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Promoter CpG Methylation Contributes to ES Cell Gene Regulation in Parallel with Oct4/Nanog, PcG Complex, and Histone H3 K4/K27 Trimethylation

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SUMMARY

We report here genome-wide mapping of DNA methylation patterns at proximal promoter regions in mouse embryonic stem (mES) cells. Most methylated genes are differentiation associated and repressed in mES cells. By contrast, the unmethylated gene set includes many housekeeping and pluripotency genes. By crossreferencing methylation patterns to genome-wide mapping of histone H3 lysine (K) 4/27 trimethylation and binding of Oct4, Nanog, and Polycomb proteins on gene promoters, we found that promoter DNA methylation is the only marker of this group present on ~30% of genes, many of which are silenced in mES cells. In demethylated mutant mES cells, we saw upregulation of a subset of X-linked genes and developmental genes that are methylated in wild-type mES cells, but lack either H3K4 and H3K27 trimethylation or association with Polycomb, Oct4, or Nanog. Our data suggest that in mES cells promoter methylation represents a unique epigenetic program that complements other regulatory mechanisms to ensure appropriate gene expression.

INTRODUCTION

Embryonic stem (ES) cells, which are derived from the inner cell mass of blastocyst embryos, have the potential to differentiate into all cell types including germ cells in vitro and in vivo, thus representing an ideal system for studying regenerative medicine (Keller, 2005). Factors influencing ES cell self renewal and differentiation include extracellular matrix, growth factors and cytokines, intracellular signaling molecules, transcription factors, and epigenetic regulators such as histone modification and DNA methylation (Keller, 2005). For example, key transcription factors such as Oct4 (encoded by *Pou5f1*), Nanog, and Sox2 form a transcription regulatory network in ES cells that activates genes essential for ES cell survival and proliferation while concurrently repressing those target genes that will be only activated during cell differentiation (Boyer et al., 2005; Loh et al., 2006), thus playing an essential role in maintaining the pluripotency and self-renewal of ES cells (Avilion et al., 2003; Chambers et al., 2003; Mitsui et al., 2003; Nichols et al., 1998).

More recently, the role of chromatin structure and epigenetic modifications in controlling gene expression during ES cell self-renewal and differentiation has been under intensive investigation (Bernstein et al., 2007; Guenther et al., 2007). For example, gene repression mediated by the Polycomb group (PcG) protein complex and the associated histone H3 lysine (K) 27 trimethylation is required for ES cell self-renewal and pluripotency (Boyer et al., 2006; Lee et al., 2006). In addition, the genome of ES cells contains domains with "bivalent" histone modifications of both H3K4 and K27 trimethylation (H3K4me3 and H3K27me3) that mark a number of differentiation genes, including many transcription factors, which are repressed in ES cells but "poised" to be activated upon differentiation (Bernstein et al., 2006; Mikkelsen et al., 2007; Pan et al., 2007; Zhao et al., 2007). However, the remaining approximately one-third of genes are not marked by histone modifications of either H3K4me or H3K27me3, and yet are mostly repressed in ES cells (Mikkelsen et al., 2007; Pan et al., 2007; Zhao et al., 2007). Our knowledge is also very limited as to how multiple regulatory mechanisms including transcriptional factors and epigenetic factors, such as histone modification and DNA methylation, are coordinated to control the "on" and "off" of pluripotent versus developmental genes in ES cells and during in vitro differentiation of ES cells.

DNA methylation in mammalian cells is postulated to play multiple roles in cell physiology, including genome stability, repression of endogenous retroviral and transposable elements, genomic imprinting, and developmental gene regulation (Bird, 2002; Jaenisch and Bird, 2003; Li, 2002; Robertson, 2005; Feng et al., 2007). During embryogenesis, levels of DNA methylation are dynamically regulated by the de novo DNA methyltransferase (Dnmt) 3a, Dnmt3b, and maintenance enzyme Dnmt1 (Chen and Li, 2004; Goll and Bestor, 2005). Failure to place or maintain the patterns of DNA methylation leads to early embryonic lethality in mice (Li et al., 1992; Okano et al., 1999) and also many human diseases including cancer, fragile-X, ICF, and ATRX syndromes (reviewed by Robertson, 2005). Interestingly, mES cells deficient

for DNA methylation can survive and proliferate in an undifferentiated state (Meissner et al., 2005; Tsumura et al., 2006) but undergo rapid apoptotic cell death upon in vitro differentiation (Panning and Jaenisch, 1996). Therefore, it is still unclear as to whether DNA methylation plays any role in gene expression and the maintenance of pluripotency in mES cells.

In mES cells, each of the highly expressed Dnmts play a specific role in the establishment and/or the maintenance of DNA methylation (Chen et al., 2003). The total level of methylcytosine in mES cells is similar to that in differentiated tissues such as kidney and liver cells in vivo (Biniszkiewicz et al., 2002). Several recent studies have attempted to identify changes in methylation patterns during long-term cultures or upon cell differentiation of ES cells. In both mouse and human ES cells, a subset of CpG islands are subject to de novo methylation during in vitro differentiation (Hattori et al., 2004 ; Kremenskoy et al., 2003; Shen et al., 2006). A more recent study demonstrated that an increase in DNA methylation occurs in selected CpG islands in a few lines of hES cells during long-term passages (Allegrucci et al., 2007). Overall, these studies suggest that methylation patterns in ES cells may be distinctly different from differentiated somatic cells and that the methylation status of ES cells or ES cell derivatives should be monitored carefully when they are used in regenerative medicine (Allegrucci et al., 2007; Shen et al., 2006).

We report here a comprehensive genome-wide mapping of promoter methylation patterns in undifferentiated mouse ES cells. We further examine the relationship between DNA methylation, histone modifications, and the promoter occupancy of pluripotent regulators such as Polycomb group proteins (PcG) and Oct4/Nanog in regulating gene expression in mouse ES cells. Our results reveal that CpG methylation patterns complement other regulatory mechanisms in maintaining the unique transcriptional program of undifferentiated mES cells.

