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Evidence for a dynamic role of the linker histone variant H1x during retinoic acid-induced differentiation of NT2 cells

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ABSTRACT

The dynamics of chromatin structure are tightly regulated by multiple epigenetic mechanisms such as histone modifications and incorporation of histone variants. In the current work, differentiation of an embryonal carcinoma cell line, NT2, was induced by retinoic acid, and total histone proteins were compared throughout this process. The results showed a significant change in expression level of a variant of H1 histone named H1x. Chromatin immunoprecipitation coupled with real-time PCR analysis demonstrated a preferential incorporation of this protein in the regulatory region of *Nanog*, a marker gene of stemness that is significantly suppressed in differentiated cells. This finding reveals a dynamic role of H1x in differentiation, and implies a repressive role for this histone variant.

1. Introduction

The H1 or "linker" histones are a family of very lysine-rich proteins that bind to the linker DNA between core nucleosomes and facilitate compaction of chromatin fiber [1,2]. To date, eleven subtypes of H1 have been identified, i.e., H1.1–H1.5 [3], $H1^0$ [4], H1t [5,6], H1T2 [7], HILS1 [8], H1Foo [9] and H1x [10–12]. In a comparison between all H1 histones, H1x has a lower molecular weight than the bulk H1 histones, which causes a higher migration rate of electrophoresis for this chromatin protein. The epigenetic role of H1x has not been defined exactly, but a nucleosome digestion assay of HeLa cell nuclei indicated that H1x is not randomly distributed in chromatin, but is enriched in a less accessible chromatin portion [11].

Changes of H1 histone variants are crucial events in modulating local chromatin arrangements. It has been mentioned that the H1 variants differ in their ability to condense chromatin [13–16]. This heterogeneity suggests a functional significance for H1 family members in accordance with the developmental status of many organisms [17,18], as well as differentiation of mammalian cell lines in vitro [19].

Embryonal carcinoma (EC) cells derived from germ cell tumors are valuable tools for investigating differentiation and developmental biology processes in vitro. The advantage of the reproducible and rapid expansion of these cell lines provides a useful alternative to embryos for the study of mammalian cell differentiation [20]. During early stages of cell differentiation, the rate of transcription of large numbers of genes is substantially altered in a time-dependent manner [21]. In developmental model systems, replacement of cognate histones by their variants has been proposed to produce epigenetic marks that cause "commitment" of cells to the differentiated phenotype [19,22], primarily according to the cell culture/induction conditions.

In our previous study, through epigenetic analysis of a human EC cell line, NT2, the dynamic interplay of core histone modifications/variations was discussed in relation to regulation of the gene expression profile during differentiation induced by retinoic acid (RA) [23].

In the current work, we investigated the contribution of linker histone variation in the process of differentiation, using the same cellular model system. The present data introduces H1x as a linker

Abbreviations: RA, retinoic acid; ChIP, chromatin immunoprecipitation; ECC, embryonal carcinoma cell; qRT-PCR, quantitative real time-polymerase chain reaction; ACTB, beta-actin; SDS–PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; FT-MS, Fourier transform mass spectrometry

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histone subtype with differential expression level during RA-induced differentiation of NT2 cells. To our knowledge this is the first time that a dynamic role in differentiation has been demonstrated for the H1x subtype.

2. Materials and methods

2.1. 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 mM L-glutamine at 37 °C in 5% CO_2. Differentiation was induced by RA as previously described [23]. The cells were harvested at 0, 3, 6, 9, 12 and 15 days of RA induction.

2.2. Electrophoresis of histone extracts and western blot analysis

Total histones were extracted using 0.2 N HCl [24], and 15 μg of extracts were electrophoretically analyzed on 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), according to the method of Laemmli [25]. Separated histones on gel were transferred to PVDF membrane, then incubated overnight with anti-histone H1x (Abcam ab31972, 1:500) as primary antibody. Peroxidase-conjugated anti-rabbit IgG (Sigma A0545, 1:15000) was used as secondary antibody and the immunoreactive proteins were visualized using chemiluminescence ECL-Plus substrate (Sigma, CPS-1-120) and detected by exposure to X-ray film. Blotting for total histone H3 (Sigma HO164) was applied as a loading control.

