Epigenetic analysis of human embryonic carcinoma cells during retinoic acid-induced neural differentiation

Maryam Shahhoseini, Adeleh Taei, Narges Zare Mehrjardi, Ghasem Hosseini Salekdeh, and Hossein Baharvand

Abstract: Differentiation of stem cells from a pluripotent to a committed state involves global changes in genome expression patterns, critically determined by chromatin structure and interactions of chromatin-binding proteins. The dynamics of chromatin structure are tightly regulated by multiple epigenetic mechanisms such as histone modifications and the incorporation of histone variants. In the current work, we induced neural differentiation of a human embryonal carcinoma stem cell line, NTERA2/NT2, by retinoic acid (RA) treatment, primarily according to two different methods of adherent cell culture (rosette formation) and suspension cell culture (EB formation) conditions, and histone modifications and variations were compared through these processes. Western blot analysis of histone extracts showed significant changes in the acetylation and methylation patterns of histone H3, and expression level of the histone variant H2A.Z, after RA treatment in both protocols. Using chromatin immunoprecipitation (ChIP) coupled with real-time PCR, it was shown that these epigenetic changes occurred on the regulatory regions of 4 marker genes (Oct4, Nanog, Nestin, and Pax6) in a culture condition dependent manner. This report demonstrates the dynamic interplay of histone modification and variation in regulating the gene expression profile, during stem cell differentiation and under different culture conditions.

Key words: epigenetic, H2A.Z, NTERA2, neural differentiation, retinoic acid.

Résumé : La différenciation des cellules souches d’un état pluripotent à un état déterminé implique des changements globaux dans les patrons d’expression du génome, déterminés de façon critique par la structure de la chromatine et les interactions des protéines de liaison de la chromatine. La dynamique de la structure de la chromatine est fortement régulée par de multiples mécanismes épigénétiques tels les modifications d’histones et l’incorporation des variants d’histones. Dans ce travail, nous avons induit une différenciation neurale des cellules de la lignée de carcinome embryonnaire humaine NTERA2/NT2 par un traitement à l’acide rétinoïque (AR), en suivant deux méthodes différentes de culture cellulaire, soit sur des cellules adhérentes (formation de rosettes) ou en suspension (formation de CE), et les modifications/variantes d’histones ont été comparées au cours de ces processus. L’analyse d’extraits d’histones en buvardage par Western ont montré des changements significatifs dans les patrons d’acétylation/méthylation de l’histone H3, et dans les niveaux d’expression du variant d’histone H2A.Z après un traitement à l’AR, dans les deux protocoles. À l’aide d’une immunoprécipitation de la chromatine (ChIP) couplée à une PCR en temps réel, nous montrons que ces changements épigénétiques surviennent dans les régions régulatrices de quatre gènes marqueurs (Oct4, Nanog, Nestin et Pax6) de façon dépendante des conditions de culture. Ce rapport démontre l’interaction dynamique de la modification/variation des histones dans la régulation du profil d’expression génique, lors de la différenciation des cellules souches et sous différentes conditions de culture.


[Intaduit par la Rédaction]

Introduction

Embryonal carcinoma (EC) cells derived from germ cell tumors are valuable tools for investigating embryogenesis and developmental biology processes. The advantage of reproducibility and rapid in vitro expansion and handling of these cell lines make them a useful alternative to embryos...
when studying molecular mechanisms of mammalian cell differentiation (Przyborski et al. 2004). The phenomenon of differentiation relies on large-scale sequential changes in expression pattern of subsets of genes involved in development. Epigenetic changes, including DNA methylation and histone modifications and variations, are the key players of gene regulation, inducing both local and global chromatin alterations of the genome (Klose and Bird 2006; Kouzarides 2007; Henikoff 2008; Ng and Gurdon 2008). In developmental model systems, covalent modifications of chromatin have been shown to act as epigenetic marks that cause "commitment" of cells to the differentiated phenotype (Dai and Rasmussen 2007; Meshorer 2007; Yeo et al. 2007), primarily according to the cell culture and (or) induction conditions.

