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Increased chromatin plasticity supports enhanced metastatic potential of mouse melanoma cells

Yael Maizels, Adi Elbaz, Rosari Hernandez-Vicens, Oshrat Sandrusy, Anna Rosenberg, Gabi Gerlitz*

Department of Molecular Biology, Faculty of Life Sciences, Ariel University, Israel

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ABSTRACT

Metastasis formation is strongly dependent on the migration capabilities of tumor cells. Recently it has become apparent that nuclear structure and morphology affect the cellular ability to migrate. Previously we found that migration of melanoma cells is both associated with and dependent on global chromatin condensation. Therefore, we anticipated that tumor progression would be associated with increased chromatin condensation. Interestingly, the opposite has been reported for melanoma. In trying to resolve this contradiction, we show that during growth conditions, tumor progression is associated with global chromatin de-condensation that is beneficial for faster proliferation. However, upon induction of migration, in both low- and high-metastatic mouse melanoma cells chromatin undergoes condensation to support cell migration.

Our results reveal that throughout tumor progression induction of chromatin condensation by migration signals is maintained, whereas the organization of chromatin during growth conditions is altered. Thus, tumor progression is associated with an increase in chromatin dynamics.

1. Introduction

In more than 90% of cancer patients in general and in particularly in melanoma, death is due to metastasis formation rather than growth of the primary tumor [1,2]. Metastasis formation is dependent on the ability of tumor cells to migrate away from the primary tumor into blood vessels and out of blood vessels into secondary tissues [2]. Tumor cell migration requires re-organization of cytoskeletal elements and adhesion complexes [3,4], as well as alterations in the shape, intracellular localization and internal organization of the cell nucleus [5,6]. Migration of tumor cells *in vivo* occurs in between normal cells and inside the extracellular matrix through confining spaces, where the nucleus can pose a major obstacle for cellular migration due to its size and relatively high stiffness. Indeed, movement of cells within this constrained environment often leads to a reduction in the nuclear diameter [7–9]. During the migration process the intra-cellular localization of the nucleus is actively determined by the actin and the microtubule networks via mechanisms that require physical associations between the nuclear envelope and cytoskeletal elements [10–14].

In addition to changes in the shape and the intracellular localization of the nucleus in migrating cells, the chromatin fibers undergo major structural changes in response to migration signals. We were able to monitor a global increase in chromatin condensation in the mouse B16-F1 melanoma cell line upon induction of migration as measured by various parameters: an increase in the levels of the heterochromatin-associated histone modifications H3K9me3, H3K27me3 and H4K20me1, an increase in DNA methylation, an increase in the chromatin residence time of histone H1, a decrease in the chromatin residence time of HMG proteins and a decrease in the chromatin sensitivity to DNase I digestion. We were also able to show that the migration process is dependent on chromatin condensation in these cells [15,16]. More recently, induction of global chromatin condensation by migration signals has been reported in primary and transformed T-cells [17] and primary tenocytes [18]. Reliance of cell migration on chromatin condensation has been reported in various types of cells including lung cancer cells [19], embryonic fibroblasts [20], breast adenocarcinoma cells [21–23], colorectal cancer cells [23], glioma cells [24], chondrosarcoma cells [25], epidermal cancer stem cells [26], primary tenocytes [18] and primary and transformed T-cells [17].

Taking into account the broad range of cell types that rely on chromatin condensation for their migration and the pivotal role of cell migration in tumor progression it can be expected to find a positive correlation between tumor progression and increased levels of chromatin condensation. Indeed, positive correlations between tumor progression and the levels of factors involved in heterochromatin

* Correspondence to: Department of Molecular Biology, Faculty of Natural Sciences, Ariel University, Kiryat Hamada, Ariel 40700, Israel.
E-mail address: gabige@ariel.ac.il (G. Gerlitz).

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formation were reported in various types of cancer including breast cancer [27–29], prostate cancer [27,30], gastric cancer [31] and melanoma [32,33]. However, opposite findings, meaning positive correlations between tumor progression and euchromatin levels have been reported as well in many types of cancer [34,35] including glioma [36,37] and melanoma [38]. More specifically, in the well-established mouse model for tumor progression in melanoma, the B16 cell lines [39], tumor progression was reported to be associated with a decrease in global chromatin condensation [38]. On the other hand, our previous data indicated on heterochromatin-dependent migration of B16-F1 cells [15,16]. To settle the apparent contradiction between the two studies we compared the chromatin organization of the B16-F1 cells to B16-F10, a highly metastatic paired cell line, and examined the importance of heterochromatin to their migration capabilities.

Here we find that upon induction of migration both the low metastatic B16-F1 cells and the high-metastatic B16-F10 cells undergo global chromatin condensation, which is required for the migration process. However, under steady-state conditions (without induction of migration) the B16-F10 cells possess higher levels of open chromatin than the B16-F1 cells, which is an advantage for a faster proliferation rate. Overall, our data suggest that melanoma progression is associated with an increase in chromatin plasticity.

