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Trichostatin-A induces differential changes in histone protein dynamics and expression in HeLa cells

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Abstract

Trichostatin-A (TSA), a histone deacetylase (HDAC) inhibitor, results in enhanced acetylation of core histones thereby disrupting chromatin organization within living cells. We report on changes in chromatin organization and the resultant alteration in nuclear architecture following treatment with TSA using fluorescence imaging. TSA triggers an expected increase in the euchromatin fraction which is accompanied by a significant increase in nuclear volume and alterations in chromatin compaction mapped using fluorescence anisotropy imaging. We observe differential changes in the mobility of core and linker histones as measured by fluorescence recovery after photo-bleaching (FRAP) and fluorescence correlation spectroscopy (FCS) methods. Further TSA induces a differential increase in linker histone transcription and increased phosphorylation of linker histone proteins accompanying an expected increase in core histone acetylation patterns. Thus subtle feedback responses triggered by changes in chromatin configurations impinge selectively on linker histone mobility and its expression. These observations have implications for understanding the role of HDAC in the dynamic maintenance of chromatin organization.

Keywords: Chromatin assembly; Histone acetylation; HDAC inhibitor; Transcription; Confocal microscopy; Fluorescence anisotropy; FCS; FRAP; Gene regulation

Histone proteins complex with DNA to form a highly organized and non-random chromatin structure within living cells [1]. Nucleosomes, the fundamental unit of chromatin, comprise a histone octamer wound around by 146 base pairs of DNA. The octamer contains two copies each of the four conserved core histones—H2A, H2B, H3, and H4. Linker histones clamp entry and exit sites of DNA around the core histone octamers and with other nuclear proteins contribute to the formation of a condensed higher order chromatin structure. Thus, 3D packaging of chromatin poses barriers to the regulatory machinery to access DNA [2]. Post-translational modifications, such as acetylation of histones therefore play a central role in regulating the structural compaction of the chromatin and hence global genome function [3]. Acetylation of lysine residues on H3 and H4 by histone acetyl transferases (HAT) leads to decompact and transcriptionally active euchromatin. Conversely, deacetylation of lysine residues by the histone deacetylase enzyme (HDAC) result in a more compact and repressive heterochromatin [4]. Thus inhibiting HDAC has been shown to alter the dynamic equilibrium between HAT and HDAC resulting in hyperacetylation and euchromatin spreading in a dosage and time dependent manner [5]. The reversible nature of acetylation and deacetylation of histone tails maintains a crucial balance between activation and inactivation states of genes [6].

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Recent studies reveal that addition of TSA, a HDAC inhibitor, induces differential changes in global gene expression patterns [7], impinging on cellular differentiation [8,9] and apoptosis [10]. Interestingly while TSA results in hyperacetylation and chromatin decondensation its correlation with changes in gene expression levels has been unclear. In some cases TSA treatment results in upregulation of regulatory genes while in many others it leads to down regulation of gene expression [11–15]. While most studies have studied the effect of TSA on regulatory genes, its role on differential changes in histone protein dynamics and expression levels that necessarily impact chromatin assembly, have not been addressed in detail. Here, we show that TSA treatment for 48 h results in swelling of the cell nucleus leading to altered chromatin compaction states imaged using fluorescence anisotropy imaging. Further, TSA induces differential changes in histone protein dynamics, where the core histone dynamics remain unaltered but linker histone mobility is enhanced as revealed by FRAP and FCS approaches. Importantly our results show that TSA induces upregulation of linker histone gene expression at the transcription level, while the core histone expression remains unaltered. Immunofluorescence data suggest possible post-translational modifications that trigger differential changes in histone protein dynamics and its expression upon TSA treatment.

Materials and methods

Cell culture. HeLa cells stably expressing H1.5-EGFP and H2B-EGFP were grown in 5% FBS in DMEM (GIBCO) and incubated in a 5% CO_2 incubator at 37 °C. Cells were plated at a density of 200,000 cells/dish in 5% complete medium and left overnight for adherence. TSA (SIGMA) was reconstituted in DMSO and was further diluted in complete medium. TSA at doses ranging from 0 to 200 ng/ml was added to cells for varying times viz 12, 24, and 48 h and at the end of the specific time points, cells were harvested for further studies.