Histone modification, specifically acetylation, methylation, and phosphorylation, can be either repressive or permissive for transcription, depending on location and context (Kouzarides 2007). Several modifications such as acetylation of lysine (K) 9 of histone H3 (H3K9ac) or H3 trimethylation on lysine 4 (H3K4me3) are highly associated with transcription activity (Zhang and Reinberg 2001; McCool et al. 2007), whereas H3K9me and H3K27me3 are correlated with inactive chromatin (Lee et al. 2007). It is established by investigators that undifferentiated ES cells possess a unique epigenetic landscape termed the "bivalent mark": a combination of the active chromatin mark H3K4me and the repressive H3K27me3 (Bernstein et al. 2006). This epigenetic mark keeps the repressed genes in a state poised for transcriptional activation during differentiation (Bernstein et al. 2006; Lee et al. 2006; Lee et al. 2007). For the genes so marked, it is thought that removal of the repressive H3K27me3 triggers transcriptional activation, although other activation signals are also needed to complete this derepression (Szutorisz et al. 2005).

### Table 1. Primer pairs used in this study.

(A) RT-PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers (5' → 3')</th>
<th>Annealing temperature (°C)</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
</table>
| OCT4   | F:GTTCTATTTGGGAAGGTATTCAGC  
        | R:GTATCAAGAACCCAATCGGGA | 60 | 323 |                     |
| NANO5  | F:CAGAAGGCTTCAGCACTCAC  
        | R:GTCAGCTTGGCAAGAATTGG | 62 | 298 | Baharvand et al. (2007) |
| NESTIN | F:CTCTGACCTGTCGAAAGAAT  
        | R:CCCACCTTCTCCATCTCGT | 56 | 475 | Baharvand et al. (2007) |
| PAX6   | F:TTCAGCACCAGTGCTCTACACAC  
        | R:GCTGACTTGTCTCGTGCTTG | 63 | 300 | Baharvand et al. (2007) |
| GAPDH  | F:AGGTTGCTCTCCCTCTGACCTCA | R:AGGGTCTCTCTCTCTCTGTCG | 60 | 223 |                     |

(B) Real-time RT-PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers (5' → 3')</th>
<th>Annealing temperature (°C)</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
</table>
| OCT4   | F:TCTATTTGGGAAGGTATTCAGC  
        | R:ATTGGTGTCGCTCGCTCCA | 60 | 124 |                     |
| NANO5  | F:AGCTCAAACAGGTAAGAC  
        | R:GGTGGTGAAGAGTAAAGG | 60 | 145 |                     |
| NESTIN | F:CTTCAGAAACTCAAGCACC  
        | R:TCTCGATTCTCTCTCTGCA | 60 | 144 |                     |
| PAX6   | F:CGGTCTCCTCCCTTCACAT  
        | R:ATCATACCTCAGCCCTATT | 60 | 196 |                     |
| ACTB   | F:TCCCTGAGAGAGACTCGAC  
        | R:GTAGTTCCTGGGATGCCACA | 60 | 131 |                     |

(C) Real-time ChIP PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers (5' → 3')</th>
<th>Annealing temperature (°C)</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
</table>
| OCT4   | F:GAGGATGGCAAGCTGAGAAA  
        | R:CTCAATCCCCAGGACAGAAC | 60 | 148 | Dahl and Collas (2007) |
| NANO5  | F:GTCTCTGTGCTCGGTTTCTTCT | R:TCCGTTCTCAGGTCCTCA | 60 | 95  | Dahl and Collas (2007) |
| NESTIN | F:CCTGAGAATTCCCCACCTCCC | R:CCTACTACACCCACACACAG | 60 | 150 |                     |
| PAX6   | F:AAAAACCCCAACCAACAAADA  
        | R:GCAAATAAAAATAAGGCGAAGGA | 60 | 102 | Dahl and Collas (2007) |

Note: bp, base pairs; ChIP, chromatin immunoprecipitation; F, forward; R, reverse; RT-PCR, reverse transcription-PCR.
and Hake 2006). Replacement of cognate histones by their variants can cause chromatin reorganization of the genome at the site where they are incorporated (Bernstein and Hake 2006). A protein from this group, a variant of histone H2A named H2A.Z, has been implicated in many diverse biological processes such as gene activation, heterochromatin silencing, and progression through the cell cycle (Zlatanova and Thakar 2008).

