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Variations in the epigenetic regulation of lineage-specific genes among human pluripotent stem cell lines

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ABSTRACT

Pluripotent stem cells (PSCs) have unique transcriptional regulatory networks and epigenetic states that are involved in maintaining pluripotency. In this study, the transcriptional levels and histone modifications of lineage-specific genes were compared for human ESC (hESC) lines and human induced pluripotent stem cell (hiPSC) lines. Expression of the pluripotency marker genes, *OCT4*, *SOX2*, and *NANOG*, was largely modulated in hESCs by permissive histone marks, whereas hiPSC lines showed differential histone modifications in the gene promoters. The permissive histone mark, H3K4me3, predominantly contributed to expression of the oncogene, *c-MYC*, in hESC lines, whereas histone modifications of the *c-MYC* promoter varied between hiPSC lines. Interestingly, the transcriptional levels and epigenetic marks in the promoters of the developmental genes such as *SOX17*, *T*, and *NESTIN* varied among individual hiPSC lines. In particular, a partially-reprogrammed hiPSC cell line showed lower frequencies of permissive and repressive histone marks in the promoters of most genes, indicating incomplete epigenetic reprogramming. Our data indicate that respective hPSCs have distinct epigenetic signatures of lineage-specific genes, thereby leading to in their propensities to follow a particular differentiation pathway.

1. Introduction

Chromatin structure is dynamic during the cell cycle, thereby controlling gene expression. Chromatin also plays important roles in embryonic development and cell fate determination. Each cell type has a unique chromatin structure, which can be modified by epigenetic marks such as chemical modifications on histone tails, DNA methylation, and chromatin-remodeling enzyme complexes. Embryonic stem cells (ESCs) are unique cells with an open chromatin structure that is essential for maintaining pluripotency and their ability for self-renewal [1]. Induced pluripotent stem cells (iPSCs), derived from differentiated cells via the ectopic expression of defined factors, are very similar to ESCs with respect to pluripotency [2,3], although some variation has been observed in chromatin structure and gene expression between ESCs and iPSCs [4].

Gene expression is closely related to specific post-translational modifications of histone tails found in the promoter regions and transcribed portions of protein-coding genes [5–7]. For example, trimethylation of histone H3 at lysine 4 (H3K4me3) as well as acetylation of histone H3 at lysine 9 (H3K9ac) are generally found in the nucleosomes of the promoter regions of actively transcribed genes [8,9], whereas trimethylation of histone H3 at lysine 27 (H3K27me3) and methylation of histone H3 at lysine 9 (H3K9me) in promoter regions are responsible for transcriptional repression [10,11]. ESCs have unique epigenetic modifications for the maintenance of pluripotency and the determination of differentiation properties [1]. Bivalent domains, consisting of both H3K4me3 and H3K27me3, are associated with developmental genes that are silent yet poised for activation once the cell is committed to a particular differentiation pathway [10,12,13]. Interestingly, it has been reported that the bivalent domains of developmental genes shift to the permissive state for gene activation during the differentiation of human ESCs (hESCs) into a specialized cell type *in vitro* [14]. Large-scale DNA methylation analyses of the epigenome have revealed certain differences between ESCs and iPSCs in humans [15–18]. Transcriptional profiles of human iPSCs (hiPSCs) are significantly different from hESCs [19–21], and it is likely that this difference is due to persistent donor cell memory in iPSCs. However, the correlation between gene expression and epigenetic signatures in ESCs and iPSCs is still poorly understood. In this study, we investigated whether the transcription of lineage-specific genes, including pluripotency marker genes, oncogenes, and developmental genes, is associated with histone modifications of promoter regions in human ESCs and iPSCs.

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2. Materials and methods

2.1. Generation of hiPSCs

Human iPSCs were derived from human foreskin fibroblasts (CRL-2097™, ATCC, Manassas, VA) via the ectopic expression of OCT4, SOX2, KLF4, and c-MYC as previously described [2].

2.2. Culture of hESCs and hiPSCs

Two hESC lines, H9-hESC and CHA4-hESC [22], and four hiPSC lines were maintained on mitomycin C (MMC)-treated STO feeder layers in Dulbecco's modified Eagle medium (DMEM)/F-12 (Invitrogen, Carlsbad, CA) supplemented with 20% Knockout Serum Replacement (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma–Aldrich, St. Louis, MO), 1% non-essential amino acids (Invitrogen), 1% penicillin–streptomycin (Invitrogen), and 4 ng/ml basic fibroblast growth factor (FGF; R&D Systems, Minneapolis, MN) at 37 °C, 5% CO_2 in air. The medium was changed daily.

