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Nuclear stiffening inhibits migration of invasive melanoma cells

Alexandre J.S. Ribeiro^{1,*}, Payal Khanna^{2,*}, Aishwarya Sukumar¹, Cheng Dong², and Kris Noel Dahl¹

¹ Department of Biomedical Engineering, Carnegie Mellon University, Pittsburgh, PA 15213

² Department of Bioengineering, The Pennsylvania State University, University Park, PA 16802

Abstract

During metastasis, melanoma cells must be sufficiently deformable to squeeze through extracellular barriers with small pore sizes. We visualize and quantify deformability of single cells using micropipette aspiration and examine the migration potential of a population of melanoma cells using a flow migration apparatus. We artificially stiffen the nucleus with recombinant overexpression of β -50 lamin A, which is found in patients with Hutchinson Gilford progeria syndrome and in aged individuals. Melanoma cells, both WM35 and Lu1205, both show reduced nuclear deformability and reduced cell invasion with the expression of β -50 lamin A. These studies suggest that cellular aging including expression of β -50 lamin A and nuclear stiffening may reduce the potential for metastatic cancer migration. Thus, the pathway of cancer metastasis may be kept in check by mechanical factors in addition to known chemical pathway regulation.

Keywords

Laminopathy; metastasis; cell mechanics

Introduction

Metastasis is currently the primary cause of death among cancer patients. The process includes a series of well-orchestrated steps including initial growth from the primary tumor followed by tumor cell intravasation, circulation, adhesion, extravasation and growth of a secondary tumor.(1) The movement and migration of cells from the circulation through the extracellular matrix (ECM) requires modulation of cell mechanics and deformation of subcellular regions.(2) Specifically, we are interested in the deformability of metastatic melanoma cancer cell lines, or the “migration potential” of WM35 and Lu1205.(3; 4) WM35 is a radial growth phase melanoma, wherein the tumor grows along the skin surface rather than in the vertical direction into the blood vessels; these melanomas cannot undergo metastatic events. Lu1205 is a vertical growth phase melanoma cell with cells below the surface and entering into blood vessels; these melanomas are more invasive and are capable

*Corresponding Authors: Kris Dahl. krisdahl@cmu.edu, Telephone: (412) 268-9609, Fax: (412) 268-7139. Cheng Dong. cxd23@psu.edu, Telephone: (814) 865-8091, Fax: (814) 863-0490.

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of undergoing metastatic events.(5) As such, WM35 is less invasive than Lu1205, but some mutations (eg. B-raf) are similar between the two cell lines.(6) These cell lines have been well characterized for their *in vivo* metastatic potential with clinical relevance and drug-therapeutic interventions.(7; 8) Generally, invasive metastatic cancer cells are less stiff than cells of the primary tumor,(9) and melanoma motility correlates with low stiffness *in vitro*.(10) In addition to motility, reduced stiffness may impact adhesion and cell deformability related to extra- and intra-vasation.

Recently, nuclear structure and mechanics has been identified to be important in cancer metastasis.(11) The cell nucleus is the largest and stiffest organelle in the cell, particularly when the cytoskeleton is capable of rapid turnover, as in cancer cells.(12) The increase in the migration potential of cancer cells correlates with a higher ability of cells to squeeze through tight spaces.(13) The length scale of the nucleus can be larger than extracellular barriers(14; 15), and the nucleus has been suggested to be a limiting mechanical factor in allowing cancer extravasation and metastatic tumor formation.(16; 17) The mechanical properties of the nucleus are strongly modulated by the lamina nucleoskeleton, which is primarily located at the inner nuclear envelope and is mainly composed of type V intermediate filaments called lamins. In some cancers lamin concentration and posttranslational modifications vary, and modulation of lamin levels can alter migration of cancer cells.(11; 18)

Mutations in lamin A are also associated with altered nucleoskeletal mechanics and a variety of different diseases.(19; 20) A cryptic splice variant of the *LMNA* gene causes production of $\Delta 50$ lamin A ($\Delta 50$ LA). Normally, small amounts of this variant are produced, and significant amounts of accumulated $\Delta 50$ LA are found only with advanced age.(21; 22) A rare, *de novo* DNA mutation in *LMNA* causes an enhanced production of $\Delta 50$ LA, which leads to the premature aging disorder Hutchinson Gilford progeria syndrome. In addition to the loss of 50 amino acids (exon 11) from the lamin A tail region and a slightly altered structure,(23) $\Delta 50$ LA retains a C-terminal farnesyl lipid moiety that enhances membrane association with the inner nuclear membrane.(24) Expression of $\Delta 50$ LA is associated with increased thickness of the nucleoskeleton as well as increased nucleoskeletal stiffness and reduced nuclear deformation in cultured cells.(25)

