Dual Roles of Histone H3 Lysine 9 Acetylation in Human Embryonic Stem Cell Pluripotency and Neural Differentiation*

Yunbo Qiao†1, Ran Wang†1, Xianfa Yang‡1,2, Ke Tang†, and Naihe Jing†2

From the §State Key Laboratory of Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 320 Yue Yang Road, Shanghai 200031, China, the ‡School of Life Science and Technology, ShanghaiTech University, Shanghai 200031, China, and the ¶Institute of Life Science, Nanchang University, Nanchang, Jiangxi 330031, China

Background: H3K9Ac is an epigenetic mark representing transcriptionally active chromatin. Results: Inhibition of HDAC activity promotes pluripotency maintenance at the initiation of hESC differentiation and inhibits neural differentiation during neural commitment stage. Conclusion: H3K9Ac plays dual roles in hESC pluripotency maintenance and neural differentiation through regulating different targets. Significance: This work provides insight into the epigenetic mechanisms underlying neural differentiation of hESCs associated with histone acetylation patterns.

Early neurodevelopment requires cell fate commitment from pluripotent stem cells to restricted neural lineages, which involves the epigenetic regulation of chromatin structure and lineage-specific gene transcription. However, it remains unclear how histone H3 lysine 9 acetylation (H3K9Ac), an epigenetic mark representing transcriptionally active chromatin, is involved in the neural commitment from pluripotent embryonic stem cells (ESCs). In this study, we demonstrate that H3K9Ac gradually declines during the first 4 days of in vitro neural differentiation of human ESCs (hESCs) and then increases during days 4–8. Consistent with this finding, the H3K9Ac enrichment at several pluripotency genes was decreased, and H3K9Ac occupancies at the loci of neurendevolmental genes increased during hESC neural commitment. Inhibiting H3K9 deacetylation on days 0–4 by histone deacetylase inhibitors (HDACis) promoted hESC pluripotency and suppressed its neural differentiation. Conversely, HDACi-elicted up-regulation of H3K9 acetylation on days 4–8 enhanced neural differentiation and activated multiple neurendevolmental genes. Mechanistically, HDACis promote pluripotency gene transcription to support hESC self-renewal through suppressing HDAC3 activity. During hESC neural commitment, HDACis relieve the inhibitory activities of HDAC1/5/8 and thereby promote early neurendevolmental gene expression by interfering with gene-specific histone acetylation patterns. Furthermore, p300 is primarily identified as the major histone acetyltransferase involved in both hESC pluripotency and neural differentiation. Our results indicate that epigenetic modification plays pivotal roles during the early neural specification of hESCs. The histone acetylation, which is regulated by distinct HDAC members at different neurodevelopmental stages, plays dual roles in hESC pluripotency maintenance and neural differentiation.

Embryonic stem cells (ESCs)†3 possess unique genetic and epigenetic characteristics that allow them to remain pluripotent and to be able to differentiate into the three germ layers (1–3). Although most human ESC (hESC) lines share concerted morphology and pluripotency genes (i.e. OCT4, NANOG, GDF3, and NODAL) (4, 5), the pluripotency and developmental plasticity of ESCs are highly regulated by specific chromatin architectures, which are associated with numerous covalent histone modifications (6, 7). Site-specific histone acetylation and methylation patterns constitute the “histone code,” leading to transcriptional activation and silencing (8–10). Histone H3 lysine 9 and/or lysine 14 (H3K9/K14) acetylation, which is an epigenetic hallmark of transcriptionally active chromatin (11–13), participates in regulating the pluripotency and reprogramming capacity of ESCs (14). In addition, the pluripotency of ESCs is characterized by a specific epigenetic profile, which is distinct from that of differentiated progeny (7). During hESC neural commitment, different sets of genes associated with pluripotent or neural fates are enriched in distinct cell states. Nonetheless, it is largely unknown how histone acetylation modulates the pluripotency and neural differentiation of hESCs.

