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REVIEW

Regulation beyond genome sequences: DNA and histone methylation in embryonic stem cells

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Abstract Embryonic stem (ES) cells distinct themselves from other cell type populations by their pluripotent ability. The unique features of ES cells are controlled by both genetic and epigenetic factors. Studies have shown that the methylation status of DNA and histones in ES cells is quite different from that of differentiated cells and somatic stem cells. Herein, we summarized recent advances in DNA and histone methylation studies of mammalian ES cells. The methylation status of several key pluripotent regulatory genes is also discussed.

Keywords Embryonic stem (ES) cells, epigenetic, DNA methylation, histone methylation

1 Introduction

Embryonic stem (ES) cells are derived from the inner cell mass of blastocysts and possess many remarkable features. On one hand, ES cells have the capability to self-renew and can replicate indefinitely if permitted. On the other hand, these cells are pluripotent and can differentiate into other cell types with diverse functions (Smith, 2001; Keller, 2005; Bibikova et al., 2008). These unique properties make ES cells a promising system for studying cell-based medicine in regenerative therapy.

The maintenance of ES cell pluripotency and the differentiation of ES cells into other cell types are both controlled by complicated genetic and epigenetic regulations. Epigenetic regulations refer to the non-sequence based changes that alter gene expression (Wu and Morris, 2001), mainly encompass DNA methylation, histone methylation and acetylation, as well as regulations by non-coding RNAs (Table 1) (Jeffares et al., 1998; Tucker, 2001; Goldberg et al., 2007). The two best understood epigenetic regulation types in ES cells are DNA methylation and changes in chromatin structure through modifications of histones. These two key epigenetic regulation processes coordinate in ES cells to maintain their unique features.

Table 1 Features and functions of major epigenetic regulation processes

epigenetic regulation	features	functions
DNA methylation	typically occur at "—C—phosphate—G—", cytosine and guanine separated by a phosphate (CpG) dinucleotide	transcription repression
histone modification	may have different effects on gene expression according to its type, position and state	acetylation: transcription activation methylation: transcription activation or repression
non-coding RNA regulation	include short interfering RNAs (siRNAs), microRNAs, etc.	mainly induce gene silencing

2 DNA methylation

DNA methylation is the first discovered epigenetic regulation mark, referring to the addition of a methyl group to the 5 position of cytosine pyrimidine ring. DNA methylation plays vital roles in various biological processes, including maintenance of genome stability, repression of endogenous retrovirus, X chromosome inactivation, genomic imprinting, and developmental gene regulation (Bird, 2002; Li, 2002). DNA methylation can be classified as *de novo* methylation and maintenance of methylation, both processes are governed by members of the DNA methyltransferase (Dnmt) family. Dnmt3a/3b methyltransferases mainly function to catalyze *de novo*

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DNA methylation, and Dnmt1 is responsible for the maintenance of methylation patterns. In mammals, DNA methylation typically occurs at "—C—phosphate—G—", cytosine and guanine separated by a phosphate (CpG) sites. However, non-CpG methylation is prevalently observed in ES cells (Haines et al., 2001; Dodge et al., 2002; Lister et al., 2009).

DNA methylation sites can be detected by bisulfite treatment, enzymes that recognize methylated CpG DNA sites, or antibodies that bind to DNA methylation associated proteins. Bisulfite treatment converts unmethylated cytosine residues to uracil, but leaves 5-methylcytosine residues unaffected. Therefore, methylated cytosines can be identified. In combination with sequencing or microarray hybridization techniques, several methods have been developed based on one or more mechanisms mentioned above to determine DNA methylation sites (Zhao et al., 2008). Especially with the recent advances of next generation sequencing technology, genome wide methylation maps can now be easily obtained (Table 2) (Fraga and Esteller, 2002; Weber et al., 2005; Callinan and Feinberg, 2006; Khulan et al., 2006).