2.3. Real-time reverse transcription-PCR (RT-PCR)

Total RNA was isolated from cells using TRIzol Reagent (Invitrogen) and reverse-transcribed using M-MLV Reverse Transcriptase (Enzynomics, Daejeon, Korea) according to the manufacturer's protocol. Gene expression levels were measured by real-time RT-PCR using 2 × Prime Q-Master Mix (GENET BIO, Daejeon, Korea). Relative expression levels were analyzed using an iCycler iQ5 Real-Time detection system (Bio-Rad Laboratories, Hercules, CA). The reaction parameters for real-time RT-PCR analysis were 95 °C for 10 min followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, with a final elongation step at 72 °C for 5 min. The primers used for gene expression are listed in Supplementary Table 1. All reactions were performed at least twice. For comparative quantification, gene expression levels were normalized to *GAPDH* (Glyceraldehyde-3-phosphate dehydrogenase) and were then expressed as a fold-change relative to the expression level of the relevant gene in H9-hESCs. The sample ΔCt (SΔCt) value was calculated as the difference between the Ct values of *GAPDH* and the target. The ΔCt value of hESCs was used as a control ΔCt (CΔCt) value. The relative gene expression level was determined using the formula, $2^{-(S\Delta Ct - C\Delta Ct)}$.

2.4. Chromatin immunoprecipitation (ChIP) analysis

The ChIP assay was performed as previously described [14]. The primer sequences used for PCR are listed in Supplementary Table 1.

2.5. Statistical analysis

The statistical significance of the real-time PCR data rate was evaluated using one-way ANOVA and Bonferroni's multiple comparison tests. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Generation of hiPSCs from fibroblasts

Two hESC lines and four hiPSC lines were employed in this study. The hESCs included one female line, H9, and one male line, CHA4-hESC [22]. The hiPSCs were generated from human foreskin fibroblasts following the ectopic expression of *OCT4*, *SOX2*, *KLF4*, and *c-MYC* genes [2]. The pluripotency of two of the hiPSC lines (hiPSC #1 and hiPSC #2) was proven previously by teratoma formation ([23] and Fig. 1D). The third hiPSC line (hiPSC #3) had an abnormal karyotype with trisomy of chromosome 12 (Fig. 1C) and the fourth (hiPSC #4) was only partially-reprogrammed. The hiPSC lines #2 and #3 expressed pluripotency markers such as OCT4, SSEA-4, and TRA-1-60 in a normal manner, whereas the partially-reprogrammed hiPSC #4 did not express TRA-1-60 (Fig. 1A). All pluripotency marker genes examined were transcribed in the normal hiPSCs; however, the partially-reprogrammed hiPSC line #4 did not express *REX1* (Supplementary Fig. 1A). Hypermethylation of the *REX1* promoter might be responsible for silencing this gene in hiPSC #4 (Fig. 1B), representing the partial reprogramming. As in the hESC lines, the promoter regions of *OCT4*, *REX1*, and *NANOG* were hypomethylated in hiPSC #2 and #3 (Fig. 1B). The ectopic genes used to induce pluripotency in the fibroblasts were silenced in all hiPSCs (Supplementary Fig. 1B).

3.2. Correlations between transcription and histone modifications of pluripotency marker genes in hPSCs

Next, we investigated whether the transcriptional expression of pluripotency marker genes was correlated with histone modifications in hESCs and hiPSCs. The expression level of a particular gene was compared with the frequency of permissive (H3K4me3 and H3K9ac) and repressive (H3K27me3, H3K9me2, and H3K9me3) histone marks in the promoter region.

The transcriptional expression level of *OCT4* in the two hESC lines (H9 and CHA4-hESC) and three of the hiPSC lines (hiPSC #1, #2, and #3) was higher than in the partially-reprogrammed hiPSC line (hiPSC #4) (Fig. 2A). The expression of *OCT4* in the H9-hESC line appeared to be regulated by both permissive histone marks, while the CHA4-hESC line seemed to be regulated only by the H3K4me3 (Fig. 2A). The hiPSC #1 line had extremely low frequencies of both permissive and repressive histone modifications for *OCT4*, although in hiPSC #2, *OCT4* expression appeared to be controlled by H3K4me3 and H3K9ac. Intriguingly, the trisomy line, hiPSC #3, retained high frequencies of both permissive and repressive histone modifications in the *OCT4* promoter, which is characteristic of bivalent histone marks. The low level of *OCT4* expression in the partially-reprogrammed hiPSC #4 might result from a paucity of histone modifications in the *OCT4* promoter in this cell line. These results demonstrated that different combinations of permissive and repressive histone modifications in the *OCT4* promoter existed in each of the hESC and hiPSC lines.