Confocal fluorescence imaging and spectroscopy. A Zeiss Confocor (Model-LSM510-Meta/Confocor2) fluorescence microscope equipped with fluorescence correlation spectroscopy and a C-Apochromat 40×/1.2 NA water corrected objective was used for imaging experiments. The EGFP fusion proteins are excited with the 488 nm line of an Argon-ion laser (Lassos) and a 500–530 nm band-pass filter was used for the collection of the emitted intensity on a PMT to form the confocal images (512 × 512 pixels, 12 bit images, pinhole aperture size ~1 airy-units). Co-localization experiments were done in the multiple-track mode to avoid the fluorescence leakage in the detectors. In the first track, the sample was excited with 488 nm (Ar-ion laser) laser line (excitation of EGFP) and fluorescence emission was collected by 510–530 nm band-pass filter and in the consequent track it was excited with 543 nm laser line (HeNe laser) and the corresponding fluorescence emission was collected with 565–615 nm band-pass filter. All imaging experiments were done in Medium 1 buffer (150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM Hepes, pH 7.3), supplemented with 1% glucose. Fluorescence anisotropy imaging, FRAP and FCS experimental methods and data analysis were as described before [16,17].

Flow cytometry. Cells (EGFP tagged stable expressing) were harvested at specific time points and then run on flow cytometer (BD FACSCAN). Cells were not fixed to avoid quenching. Live cells were gated and cells expressing H2B-EGFP or H1.5-EGFP were enumerated on the green fluorescence channel. Increase in the mean intensity in the FL1 channel, which indicates an increase in the EGFP intensity was recorded. This increase in intensity reflects in the increase in the protein level as the protein is a fusion product.

Immunofluorescence. TSA treated cells were plated on coverslip stuck dishes and after appropriate incubation time points were fixed with 2.5% para-formaldehyde for 20 min at room temperature. Cells were permeabilized with Triton X for 1 h at room temperature and were blocked with 1% BSA for 1 h. Primary antibodies were diluted in blocking buffer at a dilution of 1:200 and incubated for 1 h at room temperature. Secondary antibodies tagged to Cy3 were then used to detect the primary antibodies using confocal microscopy.

RT-PCR and analysis of H1.5 transcript levels. Total RNA was isolated from HeLa cells treated with TSA for 12 h using Quiagen RNA isolation kit and stored in −80 °C for further use. Second strand cDNA synthesis was made using cDNA synthesis kit from Bangalore Genei. Equal amounts of RNA were used for second strand synthesis. H1.5 fragment was amplified by using the following primers: fwd: 5′-GGGGTACCATG TCGGAAACCGC-3′, reverse: 5′-CGGGATCCTTCTTTTTGGCAGC C-3′. A gradient PCR was set up to establish annealing temperatures. Subsequent assays were set up at annealing temperatures of 60° and 40 cycles. Control samples for GAPDH were set up with the same cDNA samples. PCR samples were analyzed on 1% agarose gels and the bands were later quantified using Image J program.

Results and discussion

TSA results in increased nuclear volume and altered chromatin compaction states

We first mapped the effect of euchromatin spreading induced by TSA on nuclear morphology. Stable cell lines of HeLa expressing H1.5-EGFP were synchronized and treated with TSA at various concentrations and at different time points. Since TSA was observed to adversely affect cell viability in a concentration dependent manner, care was exercised to exclude dying cells from the analysis in all experiments. TSA treatment (100 ng/ml) for 48 h results in ~30% of cellular apoptosis assessed by Hoechst staining of DNA and counting apoptotic nuclei (data not shown). From live-cell images the nuclear sizes of cells treated with or without TSA were quantified. The mean area of the nucleus increased from 160 to 250 μm^2 upon 48 h of TSA treatment compared to the control cells (Fig. 1A). The enhanced hyperacetylation of histones, leading to decondensed chromatin and euchromatin spreading, perhaps resulted in the increase in nuclear volume. In order to check if the increased nuclear volume altered chromatin compaction states, we carried out fluorescence anisotropy imaging on control and TSA treated cells stably expressing H2B-EGFP. In a previous study, we had shown that fluorescence anisotropy imaging provides a detailed map of the spatio-temporal heterogeneity of chromatin compaction within living cells [16]. Here the rotational mobility of the EGFP fused to core histone proteins reflects the compaction of chromatin assembly, since enhanced packaging of the chromatin reduces the rotational mobility of H2B-EGFP. Fig. 1B is a color coded anisotropy image of a normal cell and that of a cell treated with 100 ng/ml TSA for 48 h. The mean and standard deviation was calculated from the anisotropy image pixel values where higher mean anisotropy values correspond to condensed chromatin and