Histone acetylation status is antagonistically regulated by two sets of histone-modifying enzymes: transcriptional activa-

1 Both authors contributed equally to this work.
2 To whom correspondence should be addressed. Tel: 86-21-5492-1381; Fax: 86-21-5492-1011; E-mail: njing@sibcb.ac.cn.
3 The abbreviations used are: ESC, embryonic stem cell; hESC, human ESC; H3K9, histone H3 lysine 9; H3K9Ac, H3K9 acetylation; H3K14, histone H3 lysine 14; HAT, histone acetyltransferase; HDAC, histone deacetylase; HDACi, HDAC inhibitor; TSA, trichostatin A; VPA, valproic acid; NPC, neural progenitor cell; EB, embryonic body; RNA-seq, RNA sequencing; ChIP-seq, ChIP sequencing; DOX, doxycycline; GO, gene ontology; qPCR, quantitative Real-time PCR; Dn, day n.
H3K9Ac Regulates Pluripotency and Neural Differentiation

In this study, the transcriptionally active histone mark H3K9Ac was examined during in vitro hESC neural differentiation. The acetylated H3K9 level first decreased within 4 days and increased thereafter along with neural differentiation. HDACi treatment on days 0–4 assisted in maintaining pluripotency and suppressed neural differentiation, whereas HDAC inhibition promoted neural differentiation on days 4–8. Moreover, as possible HDACi targets, HDAC3 might be involved in modulating hESC pluripotency, and HDAC1/5/8 might participate in the neural promotion effect of HDACi during the later stage of hESC neural differentiation. In addition, the HAT member p300 was preliminarily identified as a major regulator in both pluripotent stem cells and neural progenitor cells (NPCs). Our study provides an epigenetic mechanistic rationale for the regulation of hESC pluripotency and neural commitment and demonstrates that histone acetylation might play dual roles in these two cellular events through binding to specific developmental gene loci.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatment—The HUES-9 hESC line was grown on a feeder layer of mitomycin C-treated (3 h) mouse embryonic fibroblasts. The hESC culture medium (26) consisted of DMEM/F-12-containing GlutaMax, which was supplemented with 20% knock-out serum replacer, 0.1 mM nonessential amino acids, 1 mM L-glutamine in Dulbecco’s F-12 medium as aggregates for another 4 or more days. The following reagents and indicated final concentrations were used: TSA (10 ng/ml; Sigma), CTK7A (5 μM; Merck Millipore), and VPA (0.5 μM; Sigma).

Quantitative Real-time PCR (qPCR)—Total RNA was purified using TRIzol reagents (Shanghai PuFei Biotechnology). Reverse transcription of 2.5 μg of total RNA was performed using the SuperScript III reverse transcription kit (Invitrogen) (27). qPCR was performed using SYBR Green PCR Master Mix (Sigma) in 20-μl reactions. Primer sequences are available upon request.

Western Blot—Western blot analysis was conducted according to our methods described previously (28). Briefly, cells were lysed in cell lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% NaDOC, 0.1% SDS, 1% Nonidet P-40, 5 mM EDTA, 0.25 mM PMSF, and a mixture of protease inhibitors. The cell lysates were subjected to immunoblotting with the following primary antibodies: anti-H3K9Ac (1:1000; Abcam), anti-H3K9/K14Ac (1:1000; Upstate), and anti-histone H3 (1:10,000; Sigma). Antibody-reacted proteins were visualized using the ECL detection reagents. The autoradiography of x-ray film was used to capture the protein bands.

Immunostaining—EBs were fixed in 4% parformaldehyde for 2 h at room temperature. Then EBs were moved to 20% sucrose solution for up to 4 h at 4°C. EBs were embedded in OCT and sectioned into 12-μm-thick sections. Finally, the sections were immunostained as described previously (29, 30). The following primary antibodies were used: anti-Oct4 (mouse, 1:200; Santa Cruz Biotechnology, Inc.), anti-PAX6 (rabbit; Covance).

RNA-seq Analysis—The cell samples with or without TSA treatment were collected in TRIzol reagents. Then total RNA was extracted and then subjected to RNA-seq analysis by high-throughput sequencing (CAS-MPG Partner Institute for Computational Biology). We assigned FPKM 600 (fragment per kilobase per million) as an expression value for each gene using Cufflinks version 1.3.0 software. Then Cuffdiff software was applied to identify differentially expressed genes between control and TSA-treatment samples (31). Differentially expressed gene heat maps were clustered by k-means clustering using the Euclidean distance as the distance and visualized using Java TreeView software (32).