RESULTS

Genome-wide Profiling of Promoter DNA Methylation in mES Cells

With the knowledge of genome-wide patterns of histone modifications, PcG binding, and Oct4/Nanog/Sox2 binding in mouse and human ES cells (Boyer et al., 2005, 2006; Bernstein et al., 2006; Mikkelsen et al., 2007; Pan et al., 2007; Zhao et al., 2007), we wanted to map DNA methylation patterns in gene promoters to address the role of DNA methylation in ES cells in the context of other regulatory mechanisms. One high-throughput method involves the enrichment of the methylated DNA through the use of immunoprecipitation with methylcytosine antibodies or methyl-binding domain from MeCP2 in a column (Cross et al., 1994; Weber et al., 2005). By coupling methylated DNA immunoprecipitation with DNA microarray chip technology (mDIP-Chip), genome-wide methylation profiles have been examined for human normal and cancer cell lines, as well as *Arabidopsis thaliana* (Keshet et al., 2006; Weber et al., 2005, 2007; Zhang et al., 2006; Zilberman et al., 2007). These studies have provided valuable insights into the function and evolution of DNA methylation in different types of cells and organisms.

Using a previously described mDIP protocol (see the Experimental Procedures) (Keshet et al., 2006; Zhang et al., 2006), we immunoprecipitated methylated DNA from a line of male wild-type (WT) mES cells (J1 cells) as well as J1-derived mutant ES cells (*Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$; *Dnmt1* KD [TKO]) that are virtually demethylated across the entire genome (Meissner et al., 2005). We performed crosshybridization of pull-down DNA from WT and TKO cells with Agilent microarrays, which contain 15,561 annotated gene promoters with a resolution of one 60-mer probe for every 200 bp region (see Table S1 available online). To annotate the methylation status of each probe on each promoter, we first classified promoter probes into two subgroups that are either within a CpG island (>200 bp, GC > 50%, observed/expected CpG ratio > 0.6) or in a region outside of CpG island. By this classification, ~25% of total probes are within CpG islands and 75% of probes are in non-CpG island promoter regions (Figure 1A). After plotting the averaged $\log_2$ ratios for each probe (WT over TKO) from seven replicates of independent mDIP/hybridization experiments, we found a clear bimodal distribution of probe sets within or outside CpG islands (Figure 1A). A majority of probes within CpG islands showed $\log_2$ ratios less than 0, suggestive of an unmethylated state. We confirmed the unmethylated status of nine promoter regions with probe $\log_2$ ratios < 0 by using established methylation assays such as bisulfite sequencing and McrBC/HpaII genomic PCRs (Figure 1B and Figure S1). In contrast, a large fraction of non-CpG island probes exhibit $\log_2$ ratios > 0, suggesting a methylated state. As shown in Figure 1B and summarized in Table S2, we confirmed that all 32 promoter regions we analyzed with probe $\log_2$ ratios > 0 were methylated. We further examined the methylation status of the entire promoter region by using the transient receptor potential channel 1 (*Trpc1*) gene locus on chromosome 9 as an example. The *Trpc1* promoter contains probes both inside and outside of CpG islands with probe $\log_2$ ratios ranging from −0.20 to +1.06. We found that significant hybridization signals ($\log_2$ > 0.2) are over the non-CpG island promoter regions (−250 bp to −1200 bp), but not near or over the CpG island (<0.2) (Figure 1C). Bisulfite genomic sequencing confirmed that the region containing the probes with signals less than 0.2 was either unmethylated or sparsely methylated. Moreover, a gradient of increasing methylation overlays the non-CpG island region in the promoter with hybridization signals > 0.2, which is confirmed to be heavily methylated (Figure 1D). The distribution of averaged probe $\log_2$ ratios for each of 15,561 gene promoters can be visualized through an online genome browser as seen in Figure 1C.

With the above data, we made a large-scale annotation of proximal promoters as either methylated or unmethylated by setting up the following stringent criterion. To be annotated as a methylated region, the average probe $\log_2$ ratios of WT over TKO in that promoter region should be $\geq$0.2 and the statistical significance of the difference between hybridization intensities of WT and TKO should be $p < 0.01$ (t test). Using this criterion, we have annotated 6127 genes (39.4%) as methylated, which contain at least one methylated domain surrounding the hybridized probe(s) in their proximal promoter (e.g., *TrpC1*, see Table S1). To be annotated as an unmethylated promoter, all probes on a promoter region should have average probe $\log_2$ ratios < 0 with $p < 0.01$ (t test). Using this conservative criterion, we annotated 5074 unique genes (32.6%) that are unmethylated in the entire proximal promoter region (Table S1). The remaining genes (28% out of a total 15,561 promoters) that do not meet our designated criteria are excluded from further analysis.

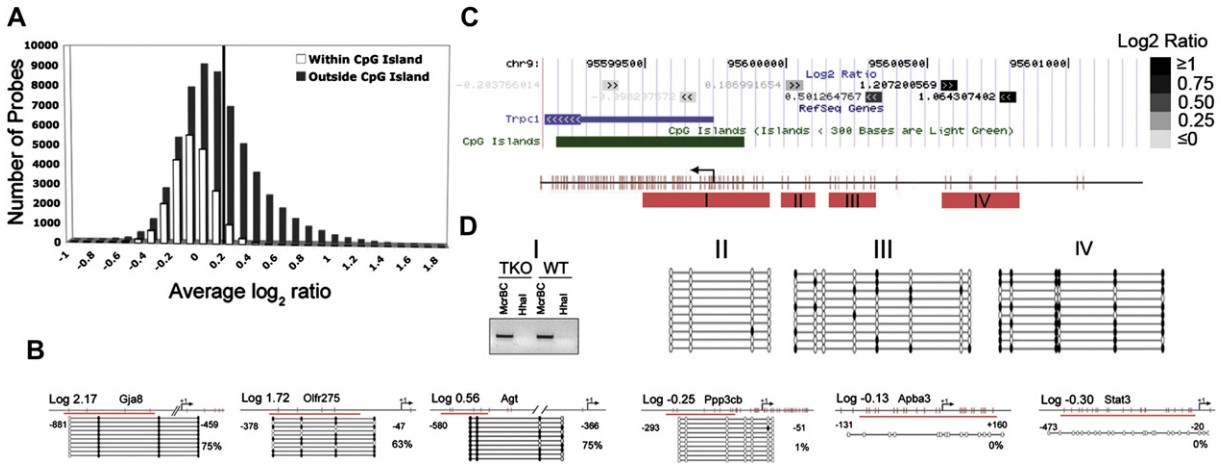

Figure 1. Using mDIP-CHIP Assay to Profile the Promoter Methylation Pattern in WT mES Cells

(A) All probes for the array are classified as either inside or within 50 bp of a CpG island (white) or outside a CpG island (gray) and then grouped by their average $\log_2$ ratio. The solid line is placed at $\log_2$ ratio of +0.2 value to annotate the proportion of methylated gene promoters.