In an ongoing effort to gain an overall understanding of the contribution of epigenetic changes to the control of cell differentiation, the current study compares chromatin modifications in human EC cell line NT2 (NTERA2), and after retinoic acid (RA) induced neural differentiation through different culture conditions. RA, the derivative of retinol, induces the EC cells to differentiate into various lineages in vitro, depending on its concentration and the cell culture conditions (Rohwedel et al. 1999; Soprano et al. 2007). To be driven to a neural lineage, undifferentiated cells may be exposed to RA under either non-adherent culture conditions (Cheung et al. 1999; Megiorni et al. 2005), or adherent culture conditions by formation of rosette structures (Horrocks et al. 2003). In this respect, we tried to compare sequential epigenetic changes of histones on the 5′-untranslated regions of 4 marker genes involved in stemness (Oct4 and Nanog) and early stages of neural differentiation (Nestin and Pax6), through neural differentiation of NTERA2 cells in either adherent and non-adherent (suspension) culture conditions. The DNA methylation status of the aforementioned stemness genes has been previously reported by investigators (Deb-Rinker et al. 2005; Yeo et al. 2007), but this is the first time that differentiation-sensitive alterations in histone code of these genes has been checked in this teratocarcinoma cell line, as well as the first time that two different culture conditions have been compared during neural differentiation. The findings presented here show the dynamic interplay of histone modifications in regulating gene expression pattern during stem cell induction and differentiation, as well as the differential expression of the H2A.Z variant, in adherent and non-adherent culture methods.

**Materials and methods**

**Cell sample preparation**

NTERA2 clone D1 (NT2.cl.D1, a gift from Dr. Peter Andrews) EC stem cells were grown and maintained in DMEM medium supplemented with 10% fetal calf serum and 2 mmol-L⁻¹ L-glutamine at 37 °C in 5% CO₂. A confluent monolayer of pluripotent cells were used as the cellular source for neural differentiation.

Neural differentiation was induced by RA in either adherent (rosette formation) or suspension (embryoid body (EB) formation) cell culture conditions, according to Horrocks et al. (2003). Briefly, RA-induced cultures were maintained for 2 weeks, changing the induction medium every 3–4 days. Adherent cultures were grown for a further 3 weeks in the absence of RA to form rosette bodies. Rosettes and EBs were separately plated onto sterile 6-well dishes (TPP 92406, Switzerland) pre-coated with poly-d-lysine (Sigma; 10 µg·mL⁻¹). Cells were grown in DMEM – 2.5% fetal calf serum for 2 weeks, in the absence of RA but in the presence of the mitotic inhibitor cytosine arabinosine (Sigma, 1 µmol·L⁻¹), for the first 7 days. A fraction of rosette or EB bodies were also plated onto glass cover slips pre-coated with poly-d-lysine and maintained for 2 weeks as described previously for the immunostaining step.

NT2 cells ready to harvest and analyze at each of the culturing steps (see Results) were synchronized using thymidine (Sigma, 3 mmol·L⁻¹) according to the method of Hake et al. (2006). After release from the thymidine block, cells were harvested, washed twice with PBS, and split into 3 samples for RNA isolation, histone extraction, and chromatin immunoprecipitation (ChIP) analysis.

**Immunocytochemistry**

Cell cultures grown on glass cover slips were stained for neural proteins using primary mouse monoclonal antibodies anti-MAP2 (Sigma M1406, 1:250) and anti-β-tubulin-III (Sigma T8680, 1:500), and goat anti-mouse IgG-conjugated fluorescein isothiocyanate (FITC, Chemicon AP308F, 1:250) as the secondary antibody in the preparation for fluorescence microscopy, as described previously (Baharvand et al. 2007).