ChIP and ChIP-seq Analysis—ChIP was performed as described previously (27) using an anti-H3K9Ac (1:1000; Abcam) antibody in hESCs or in differentiated cells (day 8). The ChIP samples were verified by qPCR or were subjected to sequencing by high-throughput sequencing platforms (Computational Biology Omics Core, PICB, Shanghai, China). The data analysis was performed using a program reported previously (33). The SOAP version 2.20 alignment tool was used to align ChIP-seq reads to the human genome. Only reads with fewer than two mismatches that uniquely mapped to the genome were used in subsequent analyses. Using FindPeaks Homer software, H3K9Ac binding peaks with 4-fold greater normalized tags were identified. The distance from the peak centers to the...
TABLE 1
Control shRNA sequences and targeting sequences for shRNAs used

| Name         | Sequences                                   |
|--------------|---------------------------------------------|
| Control shRNA| 5'-CTTGTTAAGCCTGTCGAG-3'                    |
| HDAC1 shRNA  | 5'-CCCCCTTCTTTATGAAAAACATT-3'               |
| HDAC2 shRNA  | 5'-CCGCTTACATTGGATGTTTTA-3'                |
| HDAC3 shRNA  | 5'-GCTGACAAAGATCTGAGAGA-3'                 |
| HDAC5 shRNA  | 5'-CTACAGGTTTAAACCCGATA-3'                 |
| HDAC6 shRNA  | 5'-GGTCCGCCAAGATCCTCATT-3'                 |
| HDAC7 shRNA  | 5'-GGGTCTTGTTATGAAAAACATT-3'               |
| HDAC8 shRNA  | 5'-GCCAGCCAGATCCTCATT-3'                   |
| HDAC9 shRNA  | 5'-GCCAGCCAGATCCTCATT-3'                   |
| HDAC10 shRNA | 5'-GCCGAGGTGAAGAAGTCATCGAGAT-3'           |
| HDAC11 shRNA | 5'-GCCAGCCAGATCCTCATT-3'                   |
| CBP shRNA    | 5'-GCCACGACCACCATGTTA-3'                   |
| p300 shRNA   | 5'-GCCAAAGATGTTGCGATGTTA-3'                |

Moreover, qPCR was conducted to assess the relative expression of developmental genes during neural differentiation. As expected, the early NPC markers (PAX6, SOX1, and OTX2) significantly increased along with neural differentiation, which was accompanied by the gradual down-regulation of pluripotency genes (OCT4 and NANOG) (Fig. 1B). Interestingly, the initial up-regulation of early neural genes and the semi-down-regulation of pluripotency genes were encountered on day 4 (Fig. 1B), indicating that the differentiating interval around day 4 might be an important transition stage from the pluripotent state to the neural state. Together, these data suggest that with this differentiation method, hESCs gradually lose the property of pluripotency and undergo stable and efficient neural commitment, which is suitable for investigating regulatory mechanisms underlying human neurodevelopment.

Histone acetylation is a switch between permissive and repressive chromatin and gene transcription (41). To examine the epigenetic mechanisms underlying gene activation and silencing during hESC neural differentiation, the H3K9Ac, H3K9/K14Ac, and H3Ac levels were detected by Western blot analysis. Intriguingly, both site-specific and pan-acetylated histone H3 levels initially decreased during days 0–4 and then were gradually up-regulated from day 4 to 8 (Fig. 1C). In other words, histone H3 acetylation (including H3K9Ac) exhibited relatively higher levels in both pluripotent ESCs and day 8 cells, in which the pluripotency-associated genes or early neural genes were activated, respectively (Fig. 1, B and C). Furthermore, the acetylated histone H3 level was lowest around day 4, most likely representing the transition phase of silencing pluripotency genes and initiating the transcription of early neural genes. Generally, the high H3Ac or H3K9Ac levels were correlated with the maintenance or activation of cell state-associated gene activation. Together, these results indicate that H3K9Ac down-regulation during days 0–4 and up-regulation during days 4–8 are independent events during hESC neural commitment and that histone acetylation might play distinct roles in pluripotent ESCs and NPCs.

