IL NUOVO CIMENTO **45 C** (2022) 191
DOI 10.1393/ncc/i2022-22191-7

COMMUNICATIONS: SIF Congress 2021

Investigating nanoscale chromatin alterations involved in neuroblastoma transformation by optical nanoscopy

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received 31 January 2022

Summary. — Neuroblastoma (NB) is the most common extracranial solid tumor in childhood and is characterized by remarkable heterogeneity. This work aims to characterize changes in chromatin nanoscale architecture being associated with NB transformation. We employed an NB cell line overexpressing the non-coding RNA NDM29, which promotes cell differentiation toward a neuronal phenotype. The nuclear shape, volume and architecture were assessed in both malignant and neuron-like cells by confocal microscopy. Moreover, heterochromatin and euchromatin organization was investigated by stimulated emission depletion (STED) microscopy. The results showed that the nuclei of neuron-like cells have a reduced volume and a more elongated shape compared to those of malignant cells and a different spatial arrangement of euchromatin and heterochromatin. Altogether these data point to an alteration of nuclear organization associated to NB, paving the way towards a better comprehension of the disease.

1. – Description

Neuroblastoma (NB) is a neural crest derived malignancy of the peripheral nervous system and represents the most common extracranial solid tumor in childhood. Due to its marked heterogeneity, the events that lead to the development of NB from the neural crest have not been fully elucidated [1, 2]. Recently, the non-coding portion of the genome has gained attention as it can play a fundamental role in many cancers, including NB [3]. Neuroblastoma differentiation marker 29 (NDM29) is a non-coding RNA (ncRNA) transcribed by RNA pol III that maps in a region frequently deleted in NB. Previous studies showed that NDM29 over-expression leads to differentiation and loss of malignancy in NB cells, through slowdown of cell cycle and proliferation and "recovering" toward a fully differentiated neuronal phenotype [4]. It is widely accepted that

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cancer results from an array of epigenetic and genetic alterations [5]. Since mutations and defective gene expression are strictly correlated to alterations of nuclear shape and of chromatin architecture, studying nuclear remodeling in cancer can shed light on tumorigenic processes [6,7]. Although many advancements have been done, we know little about the relationship between nuclear alterations and cancer. This work aims to characterize nuclear and chromatin architecture changes possibly correlated with NB transformation. A better understanding of the molecular and cellular mechanisms underlying NB transformation and how NDM29 can promote neuronal differentiation through remodeling the nucleus, may pave the way towards a deeper comprehension of NB onset and progression. Accordingly, it might open the way toward novel therapeutic approaches.

2. – Methods

2˙1. *Cell culture*. – SKNBE2 neuroblastoma cells were grown on RPMI 1640 medium (Sigma-Aldrich) with 10% fetal bovine serum (FBS). SKNBE2 wt cells were stably transfected with pEGFP-N1 as control (Mock) or pEGFP-N1-NDM29 (S1.1) [8]. Previous publications have shown that NDM29 can restore the functional and morphological traits of neurons, such as the expression of neuron-specific proteins, and reduce NB malignancy both *in vitro* and *in vivo*.

2˙2. *Immunofluorescence*. – Cells grown on coverslips were fixed with 4% paraformaldehyde for 15 min. Fixed cells were permeabilized with 0.15% Triton X-100 and blocked with 3% Albumin from Bovine Serum (BSA). Slides were incubated with the following primary antibodies: mouse anti-CDT1 (sc-365305, Santa Cruz), rabbit anti-H3K9Me3 (ab8898, Abcam), rabbit anti-H3K9Ac (710293, Invitrogen). The following secondary antibodies Alexa Fluor 546 goat anti-mouse IgG (A11030, Life technologies) and Aberrior Star 635P anti-rabbit (ST635P, Aberrior) were used. Then, the cells were incubated with Hoechst 33342. Images were acquired with a Nikon's A1R MP confocal microscope and Leica Stellaris 8 Tau-STED microscope and processed by using the software Leica Application Suite (LAS) and ImageJ software [9].

3. – Results

3˙1. *Image analysis to study nuclear morphology*. – Three-dimensional (3D) confocal imaging of Hoechst-stained nuclei allowed us to compare NB cells and S1.1 nuclear morphology, to identify possible alterations [10]. As chromatin organization changes during a cell cycle, we focused on a homogeneous cell sample, by immunolabeling CDT1, a G1 phase marker (fig. 1(A)) [11]. Afterward, we employed an automatic tool to obtain different morphological parameters out from the 3D fluorescence microscopy images. Different morphological parameters were extracted, including volume and elongation. Our results indicate that S1.1 cells rearrange the nuclear shape and volume compared to NB cells (fig. 1(B), (C)). Moreover, the neuron-like nuclei are smaller and more elongated, perfectly fitting with the morphological changes of the whole cell.