**RNA isolation and quantitative real-time PCR analysis**

Total RNA was extracted from 3 independent replicates using the NucleoSpin RNA II kit (Macherey-Nagel). Prior to reverse transcription (RT), RNA samples were digested with DNase I (Fermentas; EN0521) to remove contaminating genomic DNA. Standard RT was performed using 2 µg total RNA, oligo (dT)18, and the RevertAidTM H Minus First Strand cDNA Synthesis kit (Fermentas; K1622) according to the manufacturer’s instructions. The cDNA samples were subjected to PCR amplification using designed human-specific primers (Table 1A). The amplification profile was as follows: Initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 54–64 °C for 45 s, extension at 72 °C for 45 s, and a final polymerization at 72 °C for 10 min. The PCR products were analyzed by gel electrophoresis on 1.7% agarose, stained with ethidium bromide (10 µg·mL⁻¹), and visualized and photographed on a UV transiluminator (Uvidoc).

mRNA quantification was performed in triplicate by quantitative real-time PCR (qRT-PCR) on a 7500 Real-Time PCR System (AB Applied Biosystems) using SYBR Green master mix (AB Applied Biosystems), with designed primers listed in Table 1B. qRT-PCR conditions were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Gene expression data were analyzed using the ΔΔCt quantitative method (Livak and Schmittgen 2001), to estimate relative fold change values.

**Acid–urea and sodium dodecyl sulfate – polyacrylamide gel electrophoresis of histone extracts**

Total histones were extracted using 0.2 N HCl as described by Balasubramaniyan et al. (2006). Fifteen micrograms of histone extract were separated by 15% sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS–PAGE), 100 V for 1.5 h, using a Mini-PROTEAN 3 electrophoresis cell (Bio-Rad), primarily according to the method of Laemmli (1970). Acid–urea (AU) 15% gel electrophoresis was performed according to Shechter et al. (2007), also using a Mini-PROTEAN 3 electrophoresis cell (Bio-Rad).
Western blot analysis

Western blot analyses of total histones were performed twice or more, and each time 15 micrograms of histone extract were separated on 15% SDS–PAGE electrophoresis and transferred to PVDF membrane (Bio-Rad) by wet blotting system (Bio-Rad). Membranes were blocked in 1% BSA for 1 h and incubated overnight with the primary antibodies at 4 °C. The membranes were then incubated with
appropriate peroxidase-conjugated secondary antibodies, either anti-mouse IgG (Sigma A9044, 1:10000) or anti-rabbit IgG (Sigma A0545, 1:15000). The protein bands were then visualized using ECL-Plus substrate (Sigma, CPS-1–120) and detected by exposure to X-ray film (GE Healthcare). Applied primary antibodies were against H3K9ac (ab4441), H3K9me2 (ab7312), H3K4me3 (ab1012), H3K27me3 (ab6002), and H2A.Z (ab4174), all from Abcam Co., with final concentrations of 1:1000. Blotting for total histone H3 (Sigma HO164) was applied as a loading control.

**Sample preparation for mass spectrometry**

A sliced protein band from SDS–PAGE was washed twice with 50% v/v aqueous acetonitrile containing 25 mmol L⁻¹ ammonium bicarbonate, once with acetonitrile, and then dried in a vacuum concentrator for 20 min. Sequencing-grade modified porcine trypsin (Promega) with a final concentration of 0.01 mg mL⁻¹ was used for digestion of dried protein sample, 15 h at 37 °C, after dilution of the sample with 25 mmol L⁻¹ ammonium bicarbonate solution.

**Mass spectrometry**

The tryptic-digested protein fraction was applied directly to the ground steel MALDI target plate, followed immediately by an equal volume of a freshly-prepared 5 mg mL⁻¹ solution of 4-hydroxy-a-cyano-cinnamic acid (Sigma) in 50% v/v aqueous acetonitrile containing 0.1% v/v trifluoroacetic acid.