RESULTS
Alterations of Histone H3 Acetylation during hESC Neural Differentiation—Multiple methods have been used to program hESCs to differentiate into neural cells (26, 36–38). Although the neural differentiation process reported by Pankratz et al. (26) is close to innate neurodevelopment, hESCs must undergo a physical transition from floating EBs to an adherent culture according to their protocol, which might enhance the epigenetic instability for multiple cell manipulations (39). An appropriate and relatively stable differentiation method should be used to examine the histone acetylation levels during hESC neural differentiation. In our previous study, we reported efficient methods for differentiating P19 cells and mouse ESCs into NPCs under serum-free EB culture conditions (30, 40). Based on the above findings, we established a slightly modified protocol for hESC neural differentiation. Mouse embryonic fibroblast-eliminated hESCs were cultured as floating aggregates in bFGF-free hESC maintenance medium for 4 days (0–4), and then EBs were cultivated for another 4 days (4–8) in neural progenitor medium (Fig. 1A). Undifferentiated hESCs and day 8 EBs were subjected to double-immunostaining assays with anti-PAX6 and anti-OCT4 antibodies. Approximately 55% of cells in day 8 EBs were OCT4+/PAX6+ NPCs (Fig. 1A).
and ~56% PAX6+/OCT4− NPCs). Meanwhile, under the TSA-supplemented conditions on days 0–4, the proportion of NPCs declined to ~36% in day 8 EBs, whereas the TSA treatment on days 4–8 augmented the percentage of NPCs to ~72% (Fig. 2F). In contrast, the expression of neurodevelopmental genes was up-regulated, and the pluripotency genes were down-regulated with the VPA treatment on days 4–8 (Fig. 2F). Through disturbance of histone acetylation by HDACis, we conclude that histone H3 acetylation might help to support ESC self-renewal in undifferentiated hESCs and to promote neural differentiation in hESC-derived NPCs.

Global Transcriptional Changes Induced by TSA during hESC Neural Differentiation—Given the opposite effects of HDACis on the expression of the characteristic pluripotency (OCT4 and NANOG) and neural (PAX6 and SOX1) genes at different stages of hESC neural differentiation, we asked whether HDACi treatment modulates gene expression at the global transcriptional level. To answer this question, RNA-seq analysis was conducted with total RNAs prepared from undifferentiated hESCs, the day 4 differentiated cells with or without TSA treatment on days 0–4, and day 8 differentiated cells with or without TSA treatment on days 4–8. Apparently, the gene expression-based clustering analysis showed that the expression of TSA-treated (days 0–4) day 4 cells was hierarchically closer to the original hESCs and that TSA-treated (days 4–8) day 8 cells were clustered together with control day 8 cells (Fig. 3A).

Comparing the transcription profiles of day 4 cells with or without TSA supplementation, multiple pluripotency genes or hESC markers, including KLF5, LEFTY1/2, NODAL, PCDH10, GDF3, DPPA2, NANOG, and OCT4, were identified among the top-ranking TSA-up-regulated genes (Fig. 3B). Moreover, the expression of many early neurodevelopmental genes (NRF1,
NLGN3, POU3F1, ZNF521/250, OTX2, ZIC2/5, etc.) decreased upon TSA stimulation (Fig. 3B). Gene ontology (GO) analysis revealed that the TSA-repressed genes were significantly associated with neural development and that the TSA-up-regulated genes mainly correlated with signal- and glycoprotein-related cellular events (Fig. 3C).

Moreover, the transcription levels of numerous neurodevelopmental genes, such as POU3F2, NRGN, NR2F1/2, PAX3/6,
NCAM1, TUBB3, Zic1, and MAP2, were remarkably enhanced by TSA treatment during days 4–8. Correspondingly, the transcriptional levels of a set of hESC-characteristic genes (TDGF1, TRIM8, GRB7, SALL3, OCT4, and NANOG) were reduced in these TSA-treated cells (Fig. 3D). Furthermore, the enriched GO terms of TSA-increased genes were highly associated with neurodevelopment-related processes (Fig. 3E). Subsequently, the representative altered genes responding to HDACis in RNA-seq results were confirmed by qPCR analysis. Compared with control cells on day 4, TSA or VPA treatment during days 0–4 promoted the expression of pluripotency genes and inhibited neural gene activation (Fig. 3F). Consistent with the RNA-seq data, HDACi treatment on days 4–8 markedly enhanced the transcription of several essential neurodevelopmental genes (POU3F2, NRGN, NR2F1, PAX3, and Zic1) and suppressed the expression of some hESC-specific genes in day 8.
Multiple HDACs Are Involved in hESC Pluripotency Maintenance and Neural Differentiation—Because disruption of HDAC activity by TSA or VPA dually influenced hESC neural differentiation at different stages (Fig. 2), distinct HDAC family members (HDAC1–11) might be involved in this process. First, the relative expression levels of HDAC genes were examined during hESC neural differentiation. We found that the majority of HDAC expression was relatively stable, except for HDAC6/9. The expression of HDAC6 was increased along with neural progression, whereas HDAC9 transcription was gradually decreased (Fig. 4A).