(B) Using the average $\log_2$ ratio, we selected three genes thought to be methylated as well as three that were thought to be unmethylated. For the bisulfite confirmation, each line represents an individual clone and each circle represents a CpG dinucleotide. The red line indicates the region we analyzed. Filled circles are methylated CpGs, and open circles are unmethylated.

(C) The average signals ($\log_2$ ratio) for each probe (block with double arrowheads) in the promoter region are plotted in the UCSC genome browser—here we take the *Trpc1* gene as an example. Note the dark probes annotate probes with high $\log_2$ ratios, whereas light color probes represent probes with low or negative $\log_2$ ratios.

(D) The confirmation of methylation status for the probes in the *Trpc1* gene either inside or outside of a CpG island. Red bars (I–IV) indicate regions analyzed. Region I shows a gel picture of an McrBC-HhaI genomic PCR, indicating the unmethylated state of the CpG island of the *Trpc1* transcription start site. II–IV show bisulfite genomic sequencing results of methylated probes upstream of the CpG islands, indicating a gradient of methylation toward the probes with high $\log_2$ ratios (filled dots are methylated CpG sites).

Based on their entire CpG contents across the genomic region, gene promoters have been recently classified into one of three categories: HCP (high CpG promoter that contains a 500 bp region with a GC content $\geq$ 0.55 and a CpG observed to expected ratio $\geq$ 0.6), LCP (low CpG promoter containing no 500 bp interval and with a CpG observed to expected ratio $\geq$ 0.4), and ICP (intermediate CpG content promoter with CpG density between HCP and ICP) (Mikkelsen et al., 2007; Weber et al., 2007). Using the data set generated by Mikkelsen et al. (2007), we further examined the CpG content in the pool of methylated versus unmethylated promoters in mES cells. We found that in the pool of methylated genes, 51% of them belong to the HCP cluster, which is much lower than the genome average (67%) (Figure 2A). In contrast, in the pool of unmethylated genes, over 85% of genes are considered HCP promoters (Figure 2A). Detailed analysis of the distribution of methylated probes over the HCP promoters, which should contain at least a CpG island by the definition, indicated that only 3% of HCP genes have a methylated probe that overlaps with the CpG island itself (Figure 2C and Table S1). In contrast, in the pool of ICP and LCP genes, ~80% are annotated as methylated genes (Figure 2B). We conclude that DNA methylation in mES cells primarily takes place on ICP and LCP promoters or on non-CpG island regions of HCP promoters.

Gene Ontology Analysis of Unmethylated versus Methylated Promoters in mES Cells

To understand further the role of DNA methylation in mES cells, we performed gene ontology analysis of the 6127 methylated genes in mES cells. We found that methylated genes can be classified into sensory perception in response to stimuli and cell signaling molecules (Figure 3A and Table S3). This suggests that genes that exhibit DNA methylation are late differentiation-associated and signal transduction genes. Comparing these genes with gene expression data sets for mES cells in the GEO database also indicated that a majority of these genes are not expressed in mES cells (data not shown).

In contrast, gene ontology analysis on the list of 5074 unmethylated genes further indicated that over 50% of unmethylated genes are associated with transcription machinery, protein and RNA metabolic process, and other cellular machinery essential for cell survival and proliferation (Figure 3B and Table S3). This result suggests that the unmethylated status of the proximal gene promoter is a good indicator for those genes that would be expressed in mES cells. In addition, gene ontology analysis showed that approximately 10%–15% of unmethylated genes are classified as genes involved in cell differentiation and developmental process, which are not expressed in mES cells. Potentially, the repression of this small subset of development genes in mES cells could be through other mechanisms such as PcG complex- and Oct4/Nanog complex-mediated gene inhibition (see below) (Boyer et al., 2005, 2006; Lee et al., 2006; Loh et al., 2006).

Relationships between DNA Methylation and Histone Modifications in mES Cells

To understand further the role of epigenetic regulation in mouse ES cells, we compared our methylation data to recent whole-genome histone mapping (Mikkelsen et al., 2007). Mapping of histone modifications in the promoter region of both mouse and

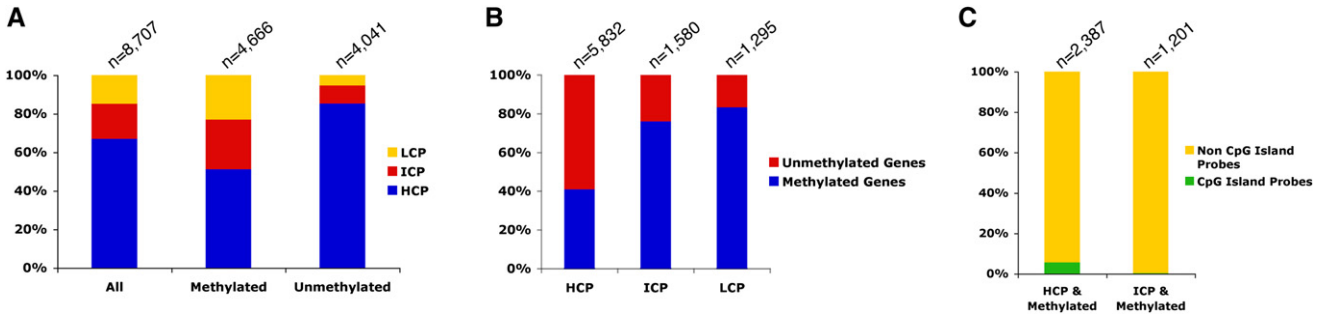

Figure 2. CpG Density of Methylated and Unmethylated Promoters

(A) Classification of all promoters, methylated promoters, or unmethylated promoters with high (HCP), intermediate (ICP), and low (LCP) CpG content. It should be noted that these classifications were assigned for V6.5 mouse ES cells, which are similar but not the same as the J1 mouse ES cells used in our study.

(B) Breakdown of methylation status for HCP, ICP, and LCP promoters.