Positive-ion MALDI mass spectra were obtained using a Bruker ultraflex III in reflectron mode, equipped with a Nd:YAG smart beam laser. MS spectra were acquired over a mass range of m/z 800–4000. Final mass spectra were externally calibrated against an adjacent spot containing 6 peptides (des-Arg¹-bradykinin, 904.681; angiotensin I, 1296.685; Glu¹-fibrinopeptide B, 1750.677; ACTH (1–17 clip), 2093.086; ACTH (18–39 clip), 2465.198; ACTH (7–38 clip), 3657.929.).Monoisotopic masses were obtained using a SNAP averagine algorithm (C 4.9384, N 1.3577, O 1.4773, S 0.0417, H 7.7583) and an S/N threshold of 2. The 10 strongest peaks of interest, with an S/N greater than 10, were selected for mass spectometry (MS) fragmentation. Fragmentation was performed without the introduction of a collision gas. The default calibration was used for MS/MS spectra, which were baseline-subtracted and smoothed (Savitsky-Golay, width 0.15 m/z, cycles 4); monoisotopic peak detection used a SNAP averagine algorithm (C 4.9384, N 1.3577, O 1.4773, S 0.0417, H 7.7583) with a minimum S/N of 3. Bruker flexAnalysis software was used to perform the spectral processing and peak list generation for both the MS and MS/MS spectra.

Combined mass spectral and tandem mass spectral data were submitted to database searching using a locally running copy of the Mascot program (Matrix Science Ltd., version 2.1) through the Bruker BioTools interface (version 3.1). Search criteria included the following: enzyme, trypsin; variable modifications, oxidation (M); peptide tolerance, 200 ppm; MS/MS tolerance, 0.8 Da; instrument, matrix-assisted laser desorption ionization time of flight (MALDI-TOF)-TOF. The search criteria also included carbamidomethyl (C) as a fixed modification for all alkylated samples. The database search was run against the NCBI non-redundant
protein database (20080218; 5947209 sequences; 2045123248 residues).

Chromatin immunoprecipitation real-time PCR analysis

Chromatin immunoprecipitation (ChIP) experiments were performed using an Orange ChIP kit (Diagenode, Belgium) according to instructions. Chromatin from $1 \times 10^5$ cells was used for each immunoprecipitation reaction. PCR amplification was performed on DNA recovered from the ChIP and the total chromatin input using designed primers listed in Table 1C. Immunoprecipitated DNA was analyzed in triplicate by real-time PCR starting from 5 μL of template DNA (from a total of 50 μL) on a 7500 Real-Time PCR System (AB Applied Biosystems) using SYBR Green master mix (AB Applied Biosystems). PCR conditions were as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 45 s. Data were expressed as fold enrichment of DNA associated with different immunoprecipitated histone modifications/variations relative to a 1/100 dilution of input chromatin.

Statistical analysis of real-time PCR

Values are expressed as means ± SEM of 3 separate experiments. All data were analyzed using repeated-measures ANOVA followed by Tukey–Kramer multiple comparisons post hoc test. Differences were considered statistically significant at $p < 0.05$.

Results

Differentiation of NT2 cells and expression of neural markers

Human EC NT2 cells were originally cultured in DMEM supplemented with 10% fetal calf serum to obtain a conflu-
ent monolayer of pluripotent cells (Fig. 1A). Two distinct methods of differentiation were performed under the induction effect of retinoic acid. These two methods focus on the formation of neurospheres under essentially two different culture conditions, allowing for the rapid production of large numbers of neural cells after plating of neurospheres. Rosettes formed in adherent culture (Fig. 1B), and EBs produced from suspension culture (Fig. 1C), were separately plated on poly-D-lysine coated culture surfaces. Although there is a delay in the formation of neurospheres in adherent culture compared with in suspension, the purified neurospheres of both culturing systems produced fine neurites soon after plating, and the difference in the amount of neurogenesis observed between the two forms of neurospheres was almost indistinguishable (Figs. 1D and 1E). Immunofluorescence analysis of the differentiated cultures was performed to evaluate the expression of two neural protein markers, MAP-II (Figs. 1F and 1G) and β-tubulin-III (Figs. 1H and 1I).

Expression analysis of marker genes in NT2 cells differentiating under different culture conditions

The expression of mRNAs of two stemness genes (Oct4 and Nanog) and two neuroectodermal marker genes (Nestin and Pax6) was determined by RT-PCR, using GAPDH (glyceraldehyde-3-phosphate dehydrogenase gene) as control for the mRNA quantity in each sample (Fig. 2). Also, to quantify the relative expression level of the marker genes through different stages of RA-induced neural differentiation of NT2 cells, with respect to its undifferentiated form, quantitative real-time PCR was performed, using the ACTB (β-actin) housekeeping gene for normalization (Fig. 3). The expression levels were analyzed 0, 4, 8, 12, 15, 21, 28, and 42 days after RA induction, according to the culture condition applied for neurosphere formation, as well as 14 days after plating of rosette or EBs on poly-D-lysine coated culture dishes. As expected, Oct4 and Nanog, which are characteristically expressed by EC and ES cells, were down-regulated after RA treatment in both culturing systems.