To study the functions of HDACs in hESC neural differentiation, lentivirus-mediated shRNAs specifically targeting HDAC1–11 were screened from commercial shRNA libraries. Then shRNAs were individually delivered into hESCs to establish stable cell lines, and the knockdown efficiencies of these shRNAs were examined by qPCR assays. Overall, HDAC1–11 genes were significantly knocked down by their corresponding shRNAs (Fig. 4B). To investigate the effect of HDACs on hESC pluripotency maintenance, shRNA-expressing hESCs were directly subjected to qPCR analysis of pluripotency genes. Interestingly, only HDAC3 depletion showed generally enhanced expression of pluripotency genes, and the deficiencies of other HDACs showed no or only minor influence (Fig. 4C). This result indicates that HDAC3 might be responsible for restricting the expression of pluripotency genes in the initiation of hESC differentiation (days 0–4). When HDACis inhibit HDAC3 activity, the restriction of pluripotency gene expression might be released. To further confirm this possibility, the response to TSA treatment was tested in HDAC3-knockdown cells on day 4. In contrast to the control hESCs, the TSA-induced up-regulation of NODAL and OCT4 was nearly abolished in the HDAC3-depleted cells (Fig. 4D), further proving that HDAC3 is the key target of HDACs at the initiating stage of hESC neural differentiation.

Next, the effects of HDAC deficiency were examined in these shRNA-containing hESCs after 8 days of differentiation. Depletion of HDAC1/5/8 enhanced the expression of some early neural genes (POL13F2, PAX3/6, NR2F1, ZIC1, and SOX1), which was similar to the observations in the TSA/VPA-treated cells during days 4–8 (Fig. 3G). Meanwhile, the expression of the pluripotency genes OCT4 and NANOG was decreased in HDAC1/5/8-knockdown cells (Fig. 4E). This phenotype of HDAC1/5/8 knockdown in hESC neural differentiation resembled the HDACi-elicited effects observed during days 4–8. In addition, HDAC2/6 depletion influenced individual neural gene expression, and, not surprisingly, the phenotype of the HDAC3 knockdown in day 8 cells was similar to that in hESCs (Fig. 4, C–E). Then we treated HDAC1/5/8-depleted cells with TSA during days 4–8. It was found that the TSA-induced promotion of neural differentiation during days 4–8 was hindered by HDAC1/5/8 depletion (Fig. 4F). Therefore, we postulate that HDAC1/5/8 are the direct and dominant targets of HDACs in hESC neural commitment (days 4–8). To further confirm the HDAC3 and HDAC1/5/8 roles at different developmental stages, we established hESC lines expressing DOX-inducible HDAC3 and HDAC1/5/8 shRNAs, and these cells were subjected to neural differentiation. Consistent with the results in stable knockdown assays (Fig. 4, C and E), HDAC3 knockdown during days 0–4 inhibited the down-regulation of pluripotency genes (OCT4, NODAL, and GDF3) (Fig. 4G), and HDAC1/5/8 depletion during days 4–8 specifically promoted early neural gene activation (NR2F1, PAX6, and PAX3) (Fig. 4H). In summary, HDAC3 is the primary target of HDACs in hESC pluripotency maintenance, and HDAC1/5/8 might be responsible for the negative regulation of neural gene expression to guarantee the appropriate progression of hESC neural differentiation.

p300 Modulates hESC Pluripotency and Neural Differentiation—Given that the histone acetylation status is negatively regulated by HDACs but also positively regulated by HATs, including CBP, p300, PCAF, and GCN5 (12, 15), the functions of HATs during hESC neural differentiation were also investigated. A water-soluble HAT inhibitor, CTK7A, was used to block HAT activity during days 0–4 or 4–8. The expression of pluripotency genes (OCT4, NANO, GDF3, and NODAL) was inhibited by CTK7A treatment (days 0–4) in day 4 cells (Fig. 5A). Furthermore, the presence of CTK7A during days 4–8 repressed the expression of the early neural genes (PAX6, SOX1, ZIC2, and ZNFS21) (Fig. 5A). Considering these results together, blocking HAT activity resulted in inhibitory effects on both hESC pluripotency and neural differentiation, suggesting that HAT activity is required for the transcriptional activation of pluripotency genes in hESCs and of neural genes in NPCs. Previously, the histone acetyltransferases CBP and p300 were reported to maintain mouse ESC identity by mediating the chromatin structure (43). To investigate their roles in hESC pluripotency and in early neural differentiation processes, CBP or p300 was knocked down using lentivirus-mediated shRNAs in hESCs (Fig. 5B). CBP or p300 depletion, respectively, resulted in reduced NODAL and LEFTY1 expression in undifferentiated hESCs. In addition, p300 knockdown led to the down-regulation of GDF3 and NANO (Fig. 5B). However, OCT4 transcription was not affected by p300 or CBP knockdown (Fig. 5B), which was consistent with a previous report (44). Then the effects of CBP and p300 knockdown were assessed in day 8 cells. The expression of some early neural genes (PAX6, SOX1, ZIC2, and ZNFS21) was inhibited by the depletion of p300 but not CBP (Fig. 5C). Because of the partially functional redundancy between CBP and p300 (45), we conclude that p300 is the primary HAT factor involved in modulating hESC pluripotency and neural differentiation.