(C) Percentage of genes with methylated CpG islands in HCP- and ICP-containing genes. By definition, there are no CpG islands in LCP-containing genes.

human ES cells (Guenther et al., 2007; Mikkelsen et al., 2007; Pan et al., 2007; Zhao et al., 2007) has shown four distinct populations of genes that are associated with either H3K4me3 or H3K27me3 alone, bivalent H3K4me3 and H3K27me3, or neither of these marks. H3K4 and H3K27 trimethylation can be explained by the actions of Trithorax (trxG) and PcG complexes and possibly the occupancy of key transcription factors, but the absence of both H3K4me3 and H3K27me3 in a significant portion of genes (27%–33% of all annotated genes) suggests the presence of other unique epigenetic marker(s) (Mikkelsen et al., 2007; Pan et al., 2007; Zhao et al., 2007). By comparing the distribution across the genome (Mikkelsen et al., 2007), we found that the subset of genes lacking both H3K4 and K27me3 marks are significantly enriched in methylated genes, but underrepresented in unmethylated genes (Figure 4A). We noticed that the majority of genes without either H3K4 or K27me3 are found in ICP and LCP genes (Mikkelsen et al., 2007), which are enriched in the pool of methylated genes in our study (Figures 4B and 2B). Moreover, examination of the overlap between methylated genes and the subset of genes lacking both H3K4 and K27me3 showed that ~87% of genes with neither of these marks are methylated in the proximal gene promoter regions (Figure 4C). In fact, the gene ontology terms for those genes without H3K4 and K27me3 are similar to the terms in our Gene Ontology analysis of methylated promoters in mES cells (data not shown; compare Figure 3A here with Figure 5 in Pan et al., 2007). Thus, for the roughly one-third of genes without H3K4 and K27me3, our analysis suggests that DNA methylation could be considered a distinguishing epigenetic mark.

To analyze further the significance of the presence of DNA methylation on the subpopulation of genes without H3K4 or K27me3, we used previously published expression array data (Mikkelsen et al., 2007) to assay whether the presence of DNA methylation is correlated with gene repression in mES cells. We found that ~80% of methylated genes lacking both histone H3K4 and K27me3 marks are not expressed in mouse ES cells (data not shown). We conclude that DNA methylation in proximal gene promoters is highly correlated with gene silencing for genes that lack both H3K4 and H3K27 trimethylation.

For the pool of genes carrying H3K4me3 only, we found that unmethylated promoters make up ~60% (Figure 4C), consistent with the possibility that these genes are the most actively transcribed in mES cells (Mikkelsen et al., 2007). For the pool of genes carrying H3K4/K27me3 bivalent marks, we found that 53% are unmethylated (Figure 4C). This result suggests that repressive mechanisms associated with bivalent H3K4/K27 such as Polycomb-mediated gene silencing may be a predominant factor in controlling gene activities for this subset of genes in mES cells (see next section). However, DNA methylation could serve as a secondary repressive mechanism to modulate further the activities of these bivalent genes in mES cells or upon cell differentiation. Finally, for genes with only the H3K27me3 mark, although the pool of genes may be too small (n = 66) to draw definitive conclusions, ~70% are methylated (Figure 4C).

Relationships between DNA Methylation and PcG Complex- or Oct4/Nanog Complex-Mediated Gene Regulation in mES Cells

In mouse and human ES cells, Polycomb proteins were found to bind and repress a subset of developmental genes, rendering them "poised" for expression upon differentiation (Boyer et al., 2006; Lee et al., 2006). To determine directly whether Polycomb-targeted genes in mES cells also show promoter methylation, we compared the PcG-targeted genes in mES cells with our list of methylated (n = 6127) and unmethylated (n = 5074) promoters and found that only 28.7% (98 out of 342) exhibit promoter DNA methylation (Figure 4D and Tables S1 and S4). This result suggests that the pool of genes targeted by DNA methylation and PcG are distinctively different. That a majority of PcG complex-repressed genes are unmethylated (71.3%) in mES cells is more compatible with the possibility that the PcG-targeted genes are poised to be activated upon cell differentiation.

It is also known that transcription factors Oct4, Nanog, Sox2, and Stat3 are required for the pluripotency and self-renewal of mES cells. The gene promoters of Oct4, Nanog, Sox2, and Stat3 are all unmethylated, allowing for high levels of expression in mES cells (Table S1 and Figure 1B) (Imamura et al., 2006). Oct4, Nanog, and Sox2 form a regulatory circuit that maintains their own expression and that of many other genes essential for ES cell self-renewal and at the same time represses differentiation genes (Boyer et al., 2005; Loh et al., 2006; Pan and Thomson, 2007; Walker et al., 2007). We therefore examined whether

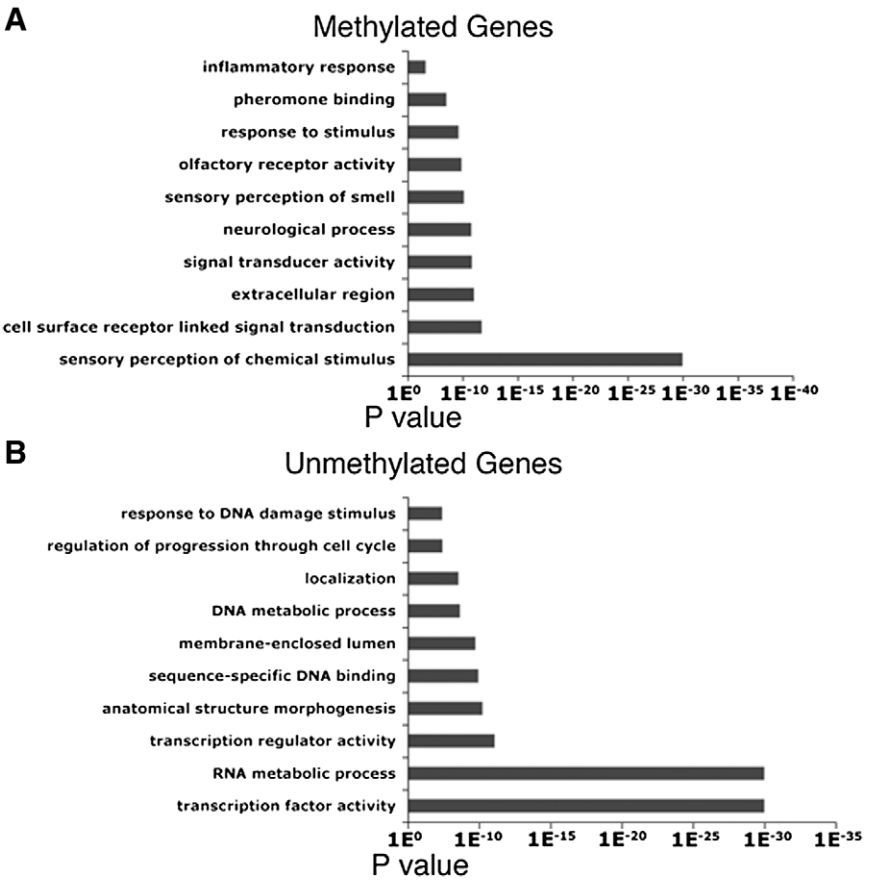

Figure 3. Comparison of Methylated and Unmethylated Genes with H3K4me3 and H3K27me3

