these high-information-content methods, several genome-wide maps of histone modifications in hES cells, mES cells, and some differentiated cell lineages have been generated recently (Table 2). These maps provide a comprehensive view of

Table 2. Genome-wide Maps of Histone Modifications

Organism	Cell Type	Histone Modifications	Locations	Method	Reference
Human	T cells	H2BK5me1, H3K9me2, H3K9me1, H3K27me2, H3K27me1, H4K20me1	Active promoters	ChIP-Seq	Barski et al., 2007
Human	T cells	H3K4me2	Active or poised promoters	ChIP-Seq	Barski et al., 2007
Human	T cells	H3K36me1, H3K79me3	Slightly enriched at active promoters	ChIP-Seq	Barski et al., 2007
Human	T cells	H3K4me1	Enhancers, upstream and downstream of TSS	ChIP-Seq	Barski et al., 2007
Human	T cells	H3K9/K14ac, H2A.Z	Active promoters, enhancers	ChIP-Seq	Barski et al., 2007
Human, mouse	hES cell, mES cell, mNPC, MEF, human T cells	H3K36me3	Along actively transcribed regions	ChIP-Seq	Barski et al., 2007; Mikkelsen et al., 2007
Human, mouse	hES cell, mES cell, mNPC, MEF, human T cells	H3K4me3	CpG islands, active or poised promoters	ChIP-chip, ChIP-Seq, ChIP-PET	Barski et al., 2007; Guenther et al., 2007; Mikkelsen et al., 2007; Zhao et al., 2007; Pan et al., 2007
Human, mouse	hES cell, mES cell, mNPC, MEF, human T cells	H3K9me3, H4K20me3	Transposons, repetitive sequences, pericentromere, telomere	ChIP-Seq	Barski et al., 2007; Mikkelsen et al., 2007
Human	T cells	H3R2me1, H3R2me2	No enrichment at promoters	ChIP-Seq	Barski et al., 2007
Human, mouse	hES cell, mES cell, mNPC, MEF, human T cells	H3K27me3	Inactive or poised promoters	ChIP-chip, ChIP-Seq, ChIP-PET	Barski et al., 2007; Bernstein et al., 2006; Boyer et al., 2006; Guenther et al., 2007; Lee et al., 2006; Mikkelsen et al., 2007; Zhao et al., 2007; Pan et al., 2007
Human	T cells	H3K79me2, H3K79me1, H4K20me2	No particular patterns	ChIP-Seq	Barski et al., 2007

the various histone modifications and their relationships with gene expression. For the most part, the recent studies confirmed some of the general principles established previously in smaller scale studies. For example, nearly all the active promoters are associated with multiple histone modifications such as H3K4me3 (histone H3 trimethylated at lysine 4; for nomenclature of histone modifications, see Turner [2005]) and H3K4me2, while H3K36me3 is located along the actively transcribed regions. In addition, H3K9me3 and H4K20me3 are mainly associated with transposons, repetitive sequences, and pericentromeres (Mikkelsen et al., 2007). The comprehensive maps also provide an opportunity for investigators to gain new insights into the combinatorial relationships between various histone modifications, and support the concept of a histone code that can be used for functional annotation of the ES cell epigenome.

While previous studies have identified H3K4me3 as a hallmark for active promoters, the most recent genome-wide analyses of this histone modification mark indicate the H3K4me3 is present at 80% of the annotated promoters in ES cells (Guenther et al., 2007). Many promoters carrying this mark do not produce full-length transcripts detectable by conventional methods. Instead, more sensitive means show that these "inactive" promoters indeed experience transcriptional initiation, but the transcript is not completed, for reasons that are still unknown. One possibility

is that an additional silencing mark, such as H3K27me3 or the Polycomb complex, may prevent the completion of transcription (Bernstein et al., 2006; Boyer et al., 2006; Lee et al., 2006). Because H3K4me3 is predominantly associated with transcriptional start sites, while H3K36me3 is associated with transcriptional elongation, the combination of these two marks allows the delineation of gene boundaries in the genome, and provides a new tool for gene annotation (Barski et al., 2007; Mikkelsen et al., 2007).