3˙2. *STED microscopy to unveil chromatin architecture*. – Stimulated emission depletion (STED) microscopy is one of the techniques that make up super-resolution microscopy [12]. We employed STED microscopy to assess the histone's modification markers typically associated with euchromatin and constitutive/facultative heterochromatin inside the 3D nuclear architecture [13-15]. In detail, we immunostained Lysin 9

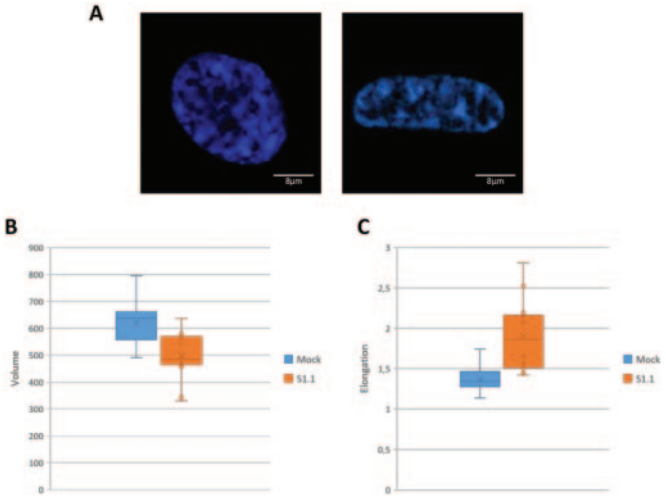

Fig. 1. – (A) Representative confocal images of mock (left) and S1.1 (right) nuclei stained with Hoechst; (B) box plot of nuclear volume; (C) box plot of nuclear elongation.

on histone 3 trymethylated (H3K9Me3) and acetylated (H3K9Ac). H3K9Me3 is a classical marker for heterochromatin, while H3K9Ac is considered a marker for euchromatin regions. We focused on G1 phase cells by means of CDT1 immunolabeling. 3D STED imaging allowed us to appreciate the different spatial arrangement of domains rich in acetylated or methylated H3K9. The H3K9Me3 staining shows a slight fluorescence accumulation in correspondence of the inner nuclear membrane. Instead, fluorescence of H3K9Ac was widely diffused in the central region of the nucleus. Moreover, a different spatial arrangement of H3K9Me3 and H3K9Ac domains and in chromatin compaction can be observed between Mock *vs.* S1.1 cells (fig. 2(A)–(D)).

4. – Discussion

Due to its rooted biological heterogeneity, NB tumor still represents a challenge to understand the mechanism sustaining malignant transformation. We explored the possible alterations in chromatin architecture and nuclear organization for a better comprehen-

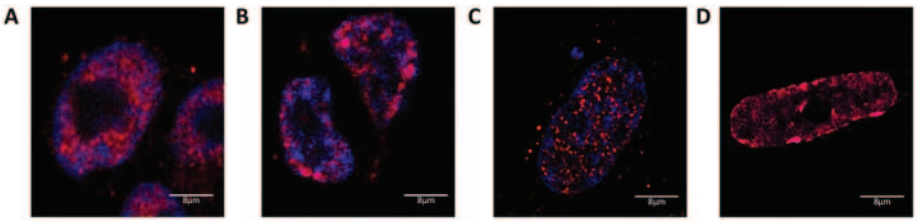

Fig. 2. – Representative STED images of mock and S1.1 cells. (A) H3K9Ac (red) and Hoechst (blue) stain of Mock cells, (B) H3K9Me3 (red) and Hoechts (blue) stain of Mock cells, (C) H3K9Ac (red) and Hoechts (blue) stain of S1.1 cells, (D) H3K9Me3 (red) and Hoechts (blue) stain of S1.1 cells.

sion of the disease mechanisms. In this work we employed a highly malignant NB cell line, SKNBE2, genetically engineered to overexpress the ncRNA NDM29, to promote the differentiation toward a neuronal phenotype [4]. We reconstructed the 3D structure of the nuclei of G1 phase cells, using confocal microscopy as the starting point. The data obtained described a modification of the nuclear structure during the NDM29-driven differentiation: S1.1 clone nuclei appeared smaller and more elongated, well paralleling the whole cellular shape remodelling process. Alterations in nuclear shape often result in modifications of chromatin organization and, consequently, genome function [16]. Considering this, we analysed histone's modification markers associated with euchromatin and both constitutive and facultative heterochromatin. Many chromatin modifications occur at length scales below the light diffraction limit. In order to uncover important information about histones modification, we exploited STED tunability to encode spatial details. Analysing 3D STED images, we assessed the different spatial arrangement of methylated and acetylated lysin 9 on histone 3. Heterochromatin domains appeared mainly clustered at the nuclear periphery, while euchromatin was more widely diffused, preferentially concentrate in the central region and far from the edges. Interestingly, H3K9 acetylated and methylated staining showed slight differences between mock and S1.1 cells, suggesting an effect of the nuclear remodeling on chromatin landscapes. In light of these observations, nuclear and chromatin remodeling during NB transformation came out as a promising direction for further studies, in order to decipher the underlying mechanisms. We also aim to integrate our approach with chromEMT [17] and label-free approaches [18, 19]. The outcome of this work, together with future analysis that will follow this direction, could unveil novel information about this malignancy and possibly lead to new prognostic and therapeutic approaches.

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