Gene ontology classifications for methylated (A) or unmethylated (B) genes. The GO term is on the y axis, and the p value indicating significance of enrichment is on the x axis.

promoter methylation is correlated with either the activation or repression of genes by the Oct4/Nanog/Sox2 complex in mES cells. Because we were focusing on the relationship between proximal promoter methylation and occupancy of Oct4/Nanog, we confined our search to genes that are bound by Oct4/Nanog within 10 kb of the transcription initiation site and either methylated or unmethylated on our promoter array (109 genes bound by Oct4 and 211 target genes bound by Nanog). We found that ~42% and 36% of Oct4- and Nanog-targeted genes, respectively, contain methylation domain(s) in the proximal promoter regions in mES cells (Figure 4D and Tables S1 and S4). Conversely, 64% of Nanog targeted genes and 58% of Oct4-targeted genes are totally unmethylated in mES cells (Figure 4D and Tables S1 and S4). To confirm further these results, we analyzed all bound loci for Nanog and Oct4, some of which are up to 500 kb away from a known transcript. Again, we found similar results for all Nanog and Oct4 bound loci (46% of genes for both Nanog and Oct4 are methylated). When we looked to see whether there was any association with gene expression, we found that 91%–93% of unmethylated Oct4/Nanog proximal bound genes were expressed (data not shown). When looking at genes that are methylated and bound by either Nanog or Oct4, we found that ~75% are expressed (data not shown). Thus, DNA methylation might play a small role in dampening the expression of Oct4/Nanog bound genes, but overall does not appear to have a strong effect. Taken together, our data favor the hypothesis that methylation-mediated repression is independent of Oct4/Nanog-mediated gene expression. This suggests that DNA methylation could be a separate epigenetic regulator that works in parallel, but generally in a nonoverlapping fashion with the action of the Polycomb-repression complexes and transcription factors such as Oct4 and Nanog in regulation of the pluripotency and differentiation program of mES cells.

Comparison of Gene Expression in Methylation-Proficient and -Deficient mES Cells

To examine whether DNA methylation directly regulates the expression of genes in mES cells, we carried out genome-wide gene expression analysis (see the Experimental Procedures) in WT and methylation-deficient mES cells that lack all three Dnmts (TKO cells) (Meissner et al., 2005). The TKO ES cells are virtually demethylated across the entire genome but exhibit cell proliferation properties and embryonic stem cell markers similar to those of the parental WT J1 mES cells. In the first round of bioinformatics analysis with a fold change cutoff of 2 (see the Experimental Procedures), our genome-wide expression profiling yielded a list of 337 genes that are upregulated and 113 genes that are downregulated in the TKO cells compared to WT (Table S5). Using a less strict filter that accounts for genes that may be turned completely off in the WT condition but slightly expressed in the TKO cells (Ohm et al., 2007), we found an additional 53 genes that were upregulated upon loss of DNA methylation. The inability of methylation-deficient ES cells to differentiate can result in potential inaccuracies when analyzing genes that are downregulated. For example, the existence of a small percentage of partially differentiated cells found in WT ES cell cultures but not in the TKO cultures (due to cell death) could contribute to the list of downregulated genes in TKO cells. Therefore, the analysis of upregulation of genes in TKO cells versus WT cells is more robust for ascertaining the effect of DNA demethylation on gene expression.

Gene ontology analysis of these upregulated genes shows an overrepresentation of tissue-specific genes, such as transcription factors and signaling molecules (Figure 5A). We then examined the tissue specificity of each upregulated gene and found that testis- and oocyte-specific genes were highly enriched in the TKO cell line (Figure 5B and Table S6). We were interested to see whether there are any genomic loci that were enriched in deregulated genes. When we mapped the list of genes that were upregulated in TKO mES cells to their loci, we found that

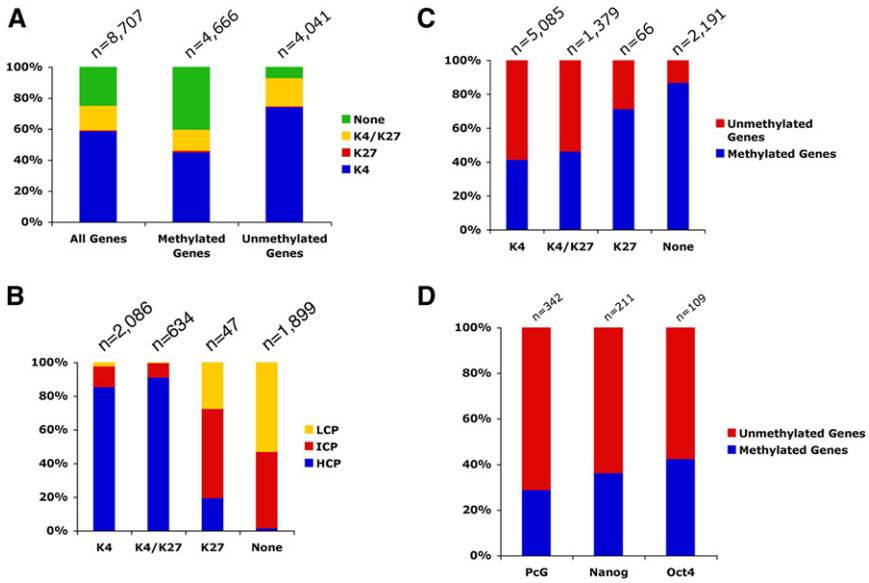

Figure 4. Comparison of Methylated and Unmethylated Genes with H3K4me3 and H3K27me3 as Well as Nanog, Oct4, and PcG Bound Genes

(A) Classification of all promoters, methylated promoters, or unmethylated promoters with H3K4me3, H3K27me3, H3K4me3 and H3K27me3 (bivalent), or neither mark. Methylated genes are enriched for genes without either histone mark.

(B) Percentage of K4, K4/K27, K27, or neither bound genes that are HCP, ICP, or LCP.

(C) Breakdown of methylation status for genes with H3K4me3 only, H3K27me3 only, H3K4me3 and H3K27me3 (bivalent), or neither mark.