Specific epigenetic marks are also useful for identifying other genomic regulatory elements, such as transcriptional enhancers. Unlike promoters, which are located at the transcription start sites and typically possess characteristic sequence motifs, enhancers are frequently found far upstream or downstream of the promoters. Finding enhancers has been a big challenge because of their long distance from promoters, and lack of identifiable marks. A recent study revealed that enhancers are generally associated with H3K4me1, but lack H3K4me3 (Heintzman et al., 2007). First reported in HeLa cells, this combination pattern has also been identified in hES cells and may indicate the locations of promoters and enhancers in the genome.

Bivalent Domains and Pluripotence

H3K27me3, a repressive chromatin mark, is found to co-occupy a large number of promoters and evolutionarily conserved

sequences together with the activating chromatin mark H3K4me3 (Bernstein et al., 2006; Mikkelsen et al., 2007). These areas of chromatin, termed "bivalent" domains, are believed to hold genes in a "transcription-ready" state. This bivalent chromatin pattern is typically found in developmentally regulated genes that are silenced in undifferentiated ES cells but are activated upon differentiation. One of the first studies comparing the chromatin modifications at a number of evolutionarily conserved regions in mES cells and adult tissues suggested a model that attributed the pluripotence of ES cells to bivalent domains (Bernstein et al., 2006). However, more recent genome-wide analyses not only confirmed the existence of bivalent chromatin signatures at promoters of developmental regulator genes in ES cells but also demonstrated that the bivalent domain signature is also present in differentiated cell types (Azuara et al., 2006; Barski et al., 2007; Mikkelsen et al., 2007; Pan et al., 2007; Roh et al., 2006; Zhao et al., 2007).

Thus, the original model that links pluripotence of embryonic stem cells to bivalent domains appears to oversimplify the mechanisms that control pluripotence. In a modification of this model, Bernstein and colleagues propose that the prevalence of bivalent chromatins in the genome may correspond to the degree of pluripotence of a given cell type, observing that the number of promoters with bivalent K4/K27 modifications appears to gradually decrease while mES cells differentiate (Mikkelsen et al., 2007). According to this view, the bivalent chromatin configuration is designed to provide a rapid induction mechanism for a class of genes, many of which are involved in cell fate determination, in ES cells and other progenitor cells. On the other hand, Thomson and colleagues observed that several key embryonic stem cell genes, including *POU5F1/OCT4*, *NANOG*, and *SOX2*, are marked by H3K4me3 alone in undifferentiated hES cells and actually adopt a bivalent domain configuration upon differentiation (Pan et al., 2007).

Generation of Histone Marks

Rapid progress has been made in understanding the factors responsible for generating, removing, and reading the H3K4me3 mark located at active gene promoters. A class of histone methyltransferases (HMTs), known as the MLL family proteins, are responsible for the trimethylation of H3K4 in mammalian cells (reviewed by Ruthenburg et al., 2007). In addition, several histone demethylases, belonging to the Jumonji domain-containing (Jmjd) protein family, have now been shown to remove this mark and reduce it to di-, mono-, or unmethylated states (Christensen et al., 2007; Iwase et al., 2007; Klose et al., 2007; Yamane et al., 2007). Furthermore, proteins containing the plant-homology domain (PHD) have now been found to specifically recognize H3K4me3. One such protein, bromodomain and PHD finger-containing transcription factor (BPTF), is a component of the nucleosome remodeling factor (NURF) complex, and its interaction with H3K4me3 facilitates the recruitment of nucleosome remodeling activities to the *HOXC8* promoter and activates its expression (Wysocka et al., 2006). On the other hand, the removal of this mark by the RBP2 H3K4me3 demethylase appears to repress HOX genes in ES cells (Christensen et al., 2007). However, H3K4me3 almost certainly plays a much broader role in mammalian transcription than regulation of *HOX* genes, as evidenced by the association of this mark with nearly 80% of promoters in hES cells (Guenther et al., 2007). The recent discovery that this mark

is recognized by the general transcription factor TFIID (via the PHD domain in TAF3, a TFIID component) further suggests that it may also be involved in the assembly of the polymerase II initiation complex (PIC) at active or poised promoters (Vermeulen et al., 2007).