In this study, we use melanoma cell lines with varying metastatic capacities to quantify how manipulation of nuclear mechanical properties affects overall cellular deformation and motility through confined spaces. Previous studies have shown that reduction of lamin A increases transmigration of cancer cells.(18) We show the converse: that effective stiffening of the nucleoskeleton by overexpression of $\Delta 50$ LA prevents deformation of the nucleus through small regions, which also correlates with reduced cell migration.

Results

To quantify the migration potential of WM35 and Lu1205, we adapted an *in vitro* flow-pore assay to measure the cell's ability to (i) escape from flow, (ii) translocate through the endothelial layer and (iii) crawl into tight interstitial spaces (schematic in Figure 1A). Previously, studies using this flow migration chamber have shown the importance of

adhesion (by α v adhesion molecules) and subsequent transendothelial migration in cancer metastasis.(26) Theoretical *in vitro* flow migration results have been validated using *in vivo* models.(8; 27)

We mimicked flow through post capillary venules by culturing a layer of endothelial cells under a parallel plate flow chamber and on top of the polycarbonate surface of a modified 48-well Boyden chamber with 8 μ m pores. Below the pores, soluble collagen IV was added as a chemoattractant for cells. We measured the number of cells able to migrate to the bottom surface over 4 hours under low shear stress (0.625 dyn/cm²). Similar to previous reports of migration potential,(8; 28) we found 44 ± 2 and 105 ± 15 cells per field of view for WM35 and Lu1205, respectively (compared with experimental data later in Figure 4C). As expected, the more metastatic Lu1205 cells showed a statistically higher degree of cellular migration.

To remove contributions from cellular adhesion and force generation, we measured the deformability of individual live cells using micropipette aspiration. Micropipette aspiration simulates the high strain deformation experienced by cancer cells invading extracellular matrix environments with micrometer size scales. There are numerous methods to mechanically characterize cells including microparticle tracking, magnetic twisting cytometry and atomic force microscopy.(29) However, micropipettes allow for simultaneous visualization of different subcellular features during cell deformation.(30; 31) Nuclei can easily be visualized in live cells using the membrane permeable DNA dye Hoeschst 33342. From visualization of deformation of the cell membrane (Lc) and nucleus (Ln), we are able to measure cell deformation and the contribution of the nucleus (Figures 1B and 2).

With increasing time after fixed aspiration pressure through the micropipette, we observe the cell deforming into the pipette (Figure 2, 3). In both the WM35 and Lu1205 cases, we observed that the cell membrane and other cellular structures deform 14 ± 2 μ m ($p = 0.08$ between WM35 and Lu1205) into the pipette before the portion of the cell containing the nucleus enters the pipette (Figure 3, x-axis). The initial deformation of the nucleus into the pipette is higher for the WM35 than for Lu1205 (Figure 3, WM35 data above Lu1205 data). This slightly higher minimum strain may be a function of nucleoskeletal mechanics or intracellular connections. Following this minimal strain, the nucleus of Lu1205 flows much more easily than WM35, as shown by the slope of nuclear deformation normalized to cellular deformation, Ln/Lc (Figure 3, inset). This suggests that after the initiation of deformation the nuclei in the Lu1205 are more easily able to deform through the small spaces. This higher contribution of the nucleus to cell deformability can also be visualized in Figure 2B versus 2A.

We stiffened the nucleoskeleton with the exogenous overexpression of DsRed 50LA (see Methods and Supplemental Figure 1). Previously, we have shown in cells expressing 50LA, both exogenous and from patients with Hutchinson Gilford progeria syndrome, that they have stiffer nuclei and less adaptable to deformation than control cells.(25) As expected, the nuclei of both WM35 and Lu1205 cells expressing 50LA were significantly less deformable than untransfected control cells (Figure 2C,D). With the 50LA-stiffened nuclei, fewer nuclei were able to enter the pipette (Figure 2, 4A). That is, in most cells the

nucleus did not deform into the pipette at all despite the cell deforming with increased aspiration pressure. Of the nuclei that were able to enter the pipette, the nuclei only deformed slightly, represented by the ratio L_n/L_c , compared with the control (Figure 4B). The data for L_n/L_c appear slightly different between figure 3 and figure 4B. Figure 3 represents the stepwise data of 4 cells for each condition with >10 measurements per cell with increasing aspiration. For figure 4, a large number of cells (>13 cells per condition) were examined with 1-2 aspiration lengths per cell for comparison of control cells with 50LA expressing cells.