H3K9Ac Regulates Pluripotency and Neural Differentiation—The above results demonstrate that histone H3 acetylation might play distinct roles in different cell states, maintaining the pluripotency of the undifferentiated hESCs and promoting hESC neural differentiation into NPCs. To further confirm this notion, ChIP-seq analysis was conducted with an anti-H3K9Ac antibody in hESCs (D0) and in the day 8 NPCs (D8). As shown in Fig. 6A, over 90% of H3K9Ac peaks were mainly located in the distal regions around transcription start sites (±1 kb) in both hESCs (n = 7522, 91%) and NPCs (n = 9625, 90%). The genes in which the enrichment of H3K9Ac is identified within ±5 kb of transcription start sites were defined as possi-
FIGURE 4. Multiple HDACs were involved in hESC pluripotency maintenance and neural differentiation. A, relative expression levels of HDAC1–11 during hESC neural differentiation from day 0 to 8. B, qPCR analysis of HDAC1–11 expression in HDAC shRNA-transfected hESCs. C, HDAC1–11 were knocked down by lentivirus-mediated shRNAs in hESCs. The transcriptional levels of multiple pluripotency genes were determined in these knockdown cells by qPCR. ↑, fold change >2; ↓, fold change <0.5. D, NODAL and OCT4 expression was detected in HDAC3-knockdown (KD) cells supplemented with or without TSA at day 4. E, the HDAC1–11-knockdown hESCs were subjected to neural differentiation for 8 days. qPCR was performed to analyze the expression of multiple neurodevelopmental genes as well as OCT4 and NANOG. F, the expression of NR2F1, POU3F2, and ZIC1 was determined by qPCR analysis with or without TSA treatment during days 4–8 in day 8 HDAC1/5/8-knockdown cells. G, HDAC3 shRNA was constructed into plvut-tTR-KRAB vector. Lentivirus-mediated HDAC3 shRNA was transduced into HUES-9 cells, and then the cells were subjected to neural differentiation. DOX (2 μg/ml) was added to the culture medium during days 0–4 (D0–4). Day 4 cells with or without DOX treatment were collected for qPCR analysis of OCT4, NODAL, and GDF3. H, a hESC line expressing plvut-tTR-KRAB-HDAC1/5/8 shRNAs was established and subjected to neural differentiation. HDAC1/5/8 shRNAs were induced by DOX during days 4–8 (D4–8), and day 8 cells were collected for examination of neural markers (NR2F1, PAX3, and PAX6). Error bars, S.D. *, p < 0.05.
H3K9Ac-regulated genes. According to this criterion, 350 H3K9Ac-enriched genes specific for hESCs, 1945 H3K9Ac-enriched genes specific for day 8 NPCs, and 9397 H3K9Ac-bound genes in both hESCs and NPCs were identified (Fig. 6B).

Moreover, the RNA-seq analysis of gene expression profile in hESCs (D0) and NPCs (D8) (Fig. 3A) showed that 2645 genes were down-regulated, and 2118 genes were up-regulated during hESC neural differentiation (Fig. 6B). Comparing the RNA-seq data with the H3K9Ac ChIP-seq result, we found that 183 of 350 D0-specific enriched genes decreased and that 1094 of 1945 D8-specific enriched genes increased during hESC neural differentiation (Fig. 6B). Therefore, we postulate that H3K9Ac-specific enriched genes may be associated with different cell identities. GO analysis further demonstrated that H3K9Ac-occupied genes in hESCs were mainly related to cell proliferation, signaling, and transport, whereas H3K9Ac-enriched genes in NPCs were closely associated with neural development (Fig. 6C), similar to the GO terms of the TSA-down-regulated genes on days 4–8 (Fig. 3C). Furthermore, the H3K9Ac enrichment at the loci of 183 down-regulated and 1094 up-regulated genes was analyzed. Clearly, compared with the intensity of the H3K9Ac ChIP signals in hESCs, the H3K9Ac enrichment of the 183 down-regulated genes (hESC-enriched) significantly decreased in day 8 NPCs, and the intensity of H3K9Ac occupancies at the loci of 1094 up-regulated genes (NPC-enriched) was strongly enhanced during the transition from hESC to the NPC state (Fig. 6D).