The factors that contribute to the modification of the repressive chromatin mark H3K27me3 have also been characterized recently (reviewed by Swigut and Wysocka, 2007). An HMT, EZH2, in the polycomb repressor complex 2 (PRC2) is responsible for the generation of this mark, while the polycomb repressor complex 1 (PRC1) recognizes and maintains this modification at the silenced promoters (see review by Cao and Zhang, 2004). Components of PRC2 HMT complex are essential for the differentiation of mES cells (Lee et al., 2006). A number of Jmjd proteins, notably UTX1, UTY1, and JMJD3, have been identified as H3K27 demethylases (Agger et al., 2007; De Santa et al., 2007; Lan et al., 2007; Lee et al., 2007). UTX1 is found to be absent from the *HOX* gene promoters in hES cells, but present in these regions in human primary fibroblast cells (Lan et al., 2007). It is interesting to point out that UTX1, a histone demethylase, is associated with MLL2 H3K4, an HMT (Agger et al., 2007; Issaeva et al., 2007; Lee et al., 2007).

The H3K9me3 is another repressive chromatin mark affecting a set of genes distinct from H3K27me3 (Barski et al., 2007; Mikkelsen et al., 2007; O'Geen et al., 2007). In mES cells, this mark is found to be mainly associated with heterochromatin regions in repeat regions and some silenced gene loci. The HMTs for H3K9me3 include Suv39H1, Suv39H2, and G9a. In addition, proteins in the heterochromatin protein-1 (HP1) family can recognize this chromatin mark. HP1 in turn interacts with histone deacetylases and DNA methylases that contribute to the silencing and maintenance of heterochromatin (reviewed in Lachner and Jenuwein, 2002). Several H3K9me3 demethylases have been discovered (Klose et al., 2006; Whetstine et al., 2006). Two of them, Jmjd1a and Jmjd2c, appear to play a role in regulating the differentiation of mES cells (Loh et al., 2007). In particular, JmJd2c is required to reverse the H3K9me3 marks at the *Nanog* promoter region and consequently prevents transcriptional repressors HP1 and KAP1 from binding, providing a mechanism of stem cell maintenance.

In summary, the recent maps of chromatin modification in ES cells and their derivatives bring us closer to understanding the molecular basis of pluripotence and differentiation. However, current studies have examined only a small fraction of the known histone modifications in the human genome, and only in a small number of cell lineages. The roles of scores of histone modifications in ES cells remain to be defined. Recognizing the need for this knowledge, the US National Institutes of Health (NIH) has recently launched the "epigenome" project (http://nihroadmap.nih.gov/epigenomics/). This large-scale project is aimed at generating reference maps of histone modifications and other epigenetic features along the human genome in ES cells and stem cells undergoing differentiation. Independently, several large-scale projects in Europe are underway to map histone modifications and DNA methylation in mES cells and other cell types. The next several years shall witness significant progress in our understanding of the epigenetics of ES cells and a better understanding of the overlap and distinct roles for DNA methylation and histone modifications in regulating pluripotence and differentiation.

Table 3. miRNA Expression Profiling in ES Cells

Organism	Cell Types	Number of miRNAs Covered	Method	Reference
Mouse	ES cell	388 miRNA species, 681 clones from three libraries (192–270/library)	Small RNA library sequencing	Houbaviy et al., 2003
Human	ES cell	36 miRNA species, 158 clones from two libraries (not tallied by library)	Small RNA library sequencing	Suh et al., 2004
Mouse	ES cell, embryoid bodies, brain, kidney, lung, liver, heart	248	qRT-PCR	Strauss et al., 2006
Mouse	ES cell, embryoid bodies, brain, kidney, lung, liver, heart	425	qRT-PCR	Strauss et al., 2006
Mouse	ES cell, brain, neuroblastoma, eye, skin, adipocytes, kidney, liver, lung, intestine, heart, T cells, B cells, spleen, thymus, pancreas, testis, placenta, embryos, germ cell tumor, MEFs	303 miRNA species, 49,518 clones from 68 libraries (11–3075/library)	Small RNA library sequencing	Landgraf et al., 2007