Fig. 2B depicts the transcriptional and epigenetic signatures of *SOX2* in hESCs and hiPSCs. The promoter region of *SOX2* in the two hESC lines and in hiPSC #2 was predominantly modified by H3K4me3 and H3K9ac, indicating that the expression of this gene was controlled through permissive histone marks. Interestingly, *SOX2* expression was higher in hiPSC #1 than in the two hESC lines, even though both the permissive and the repressive histone marks were expressed at lower frequencies than in the hESC lines. *SOX2* transcription in hiPSC #3 did not appear to be influenced by histone modifications on the promoter. However, in the partially-reprogrammed line, hiPSC #4, high levels of the repressive histone mark, H3K27me3, could explain the low level of *SOX2* expression observed. Thus, taken together, the data indicated that the histone modifications for *SOX2* transcription varied between hiPSC lines.

NANOG transcripts were enriched in both hESC and hiPSC lines (Fig. 2C), and it appeared likely that permissive histone marks regulated the transcription of *NANOG* in the two hESC lines and two of the hiPSCs (hiPSC #2 and #3). Although hiPSC #1 and #4 showed low frequencies of both permissive and repressive histone marks on the promoter, NANOG expression was not inhibited compared to the other cell lines. Thus, it is possible that histone modifications in the *NANOG* promoter are not closely associated with expression of this gene in hiPSCs.