2.3. Mass spectrometry

After running on gel, the sliced protein band was reduced, alkylated and trypsinized in gel as described before [26]. Peptides were extracted from the gel with 2% trifluoroacetic acid. Extracted peptides were desalted and acidified to a final concentration of 0.5% acetic acid and purified by STAGE tips [27].

Peptide sequencing were performed using a nano Easy LC flow system (Proxeon, Odense, Denmark) connected online to a 7-T linear ion trap ion cyclotron resonance Fourier transform (LTQ-FT Ultra) mass spectrometer (Thermo Fisher, Bremen, Germany). Raw spectrum files were processed by MaxQuant software as previously described [28]. MS/MS spectra were merged into peak-list files and searched against the human IPI protein database version 3.37.

2.4. Quantitative PCR

Total RNA extraction and cDNA synthesis were performed on three independent replicates and mRNA quantification was performed by quantitative real time-polymerase chain reaction (qRT-PCR) using primers shown in Table S1-A. Gene expression data were analyzed according to the ΔΔCt quantitative method [29] to estimate relative fold change values.

2.5. Chromatin immunoprecipitation (ChIP) – real time qPCR analysis

ChIP experiments were performed on three biological replicates, using Orange ChIP kit (Diagenode, Belgium) just according to the instruction of the manufacturer. Chromatin from 1×10^5 cells was used for each immunoprecipitation. PCR amplification was performed on DNA recovered from the ChIP and the total chromatin input, using primers listed in Table S1-B (the particular regions amplified are chosen based on our previous work [23]). Immunoprecipitated DNA was quantified by real-time PCR on a 7500 Real-Time PCR System (AB Applied Biosystems). Data were expressed as percentage of input DNA associated with immunoprecipitated histone H1x relative to a 1/100 dilution of input chromatin.

2.6. Statistical analysis of real-time PCR

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

3. Results

3.1. Analysis of total histones extracted from NT2 cells during RA-induced differentiation

To compare total histone components of NT2 cells through the differentiation process, cell samples were harvested at 0, 3, 6, 9, 12 and 15 days after RA induction, and the extracted total histones were analyzed by SDS–PAGE. As shown in Fig. 1A, a thin protein band was observed (indicated with arrowhead) in the electrophoretic pattern of histones extracted from NT2 cells after 9, 12 and 15 days of RA treatment. Localization of this protein near the area related to histone H1 suggested a linker histone. The protein was characterized by mass spectrometry analysis of Commassie-stained excised gel band, using in-gel tryptic digestion followed by Fourier transform mass spectrometry (FT-MS) analysis. The protein was thus identified as histone H1x (44.1% sequence coverage), a variant of the cognate linker histone (see Supplementary data, S2).

3.2. Differential expression of H1x during RA-induced differentiation of NT2 cells

Western blot analysis with anti-H1x antibody further confirmed the appearance of histone H1x during RA-induced differentiation (Fig. 1B), in agreement with the previous analysis.

Furthermore, quantitative real time-PCR showed a gradual increase in H1x transcript level during the course of RA-induced differentiation (Fig. 2).

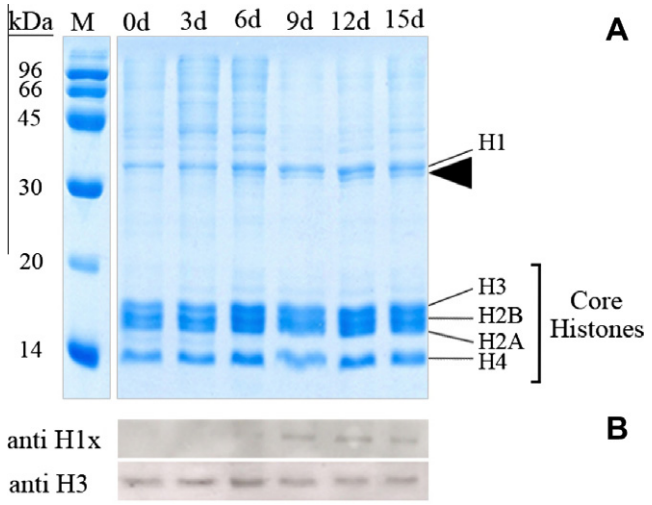

Fig. 1. (A) SDS–PAGE pattern of acid-extracted histones of NT2 cells during RA-induced differentiation, at the time points indicated. M, molecular weight marker. The arrow head shows a histone variant with differential expression pattern, identified by mass spectrometry as the H1x variant (see Supplementary data, S2). (B) Western blot analysis of total histone extracts for H1x histone. Histone H3 was used as loading control.