MicroRNAs in ES Cells

MicroRNAs (miRNAs) are small (19–25 nt) endogenous noncoding RNAs that have been shown to influence the abundance and translational efficiency of cognate mRNAs; they regulate gene expression through sequence-specific base pairing with target mRNAs, either by inhibition of mRNA translation or direct cleavage of targeted mRNA (Bartel, 2004). A large percentage of transcripts are likely to be regulated by miRNAs, with each miRNA targeting hundreds or even thousands of different mRNAs.

There has not yet been a publication of a comprehensive comparative analysis of miRNA expression in ES cells equivalent to the recently reported maps of DNA methylation and chromatin structure. Reports of miRNA expression in ES cells include two studies describing isolation and cloning of novel miRNAs, one in mES cells (Houbaviy et al., 2003) and one in hES cells (Suh et al., 2004) (Table 3). These authors used qualitative northern blotting to demonstrate differential expression of a subset of the cloned miRNAs in ES cells. Two additional studies measured miRNA expression in mES cells by qRT-PCR (Chen et al., 2007a; Strauss et al., 2006). In all of these studies, two clusters of miRNAs were found to be strongly upregulated in ES cells (mir-302 and mmu-mir-290/hsa-mir-371/372/373). (Note: miRNAs ["mir"] are assigned sequential numerical identifiers. Prefixes designate the species: "hsa" refers to human, and "mmu" designates murine.)

A recent study reporting the largest miRNA cloning and sequencing effort to date included two samples of mES cells (Landgraf et al., 2007). This study involved sequencing ~330,000 clones from 256 small RNA libraries from a wide variety of organs from human, mouse, and rat. The limited sample replication and low clone counts (only ~1000 clones per library were sequenced) make it difficult to glean statistically significant differential expression information from this data set, but these data generally support the miRNA expression results generated by other methods.

miRNA Functions in ES Cells

Dicer1 and Dgcr8 are two enzymes that are necessary in miRNA biogenesis. mES cells with reduced expression of Dicer1 (Kanellopoulou et al., 2005) or absent expression of Dgcr8 (Murchison et al., 2005) retained expression of pluripotence markers, but showed proliferation defects and were not able to differentiate normally (Kanellopoulou et al., 2005; Murchison et al., 2005; Wang et al., 2007). Recent advances in high-throughput methods (e.g., microarray and high-throughput sequencing) will enable the construction of comprehensive maps of miRNA expression and allow the understanding of the role of miRNA in the stable maintenance of a variety of cellular states, including the ES cell state. Delineation of the interplay between miRNAs and mRNAs in regulatory networks will require better strategies for large-scale analysis of functional miRNA-mRNA targeting.

Summary and Future Directions

We are still in the early stages of unraveling the epigenome of ES cells. Analysis of hES cells is especially intriguing, as epigenetic modifications may influence the safety and efficacy of these cells when used clinically. Two rapidly growing areas of research are converging to allow the opportunity for the first comprehensive investigations of the role of epigenetics in hES cell pluripotence and differentiation. One area is the improvement of methods for hES cell culture and characterization; the other is development of high-information-content analysis methods to create genome-wide maps of epigenetic factors.

The quality of data from high-throughput assays can only be as good as the quality of the samples that are analyzed, and several groups (Adewumi et al., 2007; Loring and Rao, 2006) are seeking to standardize hES cell culture and characterization methods to improve the quality of input for molecular profiling assays. Molecular analysis requires homogeneous cell populations and multiple representatives of each cell type in order to obtain reliable, significant results. Differentiation protocols for hES cells are still in the experimental stage, and there is not yet an agreement about the characteristics that should be used to define stages of differentiation. There is a need for selection tools and reagents, such as cell lines containing reporter expression systems or targeted gene modifications to make it simpler to purify cell populations. These studies will be greatly enhanced by development of more sensitive technologies for macromolecular analysis that can be used for small populations of cells.