Nestin, an intermediate neurofilament protein expressed in neural stem cells and progenitor cells, showed a low expression level in undifferentiated NT2 cells. Soon after exposure of cells to RA, expression of Nestin increased, especially in suspension culture. Down-regulation of Nestin in plated EBs was in agreement with the fact that this neurofilament protein is a neural progenitor marker, mainly expressed in early stages of neural differentiation. However, in adherent culture conditions, a continuous expression of Nestin was observed without a significant variation in quantity, a situation that continued even after plating of neurospheres. Observation of Nestin mRNA in NT2 cells before RA treatment discriminates this neuroectodermal marker gene from Pax6, at least in this embryonal carcinoma cell line.

Unlike Nestin, Pax6, is a homeobox protein expressed in neural progenitor cells, was not expressed in undifferentiated cells. This protein was immediately up-regulated after RA exposure of cells and onset of differentiation. It should be mentioned that in both adherent and non-adherent RA-induced systems, Pax6 showed the highest amounts of expression level in comparison with Nestin with respect to undifferentiated NT2 cells (Fig. 3).

Electrophoretic pattern of total histone extracts on AU- and SDS–PAGE

Total histones of cell samples were extracted and analyzed 0, 4, 8, 12, 15, 21, 28, and 42 days after RA induction, according to the culture condition applied for neurosphere formation, as well as 14 days after plating of rosette or EBs. Figure 4 displays the electrophoretic patterns of total histone extracts on two different AU and SDS–PAGE systems, at various stages of adherent and suspension cell cultures. The result declared a thin protein band in
undifferentiated NT2 cells, which disappeared after RA treatment (see arrowheads in Fig. 4). Interestingly, this protein band was discernable again in 28 day old adherent cultures, through rosette formation process, and also after plating of both rosette and EBs. To identify the aforementioned histone protein, mass spectrometry was employed on a Coomassie-stained excised band, using the in-gel tryptic digestion procedure followed by MALDI TOF/TOF MS analysis (Fig. 5). MS analysis resulted in identification of the protein as histone H2A.Z, a variant of the cognate histone H2A (see Supplemental Data).

We further analyzed the expression of H2A.Z using Western blot, which was complementary to the AU-PAGE, SDS–PAGE, and MS data already obtained. As indicated in Fig. 6, expression of histone H2A.Z in undifferentiated cells immediately disappeared after differentiation onset, especially in suspension culture. A significant increase in its expression level was also observed after 28 days of differentiation of adherent cells, as well as after plating of both rosette and EB neurospheres.

Global levels of histone modifications or variations during RA-induced differentiation of EC cells

Total levels of some histone modifications with either repressive or permissive roles in transcriptional activation were analyzed in undifferentiated hEC and NT2 cells, as well as at various stages of their RA-induced differentiation. As seen in Fig. 6, immunoblotting of acid-extracted histones was performed using antibodies against H3K9ac, H3K9me3, H3K4me3, and H3K27me3 modifications and H2A.Z variation. The results showed that during differentiation in both adherent and suspension cultures, the total pools of histone H3 decreased in K9 acetylation, whereas methylation of this residue gradually increased. Despite no significant change in tri-methylation of H3K4, another modification of bivalent mark, H3K27me3, showed a global
decrease during 7 days of RA treatment, in both adherent and suspension culturing systems.