2. Materials and methods

2.1. Cell culture and immunostaining

Mouse melanoma B16-F1 and B16-F10 were grown in DMEM (D5796, Sigma-Aldrich, St. Louis MO, USA) supplemented with 10% FCS (04-007-1A Biological Industries, Beit Haemek, Israel), 0.292 mg/mL L-glutamine (03-020-1B, 1A Biological Industries, Beit Haemek, Israel) and 40 units/mL Penicillin-Streptomycin (03-031-1B Biological Industries, Beit Haemek, Israel). For immunostaining, cells plated on fibronectin-coated coverslips (03-090-1-05, Biological Industries, Beit Haemek, Israel) and grown to confluence were scratched for the wound healing assay. After the scratch the cells were further incubated at growth conditions up to three hours. The cells were fixed in 3% paraformaldehyde-PBS at room temperature for 5 min followed by fixation in methanol at −20 °C for another 5 min. Antibodies included mouse monoclonal anti-histone H3 trimethylated on Lys27 (05-1951, Millipore, Darmstadt, Germany), rabbit polyclonal antihistone-H4 monomethylated on Lys20 (9051, Abcam, Cambridge MA, USA), rabbit polyclonal antihistone-H3 trimethylated on Lys9 (07-442, Millipore, Darmstadt, Germany), mouse monoclonal antihistone H1 (AE4, Santa Cruz Biotechnology Inc., Dallas TX, USA) and goat polyclonal anti-histone H1-N16 (34464, Santa Cruz, Dallas TX, USA). DNA was stained with Hoechst 33258 (Sigma-Adrich, St. Louis MO, USA). All images were collected using an Olympus 1X81 fluorescent microscope with a coolSNAP HQ2 CCD camera (Photometrics, Tuscon AZ, USA). The ImageJ program version 1.49S (NIH) was used to measure the intensities of the mean fluorescent signals in the nuclei of control cells (0 h) and in nuclei of cells adjacent to the scratch (first and second rows). Nuclei were encircled according to the Hoechst staining, and the mean intensities in the immunostaining channel were measured.

2.2. Wound healing assay

For the wound-healing assay, cells plated in fibronectin-coated 12-well plates were grown to confluence. Then the cells were scratched with the edge of a 10 µl tip, washed three times with DMEM and incubated in DMEM supplemented with 0.5% FCS with or without inhibitors for seven hours.

Images of the same fields were collected with a coolSNAP HQ2 CCD camera (Photometrics, Tuscon AZ, USA) mounted on an Olympus 1X81 fluorescent microscope at two time points: immediately after the scratch and seven hours after the scratch. The area covered by the migrating cells was calculated using ImageJ 1.49S (NIH) by comparison of the same fields between 0 h and 7 h. In each experiment four different fields were analyzed for each treatment (two in one well and another two in a second well). The represented results are an average of three independent experiments ± s.e., and statistical significance was determined by the Student's *t*-test. The following inhibitors were added to the medium when indicated: 0.14 µM chaetocin (C9492, Sigma-Aldrich, St. Louis Missouri, USA), 3 µM GSK 343 (SML0766, Sigma-Aldrich, St. Louis Missouri, USA) and 3 µM GSK 126 (15415, Cayman Chemical, Ann Arbor, MI, USA).

2.3. XTT assay and plasmids

To create the SUV39H1 plasmids, the open reading frames from pREV-SUV39H1 WT and pREV- SUV39H1 H324K [40] were amplified by PCR with primers that created *Xho*I and *Eco*RI sticky ends. These fragments were ligated into the pEGFPC3 plasmid (GenBank Accession #: U57607) in the multiple cloning site to create GFP-SUV39H1 WT and GFP-SUV39H1 H324K. The H2B-GFP plasmid and the GFP-NLS plasmid were kind gifts of the laboratory of Michael Bustin. Transfections were performed using jetPRIME (114-15Polyplus, Illkirch, France) and NanoJuice (71900-3, Millipore, Darmstadt, Germany) according the manufacturers protocols. Transfection efficiency 24 h after transfection was > 90% as analyzed by FACS.

For the XTT assay 50,000 cells were seeded in each well of a 96 well plate. Cell number was measured using the Cell Proliferation Kit (XTT based) (20-300-1000 Biological Industries, Beit Haemek, Israel) four hours after seeding to have a baseline cell number. Cells were transfected with: GFP-SUV39H1 WT, GFP-SUV39H1 H324K, GFP-H2B or GFP-NLS (three wells for each plasmid). Three hours after the transfection cells were washed twice with serum free DMEM and then cells were grown in low serum (0.5%) DMEM with pen/strep and L-glutamine. Cell number was measured 24 h after transfection. The represented results are an average of 9 repetitions. Statistical significance was determined using the Student's *t*-test.

2.4. Western blot analysis

Confluent B16-F1 and B16-F10 cells were scraped and washed with PBS buffer. Cell lysates were prepared by sonication in 2 X SDS sample buffer (100 mM Tris 6.8, 2% SDS, 10% glycerol, 100 mM DTT) followed by heating to 95 °C for 10 min. The proteins were separated by SDS–PAGE and transferred to nitrocellulose membranes. The following antibodies were used for western blotting: rabbit polyclonal antihistone-H3 trimethylated on Lys9 (07–442, Millipore, Darmstadt, Germany) and rabbit polyclonal antihistone-H3 (05–928, Temecula, CA, USA).

3. Results

3.1. Migration signals induce an increase in facultative heterochromatin markers in both low and high metastatic B16 cells

Histone post-translational modifications reflect the condensation level of the chromatin fibers [41–43]. We monitored these modifications to compare the global chromatin organization between low and high metastatic murine B16 melanoma cells [39] under steady-state conditions or following induction of directed-migration. Directed-migration was induced by the wound-healing assay [44]. We assessed the levels of H3K27me3 and H4K20me1, two facultative heterochromatin markers, which are enriched in repressed genes and the inactive X chromosome [45,46]. Induction of directed-migration significantly elevated the levels of both H3K27me3 and H4K20me1 in the low-metastatic B16-F1 cells already at the 30 min time point by 2.1 and