A complete epigenetic map of ES cells requires systematic analysis on multiple levels (Figure 1). For example, a gene-by-gene
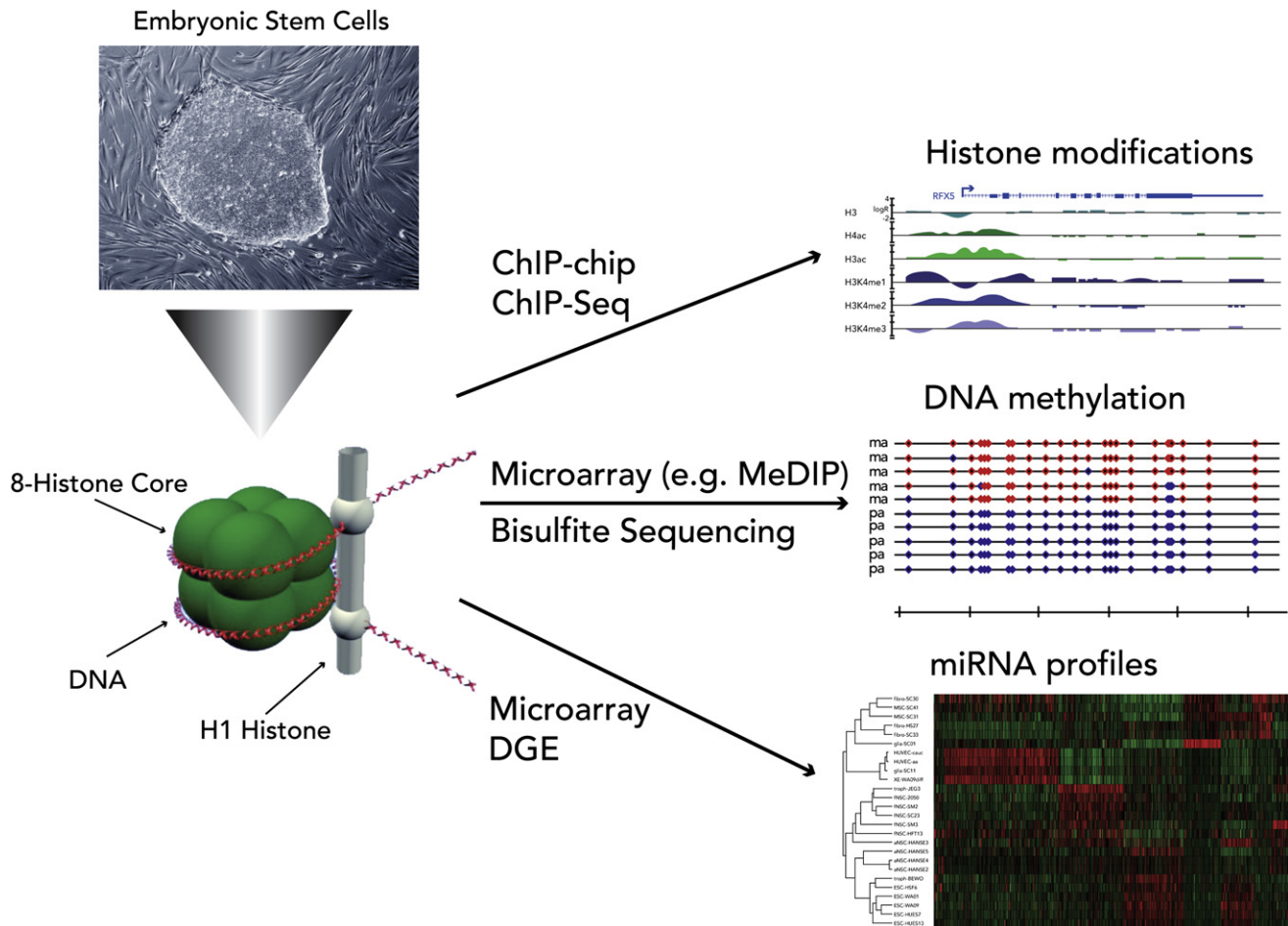
Figure 1. Comprehensive Epigenetic Characterization of ES Cells at Multiple Molecular Levels
ChIP-chip, chromatin immunoprecipitation followed by microarray (i.e., chip) readout; ChIP-Seq, chromatin immunoprecipitation followed by sequencing readout; MeDIP, methylated DNA immunoprecipitation; DGE, digital gene expression.