Table 2 Methods to detect DNA methylation sites

methods	principles
immunoprecipitation of methylated DNA (MeDIP)	Genomic DNA is randomly sheared and immunoprecipitated with an antibody that specifically recognizes methylcytidine.
methylation specific PCR (MSP)	based on bisulfite conversion, followed by traditional PCR
restriction landmark genome scanning (RLGS)	Restriction enzymes differentially recognize methylated and unmethylated CpG sites.
reduced representation bisulphite sequencing (RRBS)	Selected methylation-sensitive restriction enzymes treated fragments combined with bisulfite sequencing.
Hpall-tiny fragment enrichment by ligation-mediated PCR (HELP)	based on two restriction enzymes (Hpa*II* and Msp*I*), followed by ligation-mediated PCR
Chromatin immunoprecipitation-microarray technology (ChIP-chip)	The ChIP-enriched DNA is amplified, labeled and hybridized onto DNA microarray.
chromatin immunoprecipitation-sequencing (ChIP-seq)	The ChIP DNA is ligated to adaptors and subjected to cluster amplification by bridging PCR and massive parallel sequencing.

3 DNA methylation in ES cells

In the past few years, a number of reports have considerably expanded our understanding of DNA methylation in ES cells. The methylation patterns of mouse and human ES cells have been shown to be significantly different from those of somatic cells or other stem cell lines (Shiota et al., 2002; Bibikova et al., 2006). For example, Bibikova and his colleagues examined the methylation status of 1536 CpG sites (selected from the 5'-regulatory regions of 371 genes with important functions in regulating cellular behavior and differentiation) in 36 human ES (hES) cell populations derived from 14 independently isolated hES cell lines at various culturing stages, as well as five other cell types (including cancer cell lines, adult stem cell lines, lymphoblastoid cell lines, and cells from normal human tissues) (Bibikova et al., 2006). Clustering analysis based on the obtained methylation data revealed that all hES cells could be easily separated from other cell types, whereas adult stem cell lines are more similar to differentiated somatic cells. In addition, the methylation status of hES cells also exhibits significant differences between male and female samples (Bibikova et al., 2006).

In normal cells, CpG islands which mostly locate at promoter regions are generally hypomethylated, whereas CpG poor regions are usually hypermethylated (Jones and Baylin, 2002). In ES cells, it is widely accepted that CpG islands are protected from undergoing *de novo* methylation by their inherited common sequence elements which will be recognized by some transcription factors (Latham et al., 2008). In order to provide a genome-wide profile of promoter DNA methylation in mouse ES (mES) cells, Fouse's group immunoprecipitated methylated DNA fragments from wild-type mES cells and mutant mES cells which were depleted of DNA methylation (Fouse et al., 2008), and cross-hybridized the pull-down DNA samples with Agilent mouse promoter microarrays. A total of 6127 genes (39.4% of all genes with promoter sequences probed on the microarray) were identified to be methylated at promoter regions in mES cells. On the other hand, a set of 5074 genes were defined as unmethylated. Gene ontology analysis revealed that unmethylated genes are enriched with functions related to transcription, protein and RNA metabolism processes, as well as some house keeping and pluripotency genes, suggesting their roles in the maintenance of stem cell property. In contrast, differentiation and signal transduction related genes are enriched among methylated genes. By comparing the DNA methylation positions with CpG locations, the authors identified that DNA methylation in mES cells primarily occurs at the CpG sites located within low-GC or intermediate-GC content promoter regions, or at the non-CpG island regions of high-GC content promoters.