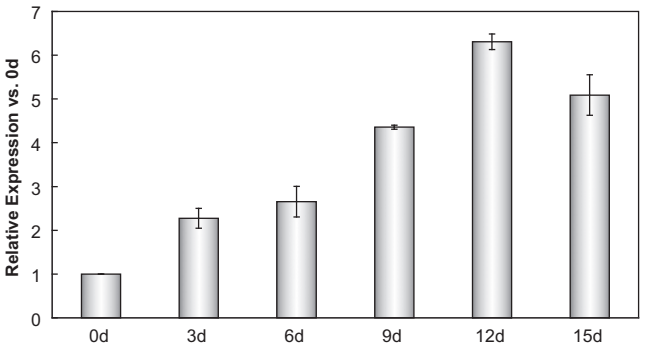

Fig. 2. Quantitative reverse transcription PCR analysis of *H1x* expression during RA-induced differentiation of NT2 cells, at the time points indicated (mean ± S.D.; $n = 3$; $^{*}P < 0.05$ compared to day 0).

3.3. Localization of the histone variant H1x on the regulatory regions of stemness/differentiation marker genes

ChIP was applied to check the presence of H1x histone variant on the regulatory regions of two stemness marker genes (*Oct4* and *Nanog*) and two neuroectodermal marker genes (*Nestin* and *Pax6*) drawn in Fig. 3A. Among the four marker genes, H1x was predominantly detected in the regulatory region of *Nanog*, the stemness gene drastically suppressed after onset of differentiation (Fig. 3B). It is interesting to note that incorporation of H1x to the up-stream region of *Nanog* was about 10-fold higher than that for the other stemness gene, *Oct4*, and also 5–10-fold more than seen with the neuroectodermal marker genes, *Nestin* and *Pax6*. A time course ChIP experiment was also performed, in order to monitor the presence of H1x on the regulatory region of *Nanog* during RA-induced differentiation. As shown in Fig. 3C, levels were significantly above the day 0 value starting at day 3, with a further increase around days 12–15.

4. Discussion

The role of linker histones in regulating chromatin structure and gene expression is well established. Although there are many reports concerning the expression of different kinds of H1 linker histone subtypes through development and also in different tissues and organs, a clear and direct correlation between individual H1 variants and chromatin structure and function is unknown [30].

In this study we demonstrated that a variant of the linker histone family, H1x, is up-regulated during RA-induced differentiation of NT2 cells. Since RA-induced differentiation of teratocarcinoma cell lines such as NT2 appears in many ways to recapitulate steps that occur during embryonic development [23,31], this finding suggests that the linker variant H1x has a dynamic role in embryonal differentiation processes.

Increased expression level of H1x through RA-induced differentiation of NT2 cells encouraged us to study the incorporation of this epigenetic mark on the 5′-regulatory regions of four stemness/differentiation marker genes *Oct4*, *Nanog*, *Nestin* and *Pax6*.

In our previous work on monitoring the expression profile of two stemness (*Oct4* and *Nanog*) and two neuroectodermal marker genes (*Nestin* and *Pax6*), it was clearly shown that these markers were reciprocally regulated through RA-induced neuronal/neuroectodermal differentiation of NT2 cells [23]. In the current study, anti-H1x ChIP coupled with real-time PCR revealed that the H1x variant was predominantly incorporated into the regulatory region of *Nanog*, a homeobox-containing transcription factor specifically expressed in pluripotent stem cells. Comparing the expression pattern of *Oct4* and *Nanog* in NT2 cells [23,32], *Oct4* shows a low