RNA interference (RNAi) approach has proved to be very effective to study specific genes implicated in regulation of self-renewal (Ivanova et al., 2006). On the other hand, high-density promoter arrays or whole-genome tiling arrays are powerful tools for ChIP-chip, DNA methylation (Weber et al., 2005), and transcriptional profiling, including noncoding RNA expression profiling (Kapranov et al., 2007). Array technologies can be adapted to provide not only genomic location but also allele-specific information for functional epigenetic analysis (Fan et al., 2006). The next generation of DNA sequencing technologies promises to provide a more efficient and cost-effective whole-genome approach to epigenetic profiling and discovery (Barski et al., 2007; Hu et al., 2005; Johnson et al., 2007).

Understanding the epigenetic processes involved in differentiation should aid in developing efficient methods to induce pluripotence, to create disease-specific pluripotent cells for research or patient-specific stem cells for cell therapy. The other promising strategy for reprogramming somatic cells, by nuclear transfer to zygotes or oocytes, is currently inefficient and often results in abnormalities that are likely due to inappropriate epigenetic patterning (Rideout et al., 2001).

Recent work on in vitro reprogramming of mouse and human fibroblasts into pluripotent ES cell-like cells (iPS, induced pluripotent stem cells), by ectopic expression of transcription factors, suggests that it is possible to effect a global epigenetic resetting of the somatic cell genome (Maherali et al., 2007; Okita et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007). Some iPS cells created from mouse fibroblasts were able to pass the gold standard test for pluripotence of mES cells: generation of germ cells in chimeras and transmission of the iPS genome through the germline (Okita et al., 2007). Germline-competent miPS cells appear to have gene expression patterns and signature chromatin modification similar to those of mES cells (Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007). Similar approaches have recently proved to work in human cells (Takahashi et al., 2007; Yu et al., 2007), and it will be interesting to ask whether the epigenetic profiles of these cells are the same as those of embryo-derived hES cells, and to compare mouse iPS cells with their human counterparts.