In another study, Meissner and colleagues used high-throughput reduced representation bisulphite sequencing (RRBS) method to generate genome-scale DNA methylation profiles at a nucleotide resolution for mES cells (Meissner et al., 2008). The obtained 21 million high quality RRBS reads almost covered every CpG dinucleotide of the mouse genome, with $> 80\%$ of reads being 'largely methylated' and $< 20\%$ of reads being 'largely unmethylated'. They further confirmed that high GC regions tend to be protected from DNA methylation by

showing that CpGs located in regions with high CpG density ($>7\%$ over 300 bp) are usually unmethylated, whereas CpGs in low CpG density regions ($<5\%$) are to the contrary. They also compared DNA methylation patterns between mES cells and ES-derived neural precursor cells (NPCs) to investigate the changes of DNA methylation during cell differentiation. The results showed that although most CpG methylation levels are highly correlated between mES cells and NPCs, still 8% of unmethylated CpGs in ES cells became largely methylated in NPCs. On the other hand, 2% of methylated CpGs in ES cells lost their methylation in NPCs (Meissner et al., 2008). This may indicate that methylation of CpGs are dynamic epigenetic marks during cellular differentiation. And it is reasonable to assume that the *de novo* methylated and demethylated genes during the differentiation processes of mES cells into various types of somatic cells might have some differences.

The expression of transcription factors Oct3/4, Nanog and Sox2 has been proven to be crucial for the pluripotency of ES cells (Silva and Smith, 2008). The Oct3/4 genes belong to the Pit Oct Unc (POU) family transcription factor, and are essential for the establishment of pluripotency during the early-implantation stages of embryos (Nichols et al., 1998). Nanog is a homeobox transcription factor that belongs to the NK-2 class. It has been shown that the expression of Nanog alone is sufficient to maintain the pluripotent stage of mES cells in the absence of leukemia inhibitory factor (Chambers et al., 2003). Sox2 is a member of the highly mobility group (HMG)-box transcript factor family, and is also essential for the maintenance of pluripotency as well as early cell fate. The expression of Oct3/4 and Nanog is restricted to pluripotent cells, whereas Sox2 is also detected in the primitive ectoderm and neural ectoderm, in addition to the inner cell mass, epiblast and germ cells (Niwa et al., 2000; Avilion et al., 2003; Chambers et al., 2003). Both Oct4 and Nanog are devoid of CpG islands in promoter regions (Yeo et al., 2007), whereas the promoter region of Sox2 is CpG rich. The promoter regions of Oct4 and Nanog are hypomethylated in ES cells, but acquire significant methylation during cell differentiation process (Fig. 1). However, the promoter region of Sox2 gene is almost completely unmethylated in both ES cells and differentiated cells. Such methylation patterns are of great importance for ES cells to set up correct gene expression patterns and, in turn, are essential for the maintenance of ES cell property.

4 Histone methylation

Histones are highly conserved alkaline proteins located in eukaryotic nuclei, which pack DNA into structural units called nucleosomes. In eukaryotes, nucleosomes are the basic repeating subunits of chromatin. The nucleosome core particle is comprised of approximately 147 bp genomic DNA wrapped around an octamer of histone proteins, including 2 copies of each of the core histones H2A, H2B, H3, and H4 (Fig. 2) (Luger et al., 1997; Strahl and Allis, 2000; Jenuwein and Allis, 2001). Modifications on histones can alter their association with DNA and other proteins, therefore affect the transcription of corresponding genes (Narlikar et al., 2002).

The types of modifications on histone proteins include methylation, acetylation, phosphorylation, ubiquitination, and sumoylation. Among them, methylation and acetylation are the two major and most studied modification types. Acetylations frequently occur at amino-terminal lysines at positions 9, 14, 18, 23 of histone H3 proteins, and lysines at positions 5, 8, 12, 16 of histone H4 proteins (Roth et al., 2001). Acetylation usually causes changes in chromatin structures to make the genes more permissive for transcription. Methylation of histone proteins can occur at either lysine or arginine residues. Three states of lysine methylation have been identified. Monomethylations