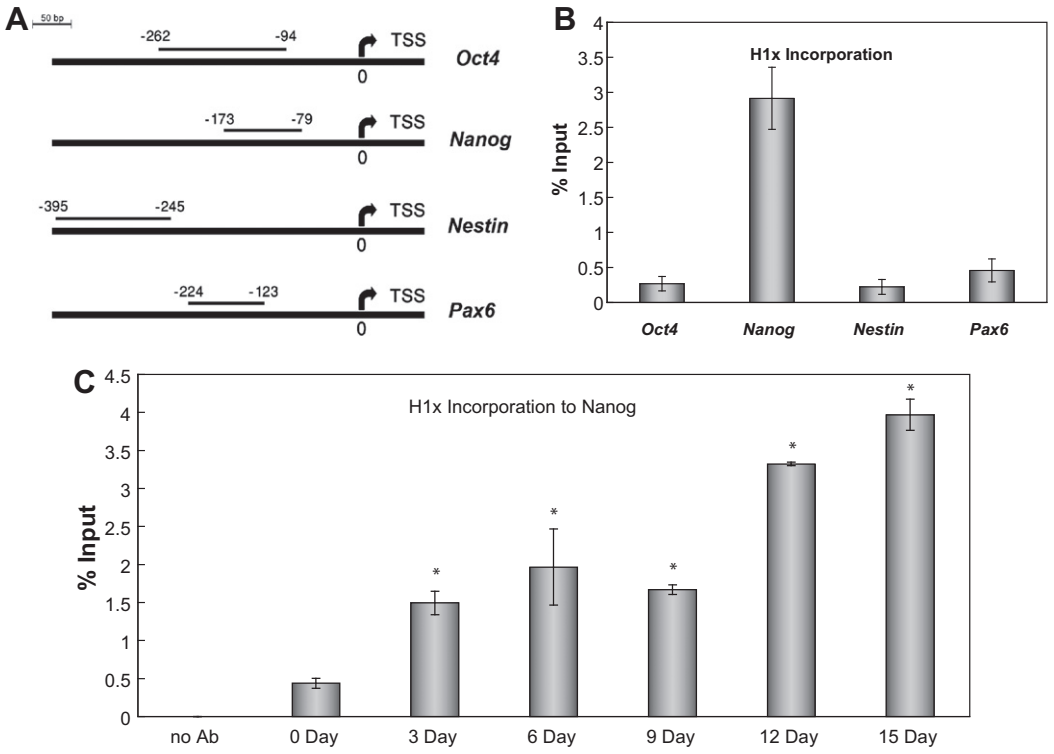

Fig. 3. (A) Regulatory regions examined by chromatin immunoprecipitation on the four marker genes *Oct4*, *Nanog*, *Nestin* and *Pax6*. Regions amplified by qPCR are indicated by bars and nucleotide number relative to the transcription start site (TSS). (B) ChIP analysis of the histone variant H1x on regulatory regions of the marker genes in 12-day RA-induced NT2 cells. (C) Time course ChIP analysis of H1x on the regulatory region of *Nanog* gene during RA-induced differentiation of NT2 cells. The results are expressed relative to a 1/100 dilution of input chromatin (mean ± S.D.; $n = 3$; $^{*}P < 0.05$ compared to 0 day).

continuous expression during RA induction, although both stemness marker genes down-regulate immediately after the onset of differentiation. With this in mind we propose that replacement of H1 histone by the variant H1x on the 5′-upstram region of *Nanog* gene has an effective repressive role in the regulation of this stemness gene. Although it was reported previously that H1x is enriched in micrococcal nuclease-resistant or less accessible chromatin portions of HeLa cell nuclei [11], the current work shows for the first time a clear link between this linker histone subtype and repression of a defined marker gene during cellular differentiation.

Concerning the numerous numbers of genes involved in differentiation of embryo into various cellular lineages, this study emphasizes the importance of elucidating the repressive role of H1x variant in the control of more developmental marker genes. It will be very interesting to use ChIP-sequencing technology to map the incorporation of this linker histone variant at the genome-wide level in this and other cellular model systems.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.10.041.

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