Epigenetic Balance Model of ES Cell States

With our current understanding of the roles of multiple epigenetic processes in ES cells in mind, it is also important to consider the
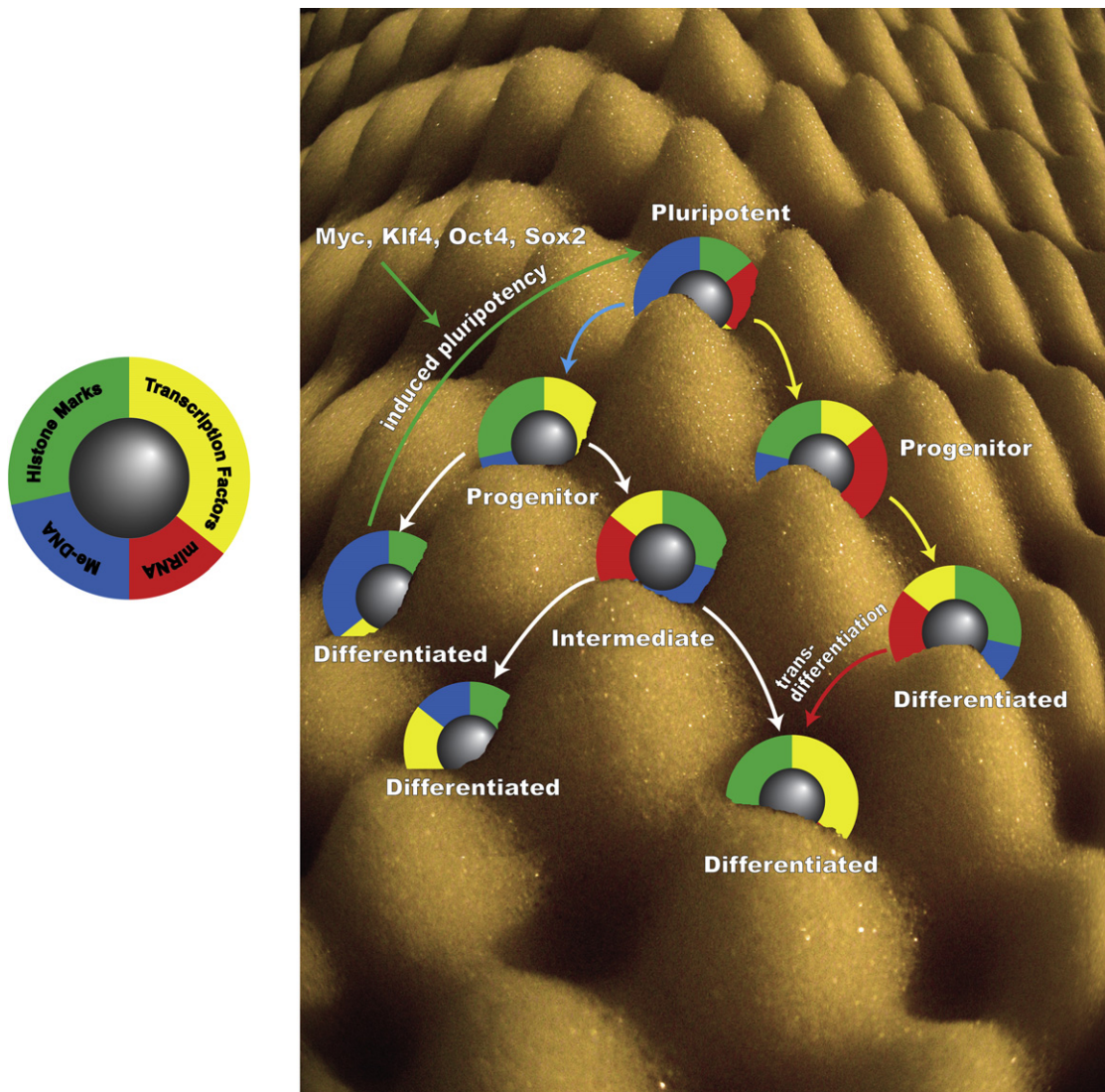
Figure 2. Model Illustrating the Contribution of Four Epigenetic Regulatory Mechanisms to Stabilization of Cellular States
This schematic represents possible transitions between cellular differentiation states, including pluripotent cells, lineage-specific progenitor cells, intermediate cells in specific lineages, and fully differentiated cells. Stable differentiation states are depicted as potential energy wells, while the transitions between them are potential energy barriers. We postulate that crosstalk between different regulatory mechanisms (transcription factors, DNA methylation [Me-DNA], miRNAs, and histone marks) stabilize each of these differentiation states. Perturbations of one or more of these mechanisms can overcome the potential energy barriers between states. Examples of transitions: in vivo differentiation of pluripotent cells → progenitor cells → differentiated cells (yellow arrows); in vitro manipulation of transcription factors resulting in mES cells → trophoblast stem cells (Niwa et al., 2000, 2005) (blue arrow); fibroblasts → iPS cells (Okita et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007) (green arrow); and transdifferentiation (hypothetical, red arrow).

contributions of other regulatory mechanisms in the maintenance of pluripotent and differentiated states, and the transitions between them. There seems to be a unique pluripotent transcriptional network, in which transcription factors clearly can stabilize or alter cellular phenotypes, acting in the context of the aggregate influences of DNA methylation, chromatin remodeling, and likely miRNA expression, as discussed. Figure 2 illustrates how we envision that this "epigenetic balance" stabilizes pluripotent cells and allows them to differentiate along different developmental pathways. We see stable cellular states as "potential energy" wells in the landscape of all possible cellular states, with each stable state maintained by a network of interactions among the different regulatory mechanisms. These are not true