Specific to individual genes, multiple pluripotency- or NPC-characteristic gene loci were occupied by acetylated H3K9 in hESCs or in neural cells, respectively. In undifferentiated hESCs, the loci of several pluripotency genes or hESC-specific genes, such as OCT4, TDGF1, DPPA5, PRICKLE1, and VLDLR, were bound by H3K9Ac, and the enrichment at these genes was impaired in day 8 NPCs (Fig. 6E). Conversely, H3K9Ac occupancies at the loci of many neurodevelopmental genes (ZNF521, ZIC2, HESX1, SIX3, and POU3F1), which were expressed with a much higher level in NPCs (D8) than in hESCs (D0) (RNA-seq data in Fig. 3A), were strikingly increased during hESC neural differentiation (Fig. 6F). Moreover, the significant H3K9Ac enrichment at other neural genes, such as LHX5, SOX5, IRX1/3, and HES1, in day 8 cells was also observed (ChIP-seq data not shown). Intriguingly, even the expression of pluripotency genes was barely detected in NPCs on day 8, whereas the H3K9Ac occupancies at the loci of these genes were impaired but still present. In contrast, the H3K9Ac-enriched peaks at the loci of the majority of neurodevelopmental genes were almost newly generated in hESC-derived NPCs (Fig. 6E and F). This suggests that the H3K9Ac occupancies at the loci of these neural genes are required for neural gene activation during hESC differentiation. These data demonstrate that high H3K9Ac levels are dually correlated with hESC pluripotency and neural fate determination. In summary, H3K9Ac is enriched at specific sets of downstream target genes associated with cell status, and the intensity of H3K9Ac is correlated with the transcriptional activation or repression of pluripotency and neural genes during hESC neural commitment.

DISCUSSION

In this study, we show that the H3K9Ac level gradually decreases (days 0–4) and then increases afterward (days 4–8) along with hESC neural differentiation. It resembles H3K9Ac alterations with a faster cadence during mouse ESC differentiation (20). Genome-wide reduction of H3K9Ac has been reported in the endoderm-like differentiation of hESCs, suggesting that histone deacetylation is required for ESC differentiation (19, 20). The present finding, as well as others (19, 20),
suggests that the down-regulation of H3K9Ac is necessary for initiating pluripotent stem cell differentiation. Given that higher levels of H3K9Ac are displayed in hESCs and NPCs (Fig. 1), H3K9 acetylation might play distinct and independent roles in the different cell states. As expected, TSA/VPA-induced inhibition of H3K9Ac reduction during days 0–4 maintains the
pluripotency gene expression and inhibits neural differentiation. Conversely, forced up-regulation of H3K9Ac by HDACis during days 4–8 promotes neural gene activation and pluripotency gene repression (Figs. 2 and 3). Our study first reveals the dual roles of H3K9Ac in hESC pluripotency maintenance and neural differentiation and indicates that distinct HDAC members and HATs are involved in regulating histone acetylation status.

Histone acetylation plays essential roles in transcriptional initiation and elongation and is known to open the chromatin structure at the respective sites to facilitate transcription (46). H3K9Ac, which is a histone modification associated with open chromatin, is enriched at gene promoters with various genomic features and highly correlates with gene expression (47). In pluripotent hESCs, the H3K9Ac patterns present at the promoters of pluripotency genes were positively correlated with the transcriptional status of these genes (Fig. 6). This result is consistent with the previous reports indicating that pluripotency genes display only permissive histone marks in hESCs (19, 25). Given that the H3K9Ac levels decrease upon the initiation of hESC differentiation (Fig. 1), we postulate that H3K9Ac down-regulation is prerequisite for ESCs to switch off the pluripotency genes and then to exit from pluripotent status. Consequently, blocking the H3K9Ac down-regulation by HDACis at the initiation stage of neural differentiation maintains pluripotency gene transcription, which has been extensively proven in previous studies (21, 22, 47).