Localized histone modification/variation patterns on gene regulatory regions

Chromatin immunoprecipitation (ChIP) was applied on the regulatory regions of the marker genes during differentiation to monitor the presence of the aforementioned global histone modifications and variation. To this end, regulatory regions of the defined genes were amplified using designed primers (listed in Table 1C), after immunoprecipitation of the chromatin with either the anti-modified or anti-variant histones H3K9ac, H3K9me2, H3K4me3, H3K27me3, and H2A.Z antibodies. As shown in Fig. 7, at the undifferentiated stage, acetylated H3K9 (H3K9ac) was the dominant modification, especially for the stemness gene Oct4, which was enriched about 80-fold over input chromatin level (no Ab control). This was in conjunction with a high level of tri-methylation on H3K4, which confirmed over-expression of this transcription factor in stem cells, as the expression data had previously shown (Figs. 3 and 4). The regulatory region of the other stemness gene, Nanog, was also epigenetically marked by hyperacetylation of H3K9 and tri-methylation of H3K4, accounting for up-regulation of this marker gene in the undifferentiated stage.

However, RA treatment caused a significant decrease in the level of H3K9ac and H3K4me3 modifications at up-stream regions of Oct4 and Nanog, confirming the remarkable decrease in expression level of these two stemness genes (Figs. 2 and 3). Rosette or EBs were signed by hypoacetylation of stemness genes Oct4 and Nanog parallel to hyperacetylation of neural differentiation genes Nestin and Pax6. In neurosphere structures, high levels of H3K9 acetylation observed on the regulatory region of Pax6 confirmed overexpression of this neuroectodermal marker gene, in both adherent and suspension systems. A remarkable difference between epigenetic marks of rosette and EBs was the presence of the H2A.Z variant in rosettes, but not in EBs, which is in accordance with Western blot analysis of total histones with anti-H2A.Z (Fig. 6).

ChIP analysis of plated neurospheres showed increasing levels of H3K9ac on Oct4 and Nanog regulatory regions, in comparison with either rosettes or EBs. This epigenetic alteration was in agreement with gene expression data (Figs. 3 and 4), showing over-expression of stemness marker genes in plated mature neurospheres.

In plated rosettes, incorporation of H2A.Z on up-stream regions of the marker genes was compatible with detection of this histone sub-type in total histone extracts of rosette bodies, as well as their plated forms (Fig. 7E). H2A.Z was also detected in plated forms of EBs produced through suspension culture of NT2 cells, although this histone variant was not observed in EBs before plating. Although the ChIP real-time data obtained for H2A.Z was in agreement with Western blot detection of this protein in total histone extracts of the sample cells, we could not deduce a relationship between the activation state of the marker genes and preferential incorporation of this histone variant to the regulatory regions of these genes.

Discussion

Differentiation is an excellent model for studying epigenetics, since the same cell with its own fixed genetic material transforms from one state to another. This transition appears to involve the combined regulation of chromatin structure via chromatin modifications, histone variants, and different chromatin-remodeling elements. As teratocarcinoma cell lines have the advantage of being a simpler, less expensive, and more robust experimental system rather than human embryos, EC stem cells derived from human germ cell tumors provide a useful tool to study molecular mechanisms through differentiation to embryonic lineages (Andrews et al. 2001).

In the current work, we used the human EC cell line NT2 to analyze and compare epigenetic changes that occurred through neural differentiation in this simple model. It has been reported by investigators that several extrinsic and in-
trinsic factors are involved in the sequential expression and (or) repression of certain subsets of genes during neurogenesis in NT2 cells (Przyborski et al. 2000; Katoh 2002; Megiorni et al. 2005). Considering this background, a marker gene set including two stemness genes (Oct4 and Nanog) and two neuroectodermal marker genes (Pax6 and Nestin) were chosen to compare epigenetic changes in neural ectoderms obtained from different adherent and suspension cell culture systems.