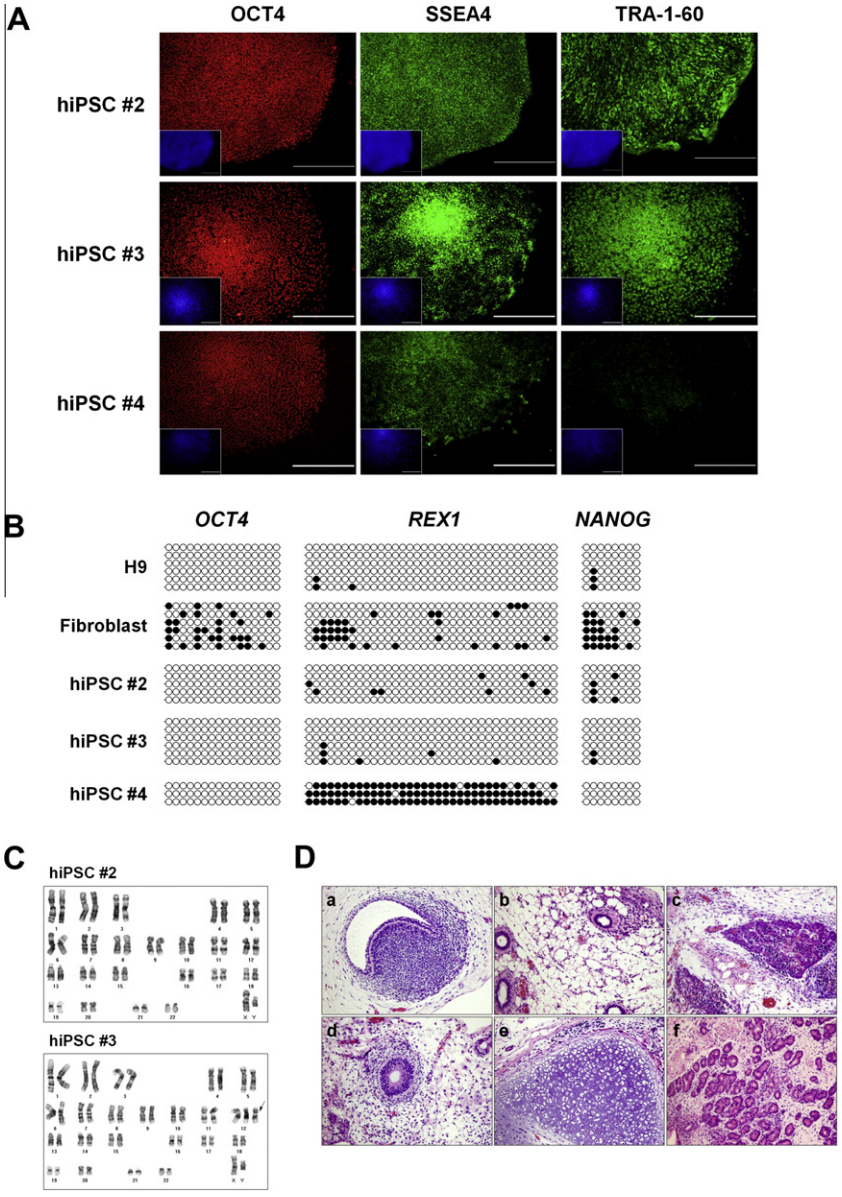

Fig. 1. Characterization of hiPSCs derived from fibroblasts. (A) Expression of pluripotency markers in hiPSC lines. Immunofluorescence detection of OCT4, SSEA4, and TRA-1-60 was performed after 5 days culture on feeder cells. Insets show DAPI (4′,6-diamidino-2-phenylindole) staining. Scale bar, 500 μm. (B) DNA methylation on promoters of pluripotency marker genes. Methylation states at CpG dinucleotides in *OCT4*, *REX1*, and *NANOG* promoters were examined in H9-hESCs, fibroblasts, and three hiPSC lines using bisulfite sequencing. Each row of circles represents the methylation status of each CpG in one bacterial clone. Open and filled circles indicate unmethylated and methylated CpG dinucleotides, respectively. (C) Karyotypic analysis of hiPSC lines #2 and #3 using G-banding analysis. (D) Teratoma formation of hiPSC #2 cells in immunodeficient mice. Hematoxylin and eosin (H and E) staining was performed on formalin-fixed teratoma sections showing ectoderm (a, ependymal structure; d, rosette), mesoderm (b, adipose tissue; e, chondroid tissue) and endoderm (c, acina cluster; f, secretory gland) tissues.

The expression levels of *REX1* showed no significant differences between the hESC and hiPSC lines except for the partially-reprogrammed hiPSC #4, which did not express this gene (Fig. 2D). In the hESC and normal hiPSC lines, it is possible that the expression of *REX1* is largely modulated by both permissive and repressive histone modifications. Intriguingly, hiPSC #3, with its abnormal karyotype, had high histone modification frequencies for both the permissive and repressive histone marks in the *REX1* promoter, although *REX1* transcripts were not enriched compared to other hiPSC lines. As expected, the partially-reprogrammed hiPSC line (hiPSC #4) did not express *REX1* due to hypermethylation of the promoter as shown in Fig. 1B, indicating incomplete reprogramming of DNA methylation. In addition, histone modifications of the *REX1* promoter were reasonably infrequent in hiPSC #4 compared to the other cell lines. These results suggest that the expression of *REX1* is, to some extent, modulated by histone modifications in hESCs and normal hiPSCs.

3.3. Correlations between transcription and histone modifications of oncogenes in hPSCs

The ectopic expression of OCT4, SOX2, KLF4, and c-MYC is needed to induce pluripotency from human fibroblasts [2], and in this study, the epigenetic regulation of *c-MYC* and *KLF4* was investigated in hiPSCs. The transcriptional activity of *c-MYC* was relatively stable in hESC lines, whereas it varied between hiPSC lines (Fig. 3A). The expression of *c-MYC* in hESCs appeared to be largely regulated by H3K4me3. The two normal iPSC lines expressed *c-MYC* at high levels compared to the other cell lines; however, the epigenetic regulation was inconsistent with this result. In