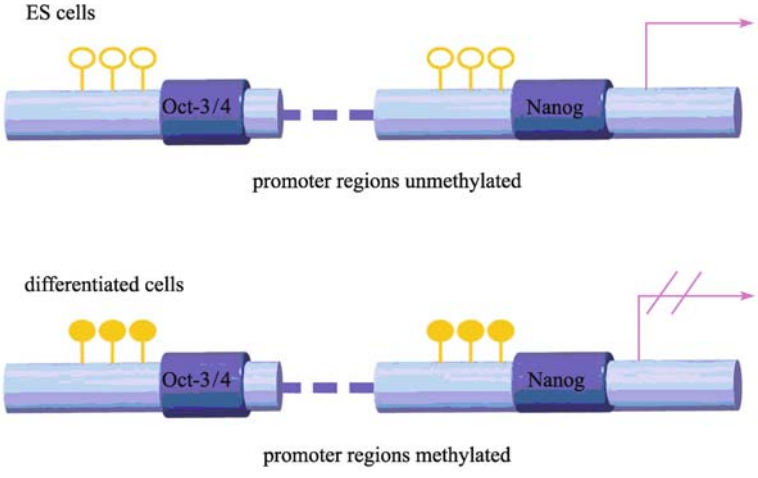

Fig. 1 The DNA methylation status at Oct3/4 and Nanog promoter regions in ES and differentiated cells

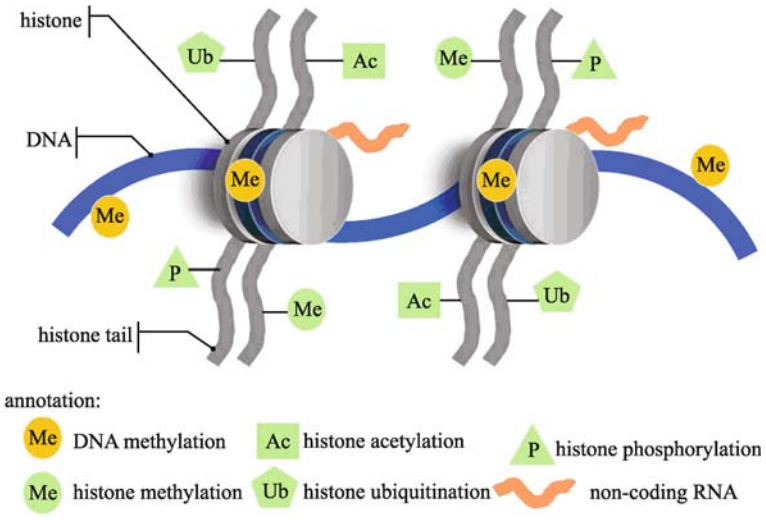

Fig. 2 Commonly observed types of epigenetic modifications

usually occur at H2BK5, H3K4, H3K9, H3K27 and H3K79 sites, and result in gene transcription activation. Dimethylations have been detected at H3K79 sites and also associated with gene activation. Trimethylations at different sites have contradictory effects. H3K4me3 serves as a gene activation mark, whereas H3K9me3 and H3K27me3 repress gene expression (Table 3) (Barski et al., 2007; Benevolenskaya, 2007; Koch et al., 2007; Steger et al., 2008). Arginine methylation on histone proteins has been detected at H3R2, H3R8, H3R17, H3R26, and H4R3 sites (Ng et al., 2009). Both active and repressive chromatin states can be regulated by arginine methylations.

Table 3 States of histone lysine methylation

monomethylation	dimethylation	trimethylation
activation (H2BK5, H3K4, H3K9, H3K27, H3K36, H3K79, H4K20)	activation (H3K4, H3K9, H3K27, H3K79)	activation (H3K4, H3K36, H3K79)
no repression	no repression	repression (H3K9, H3K27, H3K79)

5 Histone methylation in ES cells

It has been shown that the status of chromatin structures maintained by histones in nucleosomes plays a key role in regulating gene expression as well as ES cell pluripotency. Compared to differentiated cells, ES cells have overall decondensed chromatins, elevated acetylation levels of histone H3 and H4, as well as decreased H3K9 methylations (Meshorer and Misteli, 2006).