Our experiments have shown that histone modification patterns on the up-stream regulatory regions of the 4 aforementioned marker genes were drastically changed through RA treatment of cells, nearly in accordance with their gene expression profile. Comparing epigenetic patterns of neural ectoderms obtained from adherent and non-adherent cell cultures showed the significant effects of culture condition on neural commitment of EC cells. It was concluded that the epigenetic marks providing commitment of cells to neural
lineage were slightly stronger in EBs rather than rosettes. Hypoacetylation of K9 and hypermethylation of K27 on the regulatory regions of two stemness genes, Oct4 and Nanog, were more significant in EBs than in rosettes. Also, hyperacetylation of two neuroectodermal genes, Pax6 and Nestin, was about 2–3 fold higher in suspended neurospheres than in adherent rosettes (Fig. 7B and C). These results show that neural differentiation is more efficient in suspension cultures. Thus, it can be concluded that cell populations in EBs induced by RA are more homogeneous and most of them are committed cells differentiated to a neural ectoderm lineage. Also, an extensive down-regulation of them are committed cells differentiated to a neural ectoderm lineage. Also, an extensive down-regulation of oct4 and Nanog genes observed in RA-ununtreated control cells of non-adherent cultures confirms that EB structures provide a better microenvironment for differentiation of EC cells (Fig. 3). Furthermore, higher expression of Oct4 and Nanog in both forms of plated neurospheres can be attributed to heterogeneity of cells even in EBs, which amplified by propagation of the undifferentiated EC cells in the cell population after plating on an adherent surface.

Another point of view is the expression of Nestin in undifferentiated NT2 cells (Fig. 2), the data previously reported by other investigators (Andrews et al. 2001). Although Nestin is usually considered as a neural marker protein (Lendahl et al. 1990; Przyborski et al. 2003), observation of two active epigenetic markers, high H3K9ac and low H3K9me, on the regulatory region of its coding gene at the stemness stage (Fig. 7A) excludes Nestin as a specific marker in neural differentiation of this teratocarcinoma cell line.

Comparing the expression patterns of Nestin and Pax6, Pax6 can be accepted as a marker gene of differentiation in NT2 cells, owing to its specific activation by RA induction (Fig. 2 and 3). Detection of a distinct epigenetic profile for Pax6 with respect to the other 3 analyzed marker genes (Fig. 7A) was in accordance with this claim. It is interesting that among the 4 marker genes checked at the stemness stage, the repressive modification, H3K27me3, was only detected on the regulatory region of Pax6, at a high rate of about 30-fold over input chromatin. It has already been established that in embryonic stem cells the facultative heterochromatin marker, H3K27me3, is only observed in the regulatory regions of the developmental genes, which although silent in stem cells, are preferentially activated through induction of differentiation (Lunyak and Rosenfeld 2008). This “pause” state of PcG responsive genes will change to “play” in the presence of H3K4me3 and other transcriptionally active epigenetic markers such as H3K9ac at the onset of differentiation (Zhang and Reinberg 2001). Detection of the “bivalent mark” (H3K4me3/H2K27me3) on the regulatory region of the neural progenitor marker gene, Pax6, supported the developmental regulatory role of this transcription factor, which was specifically up-regulated in response to differentiation signals.

Histone variant H2A.Z can be considered as novel epigenetic marker in teratoma cells

Expression of a variant of histone H2A, identified as H2A.Z, in teratocarcinoma cell line NT2 is a considerable finding in this study. With this in mind, i.e., that EC cells are originally malignant but their differentiated derivatives are not, the presence of H2A.Z in total histone extract of undifferentiated NT2 cells and absence of this histone variant after RA-induced differentiation of this cell line (Fig. 4) suggests H2A.Z as a novel epigenetic marker in teratoma cells. This claim can be supported by a recent finding reported by Hua et al. (2008), correlating the expression of H2A.Z with increased probability of progression and metastasis of tumor cells in patients suffering from breast cancer. It is interesting to note that this property was suggested by the authors as a biomarker of cancer in a wide range of teratoma systems.

Detection of H2A.Z in histone extracts of EBs after plating (Figs. 7D and 7E), may be related to adhesion of undifferentiated NT2 cells remaining in the cell population, and possibly of the propagation of these teratocarcinoma stem cells on the adherent surface of culture dishes.

In conclusion, it should be mentioned that differential incorporation of H2A.Z on the regulatory regions of the four marker genes obtained from ChIP real-time analysis (Fig. 7) encouraged us to study whole-genome mapping of this histone variant protein in teratocarcinoma cell line NT2.

Acknowledgements

We would like to thank Mansoureh Shahsavani and Azam Samadian for their technical support in the molecular biology analyses. The authors would like to dedicate this paper to the memory of Dr. Saeid Kazemi Ashitani, the late founder of the Royan Institute. This project was financially supported by the grant No. 630-3 of the Royan Institute.

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