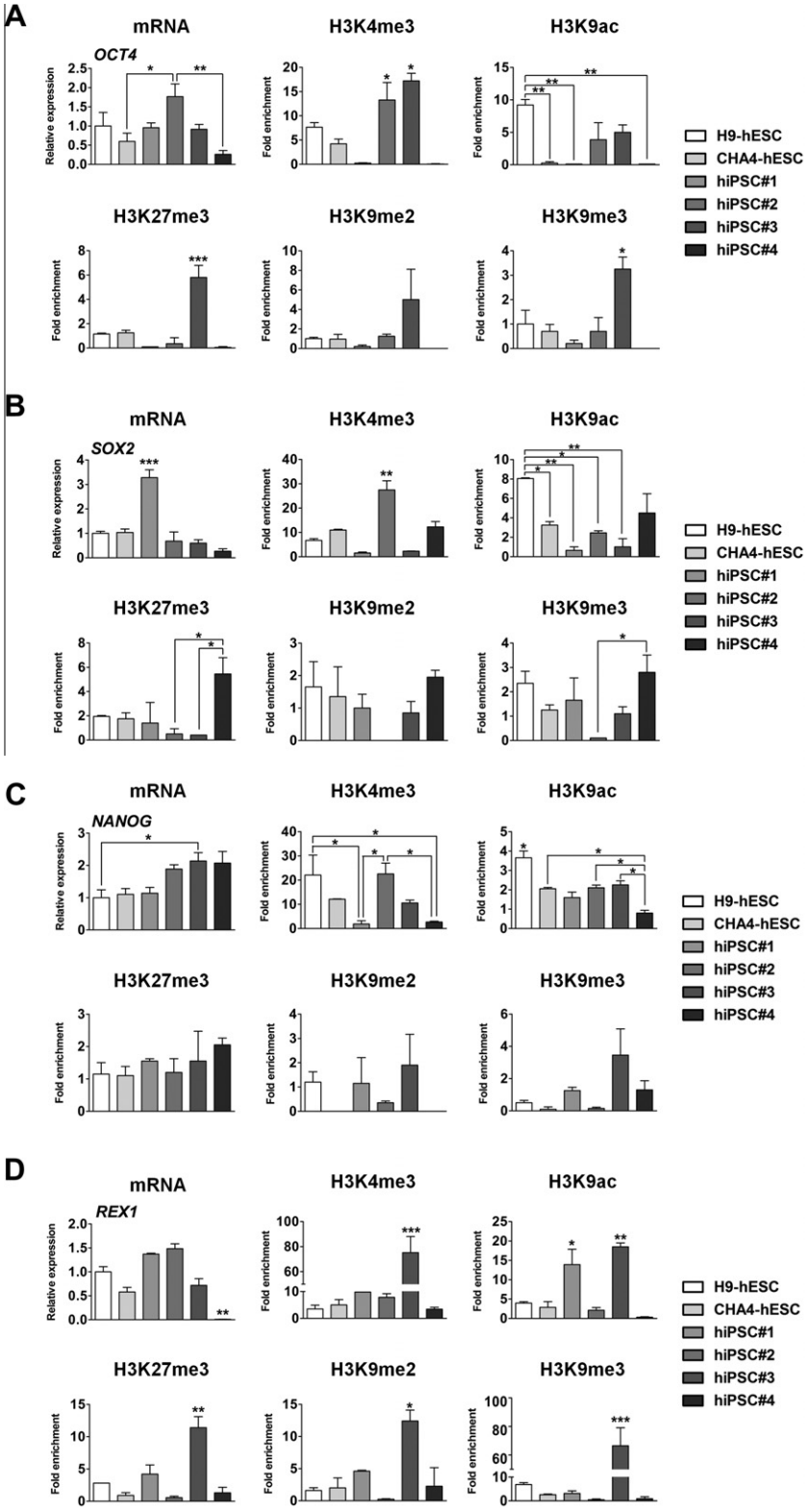

Fig. 2. Expression and histone modifications of pluripotency marker genes in hESC and hiPSC lines. The relative expression of *OCT4* (A), *SOX2* (B), *NANOG* (C), and *REX1* (D) in hESC and hiPSC lines was determined by real-time RT-PCR. Gene expression was normalized to *GAPDH*. ChIP analysis of histone modifications in the promoter regions of *OCT4* (A), *SOX2* (B), *NANOG* (C), and *REX1* (D) was performed on hESCs and hiPSCs. Data, validated by real-time PCR, are presented as fold enrichments of precipitated DNA associated with a given histone modification relative to a 100-fold dilution of input chromatin. The data are presented as the mean ± SEM ($n = 2$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

contrast to low frequencies of both permissive and repressive histone marks in hiPSC #1, hiPSC #2 exhibited very high frequencies of both types of histone mark on the *c-MYC* promoter. Conversely, the *c-MYC* promoter in the trisomic line, hiPSC #3, was predominantly modified by the permissive marks, H3K4me3 and H3K9ac, in spite of a low level of gene transcription. The extremely low le-