Most studies on ES cell histone methylation focused on examining the methylation status of H3K4me3 and H3K27me3 due to their association with promoter regions and controversial effects on regulating gene expressions. In 2006, Bernstein's team examined histone H3K4 and H3K27 methylation patterns in mouse ES cells across a subset of highly conserved noncoding element rich loci using a combination of ChIP and tilling arrays covering 60.3 Mb genomic regions. Besides the identification of H3K4me3 associated active chromatin regions as well as H3K27me3 associated repressive chromatin regions, the co-existence of these two adverse modifications was observed at 109 genomic regions (Bernstein et al., 2006). Such H3K4me3 and H3K27me3 co-existence regions were named "bivalent domains" for the presence of both active and repressive histone modifications. Genes with "bivalent domain" at their promoters are mainly key developmental regulators and their expression is poised in ES cells (Fig. 3) (Pan et al., 2007). Silencing of these genes by "bivalent domains" is thought to be a key factor for ES cell pluripotency. During the differentiation process of ES cells, the presence of H3K4me3 and H3K27me3 bivalent modification decreased gradually (Mikkelsen et al., 2007).

Follow up studies with genome-wide analysis of H3K4me3 and H3K27me3 modification sites in mouse ES cells confirmed the presence of "bivalent domains", and identified many novel features of histone modification in ES cells. Mikkelsen and colleagues immunoprecipitated DNA fragments with various types of histone methylations and performed Illumina sequencing to resolve the methylation locations (Mikkelsen et al., 2007), therefore obtained a single-nucleotide based genome-wide histone methylation profile. They found that about 22% out of 2525 high CpG promoters are bivalently modified by H3K4me3 and H3K27me3. Comparison of ES cells with neuronal progenitor cells (NPCs) and embryonic fibroblasts (MEFs) revealed that a majority of bivalently modified CpG rich promoters lost one of the modifications and become either H3K4me3 or H3K27me3 modified in differentiated cells. Phylogenetic analysis revealed that more than half of "bivalent domains" contain conserved

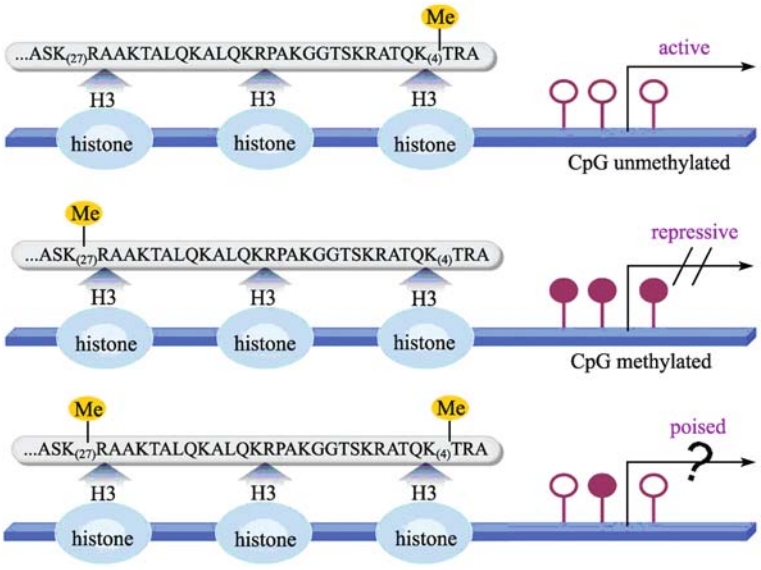

Fig. 3 Effects of histone modifications on "bivalent domains" in regulating gene expression.

sequence elements across different species, suggesting their functional importance (Zhao et al., 2007). It is worth to note that although bivalently modified genes are enriched of transcription factors, the above mentioned core regulators for ES cell pluripotency, Oct4, Nanog and Sox2, are only marked by H3K4me3.