energy barriers, of course, but are systems-based barriers where the system has a built-in capacity to buffer changes. In Figure 2 the four mechanisms are represented by colors on a wheel. Transitions between stable states are shown as "potential energy" barriers of different heights. We postulate that in any stable cellular state small perturbations in any one of these mechanisms (caused, for example, by minor changes in the environment) will be absorbed by the network of interactions, and therefore will not lead to fundamental changes in cellular phenotype. However, if there are coordinate changes in more than one mechanism, or a marked change in one mechanism (for example, over-expression of several transcription factors), a cell may be pushed over the barrier into another cellular state, which is stabilized by

a new equilibrium among the four elements. The stimulus for transition could be an accumulation of epigenetic changes in response to several different signals or could be due to a single signal that starts a cascade of events. The conclusions from the Fan lab presented in this issue (Fouse et al., 2008) support these concepts. Like many other groups, they found that differentiation-related genes were repressed in mES cells. However, of the genes that appeared to be repressed by DNA methylation, only 5.7% were also bound by the Polycomb group complex. Similarly, only 1.7% of the promoters bound by Nanog and/or Pou5F1/Oct4 were repressed by DNA methylation, and 87% of the promoter regions devoid of both H3K4 and H3K27 trimethylation marks were methylated. These findings suggest that multiple regulatory mechanisms, including DNA methylation, chromatin remodeling, and binding of transcription factors, contribute to the maintenance of the undifferentiated state. It also appears that individual genes may be regulated by several mechanisms, but these may be the exception rather than the rule.

This model suggests that there are multiple possible routes to the same stable phenotype. The route followed by cells during embryonic development is driven by a complex series of cell-cell signaling events during embryogenesis that results in appropriate cellular phenotypes. But experimental modulation of an epigenetic element does not need to recapitulate normal embryogenic processes in order to direct ES cells into the same cellular phenotype. In Figure 2, yellow arrows indicate an example of a series of transitions that occurs in vivo: pluripotent cells in the inner cell mass differentiate into progenitor cells (such as neural progenitors), which then differentiate into definitively differentiated cells (such as neurons). Alternatively, similar cells can be generated in vitro by following a different path (blue and white arrows). An example of experimentally induced transition from undifferentiated to differentiated cells is the generation of trophoblast stem cells from mES cells by either repression of the transcription factor Pou5F1/Oct4 or overexpression of Cdx2 (Niwa et al., 2000; Niwa et al., 2005). The model also illustrates transitions that, as far as we know, never occur in vivo. The green arrow in Figure 2 illustrates the generation of pluripotent cells by experimental overexpression of four transcription factors (Myc, Klf4, Pou5F1/Oct4, and Sox2) in fibroblasts, producing induced pluripotent cells (Okita et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007). The red arrow illustrates the hypothetical process of "transdifferentiation" that has been postulated to occur between different types of differentiated cells.

Future Directions

Currently, research in the epigenetics of ES cells is motivated not only by interest in the fundamental mechanisms of control of differentiation but also, in the case of hES cells, by a desire to make these cells clinically useful. One of our greatest challenges is to develop strategies to generate hES cell-derived cells that can be both safe and efficacious for cell replacement and repair. One critical issue is immune rejection; attempts to generate patient-specific pluripotent cells by somatic nuclear transfer have so far been unsuccessful, both because of technical difficulties and the scarcity of human oocytes to use as hosts for somatic nuclei. The recent success of molecular biology-based reprogramming methods makes it seem possible to take any person's somatic cells, make them pluripotent, and turn them into the cell types—pancreatic islets, neurons, glial cells, and cardiac myo-

blasts—that are needed to restore that person's damaged tissues. Currently, this reprogramming method requires genetic engineering—insertion of transgenes into the cells. However, technical advances in analysis of epigenetic regulatory mechanisms may provide the knowledge that will lead to new simpler methods to reprogram cells. If we can directly reset the epigenetic state of cells without disturbing their genetic integrity, the many currently imagined therapeutic approaches to incurable human diseases may come closer to becoming realities.

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