When ESCs differentiate into an intermediate state with low H3K9Ac levels and reduced expression of the pluripotency genes (around day 4), these cells are in the transition stage to be programmed into NPCs. In the subsequent neural progression stage (days 4–8), the early neurodevelopmental genes must be activated with permissive chromatin. Consequently, the H3K9Ac level is expectedly increased or newly displayed (Figs. 1 and 6). Importantly, H3K9Ac is mainly enriched at the promoter of some key neural genes in NPCs (Fig. 6). The forced up-regulation of H3K9Ac by HDACis at the neural commitment stage (days 4–8) promotes hESC neural progression, accompanied by decreased expression of pluripotency genes (Figs. 2 and 3). Furthermore, blocking HAT activity results in impaired neural differentiation (Fig. 5A), demonstrating that H3K9Ac is required for the early hESC neural commitment to NPCs. Our findings are coincident with the effects of the HDAC inhibition on neuronal differentiation of adult NPCs (48, 49). Examination of H3K9Ac enrichment reveals the gradual and progressive changes of the different genomic features during the transition from pluripotent hESCs to NPCs (Fig. 6). High H3K9Ac levels are identified in hESCs to maintain the expression of pluripotency genes and in NPCs to activate the transcription of the early neural genes (Figs. 1–3). Thus, H3K9Ac is one of the essential mechanisms of epigenetic regulation that determines the different cellular properties, and this modification is required for both hESC pluripotency maintenance and early neural commitment.

The timing of HDACis exposure appears to markedly influence the final effects of HDAC inhibition on hESC neural differentiation (Fig. 2). It has been revealed that VPA-elicited epigenetic changes suppress hESC neural differentiation (22). We argue that HDACis here might act by promoting mouse ESC pluripotency to block its neural differentiation, because pluripotency gene expression is concurrently up-regulated by VPA treatment during the early phase of ESC neural differentiation (22), which is similar to our results (Fig. 2).

Histone acetylation is antagonistically modulated by HATs and HDACs (15, 16). In the present study, HDAC1–11 knockdown assays demonstrate that HDAC3 is the possible HDAC protein involved in silencing hESC pluripotency genes (Fig. 4). Most likely, HDAC3 is the main target of TSA during days 0–4, and the inhibition of HDAC3 blocks TSA-induced up-regulation of pluripotency genes during the initiation stage of hESC differentiation (Fig. 4D). Moreover, HDAC1/5/8 are identified as HDACis targets during the later neural commitment stage, and the repression of these HDACs relieves the inhibition of early neural gene activation (Fig. 4). These findings indicate that the different HDACs participate in the epigenetic regulation of the histone deacetylation, which, in turn, determines the cell properties during hESC neural commitment. In addition, p300 is extensively implicated in modulating H3K9 acetylation (44, 47, 50, 51), and it also plays imperative roles in mouse ESC differentiation and Nanog expression (44). Consistent with these findings, p300 is involved in the transcriptional regulation of both pluripotency genes and neural genes (Fig. 5), which may rely on regulating H3K9 acetylation modifications. In view of the redundancy of CBP and p300, as well as the possible involvement of GCN5/PCAF, which are also able to mediate H3K9Ac (52), the effects of CBP or p300 knockdown are relatively milder (Fig. 5). In summary, both HATs and HDACs participate in establishing cell status-featured histone modification patterns. Additionally, the initial effects of HDAC inhibition on histone acetylation might be linked to disrupted secondary epigenetic changes, such as DNA methylation and histone methylation (21, 22).

In summary, the global investigation of H3K9Ac-representative permissive states suggests that H3K9 acetylation plays dual roles in hESC pluripotency and neural commitment (Fig. 7). H3K9Ac is present at different sets of gene promoters associated with cell identities and enables active transcription with cell type-specific patterns. The preference of acetylated histones may rely on the integrated network of histone chaperones, transcription factors, and DNA modifications (53, 54). p300-activated H3K9Ac is enriched at the loci of pluripotency genes in hESCs. HDAC3 might be responsible for the repression of pluripotency genes (days 0–4). When HDAC3 is inhibited by TSA, it results in the increased H3K9Ac levels and up-regulated expression of pluripotency genes. During the neural commitment stage (days 4–8), HDAC1/5/8 probably restrict neural differentiation to guarantee the appropriate neural commitment process. TSA derepresses the repressive effect of HDAC1/5/8 to promote H3K9 acetylation and neural differentiation. Overall, the global alterations of the H3K9Ac levels during hESC neural differentiation lead to a meaningful shift of the chromatin state from pluripotent stem cells to NPCs, which is epigenetically controlled by multiple HDACs and HATs. This study will help us to understand the epigenetic mechanisms...
underlying cell identities and directed differentiation of ESCs associated with histone acetylation patterns.

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