For other histone methylation types, H3K9me3 and H4K40me3 are shown to be highly correlated in terms of methylation loci and specifically target repetitive sequences, and H3K4me3 or H3K9me3 overlap with each other at imprinted loci (Mikkelsen et al., 2007). In addition, some histone methylation sites exhibit allele specificity (Mikkelsen et al., 2007).

No systematic studies on histone arginine methylation of ES cells have been reported yet. In an attempt to investigate the effects of histone arginine methylation in regulating cell fates of mouse embryos, Torres-Padilla and colleagues showed that in four-cell stage blastomeres, cells that contribute to inner cell mass (ICM) and polar trophectoderm had higher arginine methylation levels than cells contribute to mural trophectoderm (Torres-Padilla et al., 2007). In addition, increment of arginine methylation levels by overexpression of an H3-specific arginine methyltransferase turned the progenies of blastomeres into the ICM, further proved that higher levels of histone H3 arginine methylation contribute to the acquirement of cell pluripotency in early embryo development (Torres-Padilla et al., 2007).

6 The relationship between DNA methylation and histone methylation

The functional link between DNA and histone methylations have been proved in many organisms (Lachner and Jenuwein, 2002). The mutation of a histone H3K9 specific methyltransferase gene, dim-5, caused almost complete loss of cytosine methylation in Neurospora crassa (Tamaru and Selker, 2001). Similar results were also obtained in Arabidopsis thaliana by mutating the H3K9 specific histone methyltransferase related kryptonite gene (Jackson et al., 2002). Interplay of different epigenetic regulators is also essential for ES cells. It has been shown that DNA (cytosine-5)-methyltransferase 3-like (DNMT3L) gene regulates *de novo* DNA methylation according to the states of methylation at H3K4 sites, suggesting that histone methylation can direct DNA methylation (Ooi et al., 2007). Demethylation at H3K9 and H3K27 sites induced loss of DNA methylation in ES cells (Ikegami et al., 2007). In addition, the lack of H3K9 methyltransferases (Suv39h1 and Suv39h2) can cause demethylation of satellite DNA in mES cells (Lehnertz et al., 2003).

7 Perspectives

Methylation marks are important epigenetic factors for maintaining ES cell properties. With the availability of genome-scale methylation detection methods developed in recent years, more comprehensive epigenetic landscapes are expected to emerge in the coming years. Besides providing more complete and dynamical methylation maps of ES cells, other remaining questions in ES cell methylation studies include, but are not limited to, what are the upstream regulatory signals and factors that trigger the establishment of methylation marks on either DNA or histones? How do methylations on different sites or on DNA and histones coordinate or compete with each other? Similarly, how does methylation mark function in concert with other epigenetic regulations to control gene

expression? Given so many methylation sites identified in ES cells, what are their positions in gene regulatory network and how do they act with other regulatory factors to control the unique properties of ES cells?

With the appearance of induced pluripotent stem (iPS) cells, whether the properties of iPS cells are to a large extent identical or different from ES cells remains to be an important issue with regard to the feasibility of medical applications of iPS cells. Although several groups have tried to address this question at epigenetic modification level, there are some discrepancies in their results (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Brambrink et al., 2008; Shi et al., 2008; Chin et al., 2009; Hochedlinger and Plath, 2009), probably due to the differences of cell resources they used. A better understanding of the epigenetic modification profiles in iPS cells, as well as how epigenetic modifications influence the cell reprogramming process will not only help to reveal the mechanisms underlying iPS technology, but also shed light on the improvement of pluripotency induction efficiency. Undoubtedly, the study and application of iPS techniques is one of the most rapidly-developed and promising field for research and medicine. It is expectable that in the coming years we will witness many exciting new discoveries that can greatly influence scientific researches and our lives.

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