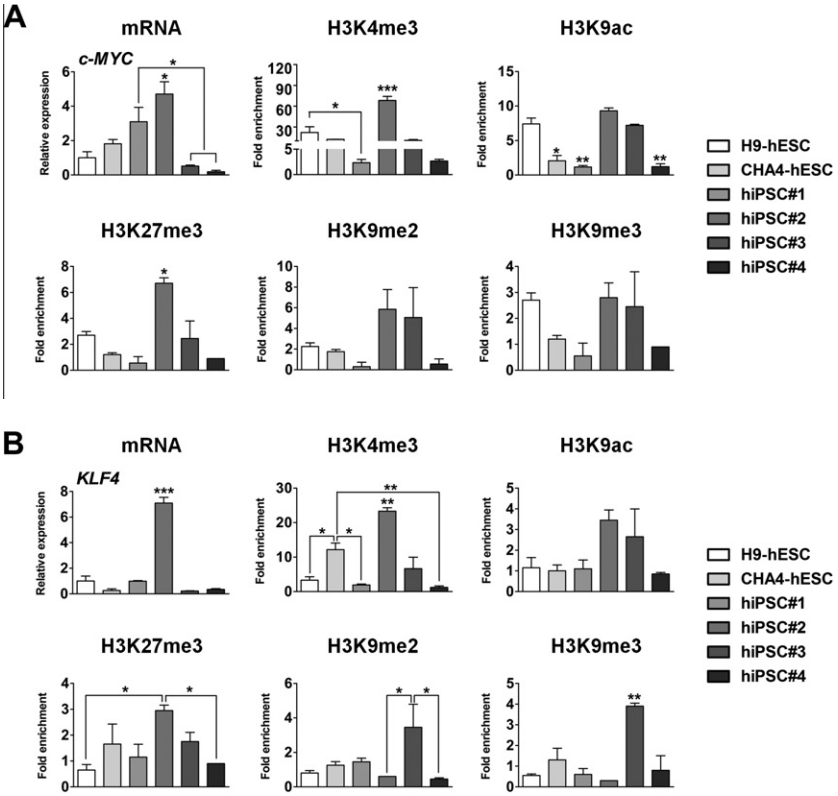

Fig. 3. Expression and histone modifications of oncogenes in hESC and hiPSC lines. The relative expression of *c-MYC* (A) and *KLF4* (B) in hESC and hiPSC lines was determined by real-time RT-PCR. ChIP analysis of histone modifications in the promoter regions of *c-MYC* (A) and *KLF4* (B) was performed on hESCs and hiPSCs. The data are presented as the mean ± SEM ($n = 2$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

vel of *c-MYC* expression in hiPSC #4 might be due to the low frequencies of both types of histone mark. From these results, it was concluded that the expression of *c-MYC* is largely regulated by the permissive mark, H3K4me3, in hESC lines, and by bivalent histone modifications in hiPSC lines.

Fig. 3B depicts the transcriptional expression and histone modifications for *KLF4* in the hESC and hiPSC lines studied. Despite a relatively high frequency of the permissive histone mark, H3K4me3, the expression level of *KLF4* was relatively low in the hESC line, CHA4-hESC, compared with H9-hESC. Interestingly, the high level of *KLF4* expression in hiPSC #2 appeared to be largely modulated by the permissive histone mark, H3K4me3, whereas the very low level of *KLF4* transcription in hiPSC #3 might be associated with repressive histone marks such as H3K27me3, H3K9me2, and H3K9me3 in hiPSC #3, which were all expressed at high frequencies in this cell line. Thus, these results clearly demonstrated variations in histone modifications related to *KLF4* expression were detected between hiPSC lines.

3.4. Epigenetic signatures of developmental genes in hiPSCs

The final experiment was performed to examine the epigenetic signatures of developmental genes in hESCs and hiPSCs. A number of inactive developmental regulatory genes in ESCs retain the bivalent chromatin structure in which the permissive mark, H3K4me3, colocalizes with the repressive mark, H3K27me3 [12,24–26].