(D) Bar graph showing the breakdown of methylation status for PcG, Nanog, or Oct4 bound genes in mES cells. The Nanog, Oct4, and PcG bound genes that are either methylated or unmethylated are listed in Table S1.

a high percentage are located on the X chromosome (14.5% of observed values versus 4% of expected) (Figure 5C). We confirmed the microarray data by real-time PCR for a few genes, including the X-linked genes *Rhox2* and *Magea3* (Figure 5D and S.D.F. and G.F., unpublished data). The upregulation of many X-linked genes was also found in a separate line of methylation-deficient mES cells due to *Dnmt1* gene deletion (Lei et al., 1996) (Figure S2), consistent with the notion that DNA demethylation is linked to the upregulation of this subset of X-linked genes.

We next looked to see what histone marks the TKO upregulated genes had and whether they were also marked by DNA methylation. When we look at the breakdown of histone marks among the TKO upregulated genes, we find that 40% are lacking both H3K4 and K27me3, 35% are bivalent K4/K27me3, 23% are K4me3, and 2% are K27me3 only. Of the TKO upregulated that are not marked by either histone mark, we find that 85% are methylated in WT mES cells (Table S7). In fact, the X-linked *Rhox* family genes that are upregulated in the absence of DNA methylation fall in this category (Figure 5D). This result suggests that DNA methylation is one of the major repressive mechanisms for a subset of genes that lack both H3K4 and K27me3 in mES cells. In contrast, when we examined the methylation status for the subset of genes with bivalent H3K4/K27me3 or H3K4me3 only in WT mES cells that would be upregulated in TKO cells, we found they are not as frequently methylated as genes without H3K4 or K27me3 (37% and 50%, respectively).

To determine whether DNA demethylation also affects the expression of any genes that are repressed by PcG or Oct4/Nanog/Sox2, we compared the genes that are upregulated in the DNA methylation-deficient mouse ES cells to the list of genes that are bound by the PcG complex and Oct4/Nanog (Boyer et al., 2006; Loh et al., 2006). Interestingly, only 5.7% (29/512) of upregulated genes in TKO mES cells overlap with the PcG bound genes (Figure 6 and Table S7). This result provides further evidence that the genes repressed by PcG are distinct from the DNA-methylated gene set. Similarly, we found that only 1.7% (9/525) of Nanog and/or Oct4 proximal promoter (within 10 kb) bound genes (or 1.2% [37/3080] of all Nanog and/or Oct4 bound genes [up to 500 kb from the transcript]) (Loh et al., 2006) overlap with the upregulated genes in demethylated ES cells (Figure 6 and Table S7), although it should be noted that the majority of genes that are bound by Oct4/Nanog and methylated are expressed even in WT cells. Nevertheless, these results also support the conclusion that DNA methylation and the transcriptional factor complex containing Oct4/Nanog play distinct roles in gene regulation in pluripotent mES cells.

DISCUSSION

ES cells represent a unique type of stem cell that can undergo indefinite cycles of self-renewal while maintaining pluripotency. Previous studies have identified many crucial gene transcription factors and regulatory networks that are required for maintaining the "stemness" of ES cells (Avilion et al., 2003; Chambers et al., 2003; Ivanova et al., 2006; Mitsui et al., 2003; Nichols et al., 1998; Walker et al., 2007). One of the insightful conclusions is that Oct4, Nanog, and Sox2 form transcriptional circuitry to activate their own expression in a forward feedback manner; furthermore, the Oct4/Nanog/Sox2 complex promotes expression of those genes required for the self-renewal of ES cells, but represses the developmental genes that will only be activated upon cell differentiation (Boyer et al., 2006; Loh et al., 2006). In addition, along with Oct4/Nanog/Sox2, Trithorax complex- and Polycomb complex-mediated histone modifications are also involved in the activation or repression genes, as well as in maintaining the "poised" nature of some genes (Mikkelsen et al., 2007; Walker et al., 2007; Pan et al., 2007; Zhao et al., 2007). In this report, we have comprehensively mapped CpG methylation in the proximal gene promoter regions and identified 6127 methylated and 5074 unmethylated proximal gene promoters. Our data help refine the emerging epigenetic landscape of mES cells. By comparing promoter DNA methylation with histone modifications, we can gain insights into how overlapping or independent epigenetic regulators regulate particular sets of genes (Figure 7). One of the interesting findings in this study is that DNA methylation