In this study, *SOX17*, *T*, and *NESTIN* were selected as representative genes for the definitive endodermal, mesodermal and ectodermal lineages, respectively (Fig. 4). In the hESC line, H9-hESC, and the hiPSC line, hiPSC #2, the promoter regions of these three developmental genes were predominantly occupied by both permissive and repressive histone marks. The partially-reprogrammed line, hiPSC #4, had very low frequencies of both types of histone modification for all three genes compared with the other hiPSC lines (Fig. 4), suggesting incomplete epigenetic reprogramming of developmental genes. The *SOX17* promoters in the lines, CHA4-hESC and hiPSC #1, were largely modified by the repressive mark, H3K27me3 (Fig. 4A), while the trisomic line, hiPSC #3, showed a high frequency of bivalent marks in the *SOX17* promoter. With respect to histone marks in the T gene promoter, the lines CHA4-hESC, hiPSC #2 and hiPSC #3 all had bivalent domains (Fig. 4B). However, hiPSC #2 had very high level occupancy of the permissive mark, H3K4me3, which might be responsible for the enhanced expression of *T* in this line compared to the other lines. The line, hiPSC #1, was enriched for all three repressive histone marks in the *T* promoter. Finally, with regard to the NESTIN promoter, CHA4-hESC had very low levels of both permissive and repressive histone marks (Fig. 4C). The *NESTIN* promoter region in hiPSC #1 was largely modified by bivalent histone marks whereas in hiPSC #2, it was enriched for the permissive mark, H3K4me3. Like the *SOX17* promoter, the *NESTIN* promoter showed high occupancy levels of both permissive and repressive histone marks in the abnormal karyotype line, hiPSC #3.

4. Discussion

PSCs have an open chromatin configuration and exhibit transcriptional hyperactivity, features that are required for the maintenance of pluripotency [27]. Nonetheless, gene transcription and epigenetic modifications, such as DNA methylation, may not be equivalent between ESCs and iPSCs [15–21]. In the current study, our data revealed that the transcriptional levels and histone modifications of lineage-specific genes are more variable in hiPSCs than hESCs.

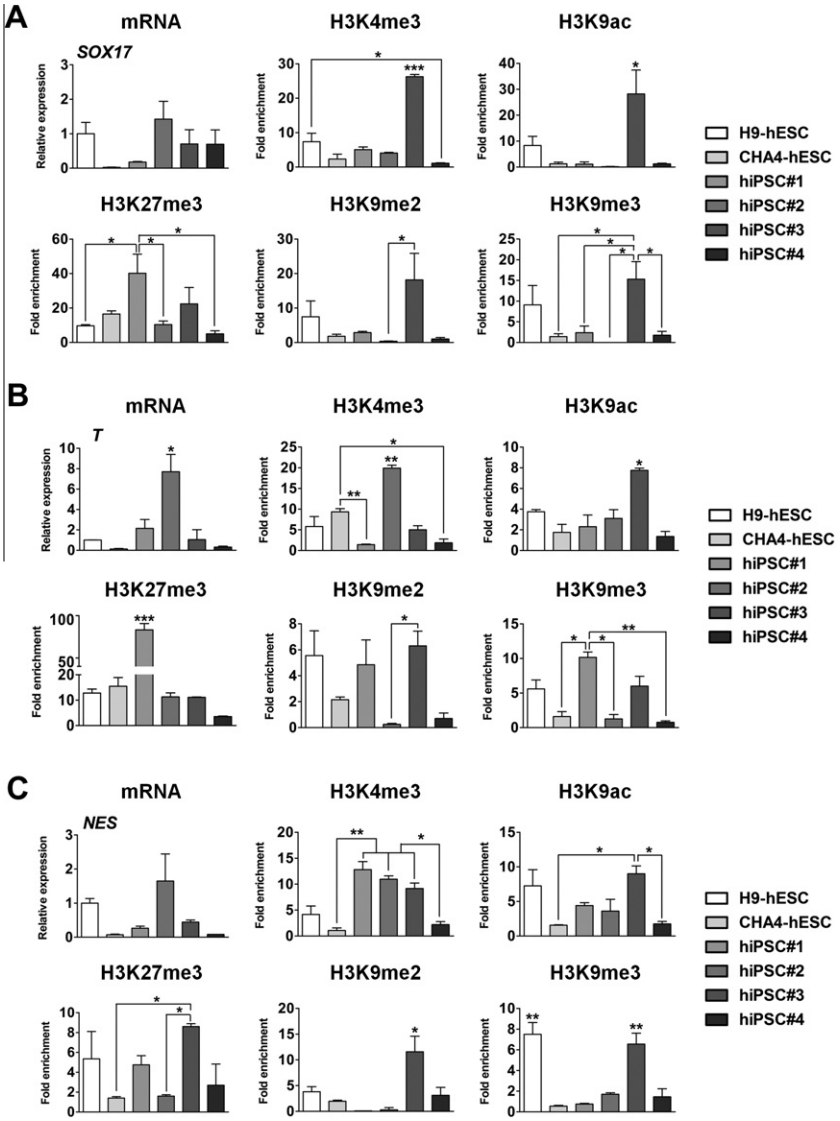

Fig. 4. Expression and histone modifications of developmental genes in hESC and hiPSC lines. The relative expression of *SOX17* (A), *T* (B), and *NESTIN* (C) in hESC and hiPSC lines was determined by real-time RT-PCR. ChIP analysis of histone modifications in the promoter regions of *SOX17* (A), *T* (B), and *NESTIN* (C) was performed on hESCs and hiPSCs. The data are presented as the mean ± SEM ($n = 2$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

In hESCs, the consistent expression of pluripotency marker genes is regulated by canonical epigenetic mechanisms, which are essential for maintaining pluripotency. Similarly, the promoters of *OCT4*, *SOX2*, and *NANOG* were largely occupied by permissive histone marks such as H3K4me3 in hESCs (Fig. 2). However, the frequencies of the permissive histone marks varied between hiPSC lines. Transcription of the three marker genes in one of the normal hiPSC lines (hiPSC #2) seemed to be regulated by canonical histone modifications as in hESCs, whereas the other normal line (hiPSC #1) showed unusually low occupancies of H3K4me3 in the promoters of *OCT4*, *SOX2*, and *NANOG* (Fig. 2), illustrating variations in epigenetic modifications between hiPSC lines. These results disagree with a previous report that nucleosomes bearing a H3K4me3 permissive mark on the *OCT4* promoter showed no significant differences between hESC and hiPSC lines [4].

Greater variation in epigenetic modifications was observed in the two abnormal hiPSC lines. In the trisomic line, hiPSC #3, transcriptional expression of *OCT4* and *NANOG* were modulated by high levels of bivalent histone modifications and permissive histone marks, respectively; however, *SOX2* expression did not appear to be modulated by histone modifications in this cell line. Intriguingly, hiPSC #3 exhibited high levels of both permissive and repressive histone marks in the *REX1* promoter. Suppression of *REX1* transcription in the partially-reprogrammed line, hiPSC #4, could be due to the observed hypermethylation of CpG sites and lower occupancies of all histone marks in the promoter (Figs. 1B and 2D). Since it is known that unmethylated CpG dinucleotides bind to CXXC domains in H3K4 methyltransferase complexes [28], this result implies that hypermethylation may hinder the activity of histone modifying enzymes in the promoter. The *REX1* gene product is a zinc finger protein, required for the maintenance of pluripotency and capable of interactions with core transcription factors such as OCT4, SOX2, and NANOG in ESCs [29–32]. Thus, hypermethylation of the promoter may lead to inactivation of *REX1* by preventing access of the core transcription factors, thereby resulting in repression of the gene. The low transcriptional level of *SOX2* in hiPSC #4 seems to be regulated by the repressive histone mark, H3K27me3; however, histone modifications in *OCT4* and *NANOG* promoters may not be required for gene expression in this cell line. Thus, our findings demonstrated that epigenetic regulation might act randomly on the expression of pluripotency marker genes in partially-reprogrammed hiPSCs.

It is known that c-MYC and KLF4 increase the proliferative capacity of somatic cells during reprogramming to the pluripotent state [2,33]. However, in this study, variations in the histone modifications controlling the transcription of *c-MYC* and *KLF4* were observed even between the two normal hiPSC lines (Fig. 3). The expression of *c-MYC* was modulated predominantly by very low levels of the three repressive histone marks on the *c-MYC* promoter in hiPSC #1, but was largely activated by very high levels of the permissive mark, H3K4me3, in hiPSC #2. By contrast, the transcriptional level of KLF4 appeared to be dependent on the frequency of the permissive histone mark, H3K4me3, in both hiPSC #1 and #2 (Fig. 3B).

Bivalent domains, in which the permissive mark, H3K4me3, and the repressive mark, H3K27me3, co-exist, repress the activation of developmental genes in ESCs, thereby maintaining pluripotency [12,34]. In addition, it is reported that these domains facilitate the cell fate determination of ESCs during differentiation through a shift, from the bivalent state to the permissive one following the loss of suppressive histone marks in the promoters of developmental genes [14]. In the present study, certain variations were observed in the histone modification of developmental genes between hESC and normal hiPSC lines (Fig. 4). Our findings suggest that each of the respective hiPSC lines may have a different competence for cell fate determination during *in vitro* differentiation.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.06.122.

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