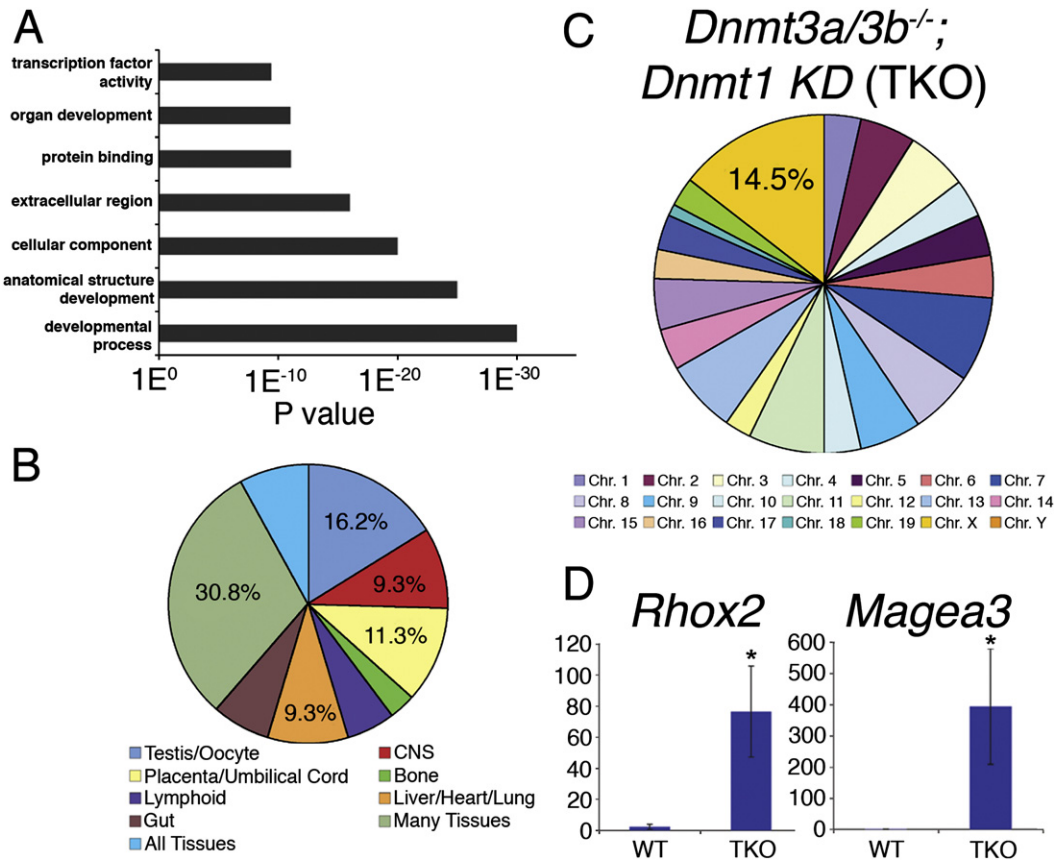

Figure 5. Gene Expression Profiling of DNA Methylation-Deficient mES Cells

(A) Gene ontology analysis for upregulated genes in TKO cells shows enrichment of transcription factor activity, protein binding, extracellular region, and developmental genes.

(B) Tissue specificity of overexpression genes in TKO cells. Upregulated genes were analyzed for the tissues that are normally expressed in using the GNF database. Note the overrepresentation of genes that are expressed in reproductive tissues including ovary/testis (16.2%) and placenta/umbilical cord (11.3%).

(C) The chromosomal location of the 390 genes that are upregulated in TKO mES cells compared to WT mES cells (clockwise from chromosome 1 to X and Y sex chromosomes). The percentage of genes upregulated on the X chromosome is 14.5%.

(D) Confirmation of *Rhox2* and *Magea3* that are upregulated in DNA methylation-deficient mES cells by qPCR analysis. *$p < 0.05$. Error bars represent standard deviations.

occurs in ~87% of the genes in ES cells that lack either H3K4me3 and H3K27me3 (Figure 4B). This population of methylated genes could potentially constitute close to one-third of all annotated genes in mouse and human ES cells (Mikkelsen et al., 2007; Pan et al., 2007; Zhao et al., 2007). Therefore, we conclude that DNA methylation in proximal gene promoter regions represents another major epigenetic marker that can distinguish different classes of genes in undifferentiated ES cells (Figure 7).

In this study, we provided evidence that DNA methylation is causally linked to the silencing of a cluster of X-linked genes and a subset of developmentally regulated genes. It should be noted that the mES cells used in these experiments are male (XY) and lack X inactivation; therefore, upregulation of X-linked genes in TKO mES cells cannot be related to deregulation of X inactivation. However, it is known that genes involved in germ cell differentiation and sex development are overrepresented on the X chromosome (Wang et al., 2001), and many of these genes tend to be duplicated on the X chromosome. Furthermore, DNA methylation is proposed to directly silence many genes involved in germ cell development (Maatouk et al., 2006). Indeed, *Rhox* family genes that have been shown to be duplicated extensively on the X chromosome are repressed by DNA methylation in somatic cells and ES cells (MacLean et al., 2005, 2006; Oda et al., 2006). Similarly, DNA methylation represses the *Mage* gene family and the *Dazl* gene that are related to germ cell development (Chuang et al., 2005; De Smet et al., 1999; Maatouk et al., 2006). Finally, demethylation-induced overexpression of *Mage* family genes is also observed in somatic cells treated with 5′azacytodine to inhibit Dnmts (Chuang et al., 2005). It is worth noting that the upregulation of a subset of X-linked and development genes apparently does not interfere with the self-renewal of demethylated ES cells. Thus, ES cells can tolerate the overexpression of a subset of genes that serve a specialized function in germ cell or other types of somatic cells.

Although our study provides direct evidence that DNA demethylation can induce gene activation for a number of cell differentiation genes, we also found many genes are not upregulated in the absence of DNA methylation. This result is in contrast to the result observed in demethylated primary fibroblasts, in which up to 10% of expressed genes can be upregulated compared to WT

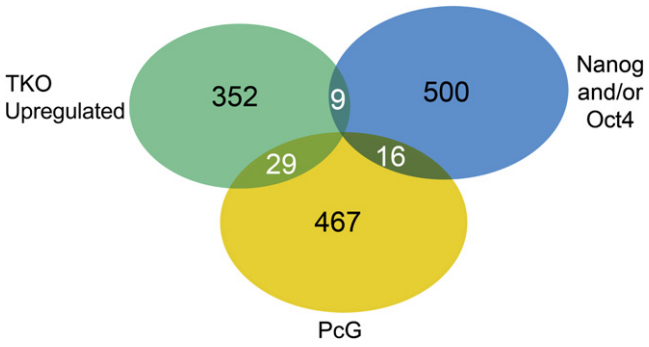

Figure 6. Comparison of Upregulated Genes in Demethylated mES Cells with Oct4, Nanog, and PcG Bound Genes

Venn diagram showing the minimal overlap between the TKO upregulated genes (green), Nanog and/or Oct4 bound genes (within 10 kb proximal promoter region) (blue), and PcG bound genes (yellow). It should be noted that there is one gene, *Podxl*, which overlaps with all three categories. This analysis includes all genes, not just those present on the promoter methylation array. The Nanog, Oct4 (Loh et al., 2006), and PcG (Boyer et al., 2006) bound genes that overlap with TKO upregulated genes are listed in Table S7.

primary fibroblasts (Jackson-Grusby et al., 2001). Genome-wide mapping of histone modifications indicated that bivalent H3K4/K27me3 is more widespread than the association of Polycomb proteins (Boyer et al., 2006; Mikkelsen et al., 2007; Figure 7). Moreover, many methylated genes also contain H3K27me3 or bivalent H3K4/K27me3 markers in mES cells, raising the possibility that repressive histone marks can to some extent compensate for loss of DNA methylation in gene repression. It is also possible that compensatory repressive mechanisms have become activated during generation and culture of the TKO cell line. In addition, a lack of proper gene transcription activators in mES cells for those demethylated genes may also account for the continued inactive status of a majority of demethylated genes in TKO mES cells. Our results are consistent with the notion that DNA demethylation is necessary but not sufficient for gene activation. Conversely, methylation of a promoter is not always sufficient for gene repression. Using previously published gene expression data for mES cells, we found that up to 36% of genes are still expressed even if methylated in the proximal promoter. However, 80% of the expressed genes that exhibit promoter methylation are marked by the active histone H3K4me3 mark and are HCP genes. These observations are consistent with the suggestion by Weber et al. (2007) that a low density of DNA methylation in a gene promoter may not be sufficient to silence gene transcription by itself.

The mapping of promoter methylation patterns in mES cells could provide insights into why mES cells are a good cell source for somatic nuclear transfer experiments when compared to differentiated somatic cells (Yamanaka, 2007). One of the major hurdles in somatic nuclear transfer experiments is the efficiency of epigenetic reprogramming. It has been shown that a panel of genes including *Oct4* and *Dppa4* are only partially reactivated in somatic nuclear reconstituted blastocyst embryos, which could be attributed to the incomplete demethylation of *Oct4* and *Dppa* family genes in somatic nuclei during reprogramming (Bortvin et al., 2003). Our methylation mapping indicated that both *Oct4* and *Dppa4* genes are demethylated in mES cells, supporting

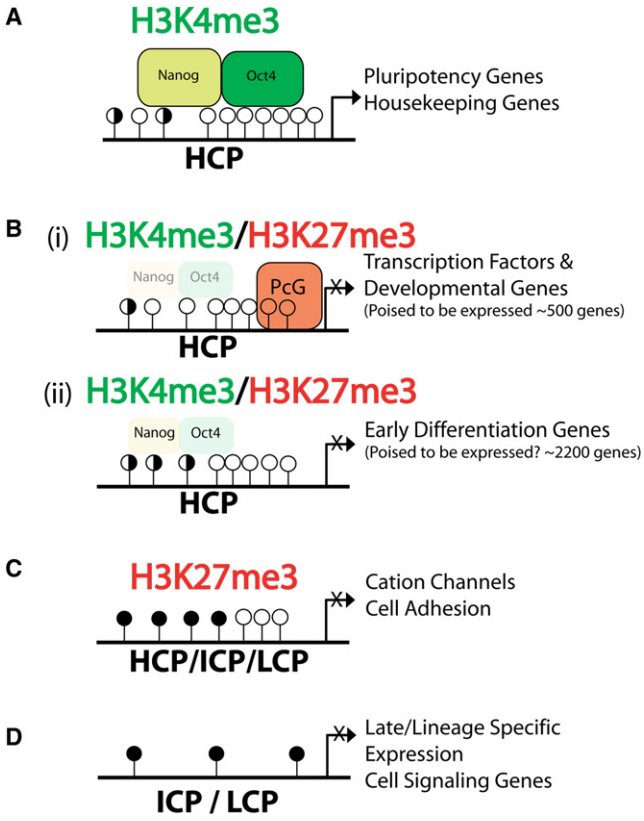

Figure 7. A Schematic Summary of Epigenetic and Transcriptional Regulation in mES Cells

The patterns of DNA methylation and histone modifications are sorted into four subclasses of gene promoters with H3K4me3 (A), bivalent genes (B) with (i) or without (ii) Polycomb binding, H3K27me3 only (C), and no histone marks (D). Gene promoters are further annotated as HCP, ICP, and LCP that correspond to different density of CpG dinucleotides and DNA methylation. Open circles designate unmethylated CpG sites, filled circles represent methylated CpG sites, and half-filled circles represent promoters that can be found either methylated or unmethylated. Unmethylated CpG islands are enriched in HCP promoters with H3K4me3 only or bivalent H3K4/K27 marks. Methylated CpGs are enriched in LCP and ICP gene promoters without H3K4 and H3K27 trimethylation or with H3K27me3 only. The preferential interaction of Polycomb proteins and Oct4/Nanog complex with different classes of gene promoter is also illustrated. Different classes of genes that are either expressed or repressed in mES cells are listed next to each type of promoters.

the notion that ES cells are more easily reprogrammed than somatic nuclei.

In summary, our comprehensive mapping of DNA methylation of gene promoters in mES cells provides a valuable resource for understanding the function of DNA methylation in the maintenance of self-renewal and pluripotency. Furthermore, the methylation patterns in gene promoters may also represent an epigenetic code that underlies the program of lineage-specific differentiation.

EXPERIMENTAL PROCEDURES

ES Cell Cultures

ES cells were maintained as previously described (Meissner et al., 2005). RNA was isolated using Trizol (Invitrogen) while DNA was isolated using DNA lysis buffer, and then phenol:chloroform extracted.

mDIP

The methylated DNA immunoprecipitation (mDIP) method was adapted from a recent study (Zhang et al., 2006; Keshet et al., 2006). Briefly, 2 ug of DNA was immunoprecipitated with 20 ug of a monoclonal antibody to 5-me-cytosine. The immunoprecipitated DNA was washed, eluted, and quantitated for microarray hybridization. For details, see the Supplemental Data.

Microarray Hybridization

Gene expression microarrays were done with Agilent Whole Genome microarrays (G4122A) using the suggested protocol. These arrays were performed in triplicate. Methylation microarray hybridizations were done with Agilent custom mouse promoter microarrays that covered approximately −800 to +200 bp of 15,561 genes. We labeled 250 ng of J1 and TKO mES cell DNA for each array. We performed seven replicates of the methylation arrays. For details, see the Supplemental Data.

Bisulfite Conversion and Sequencing

Bisulfite conversion was performed as described (Shen et al., 2006). Briefly, we digested genomic DNA with BglII overnight. Digested DNAs were then incubated with a sodium bisulfite solution for 16 hr. Bisulfite-treated DNA was then desalted and precipitated. We used 1/10 of precipitated DNA for each PCR. For PCR, we used nested primers to generate our products. PCR products were gel purified and used for either Topo Cloning (Invitrogen) or direct PCR sequencing.

Quantitative Reverse Transcription PCR

RNA was DNase I treated (Invitrogen) and then quantified again. cDNA conversion was done using the iScript kit (Bio-Rad). Quantitative PCR was done on a MyIQ Thermocycler (Bio-Rad) using the Sybr Green Supermix (Bio-Rad).

Statistical Methods

Detailed descriptions are found in the Supplemental Data.

UCSC Genome Tracks

Methylation data can be viewed at http://epigenomics.mcdb.ucla.edu/mESC/.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, eight tables, and three figures and can be found with this article online at http://www.cellstemcell.com/cgi/content/full/2/2/160/DC1/.

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Accession Numbers

Microarray data are available at GEO (Gene Expression Omnibus), http://www.ncbi.nlm.nih.gov/projects/geo/index.cgi, with the GEO series accession number GSE9172.