Once we determined changes in single cell deformability, we re-examined metastasis using the flow chamber setup from Figure 1. Similar to the single cell experiments, we observed a statistical reduction in migration potential of both WM35 and Lu1205 cells with the overexpression of 50LA (Figure 4C). However, in every experimental condition the overexpression of lamin A had no statistical effect compared with untransfected control cells (Figure 4A, B, C and see Discussion).

Discussion

Cancer arises from genetic instability and expression of oncogenes, which include genes that regulate cell motility, migration, proliferation, and survival.(32) Measuring changes in the expression of these oncogenes can be used for cancer detection and mapping of intracellular protein pathways involved with cancer progression.(1; 33; 34) Simultaneously, nuclear structure and morphology are usually altered in cancer cells.(35) Nuclear morphology serves as a key diagnostic tool to identify the presence and type of cancer,(36; 37) and lamins are differentially expressed and localized in some cancers.(38; 39) Further, the altered nuclear architecture may allow for the increased deformability of the cancer cell as indicated by our results. Studies of cancer cell mechanics suggest that most cancer cells are more deformable than healthy cells and stiffness is related with their metastatic potential.(36) Obviously, a deformable nucleus allows for cellular deformation and migration through tight interstitial spaces.(40) Also, our previous work has shown that melanoma deformability enhances adhesion to endothelial cells under shear flow by increasing contact area.(41; 42)

While expression of exogenous wild type lamin A had little effect on migration of melanoma cells, expression of 50LA decreased migration potential of both WM35 and Lu1205 cells through a layer of endothelial cells by nearly 80% under low shear stresses. We were surprised that the exogenous overexpression of lamin A had no effect, but we suggest that the added lamins were insufficient to determine differences using these methodologies; perhaps more sensitive biophysical characterization would yield a statistical difference. Expression of 50LA pathologically increases the stiffness of the nucleoskeleton(25) resulting in stiffening and reduced responsiveness of the nucleus.(43) Overexpression of 50LA generally results in over-accumulation of lamins within the nucleoskeleton as well as other structural nuclear, cytoplasmic and cytoskeletal defects.(25; 44; 45) Alteration of nuclear structure and stiffness with the expression of 50LA may impact other cellular processes including intracellular force generation and transduction, cell adhesion and gene expression. Our own work in fibroblasts has shown that expression of 50LA reduces motility of cells through tight spaces from an increased nuclear resistance

and from decreased actin-myosin force generation.(40) Independent of nuclear structure, altered cytoskeletal organization mediated by substrate stiffness has been shown to affect the movement of tumor cells into tight interstitial spaces.(15) Thus, the coupled intracellular mechanisms of invasiveness and motility as they relate to resistance and driving force of cell movement will need to be further elucidated. However, we find that nuclear deformability is drastically impacted in single suspended cells with the expression of 50LA measured by micropipette aspiration where internal cytoskeletal stress fibers are lost.(46) Thus, micropipette aspiration measures cell deformation independent of adhesion and force generation. This agrees with previous studies of nuclear stiffening with the expression of 50LA found in isolated nuclei.(25)

In measuring bulk migration potential we observe large differences in invasion between control Lu1205 and WM35 cells that are less obvious in single cell deformability assays. The increased invasion of the Lu1205 over WM35 is consistent with the increased metastatic pathogenesis described in the introduction. There is a small increase in the ability of Lu1205 nuclei to deform within cells after a minimum strain compared with WM35 (quantified as Ln/Lc in Figure 3), but the differences from applied extracellular forces are substantially less than the bulk migration assay. As such, we suggest that the cell-type differences in bulk invasion and transmigration between Lu1205 and WM35 cells may reflect altered adhesion, force generation, etc. Flow cytometry studies by Zhang et al. have shown lower expression of adhesion molecules such as ICAM-1 and $\alpha v \beta 3$ on WM35 cells compared to Lu1205 melanoma cells.(3) Collectively, these results show that there is a change in cell migration between these WM35 and Lu1205 cell lines dependent on the expression of adhesion molecules. However, modulating nuclear stiffness with 50LA reduces invasiveness of both cell types in our migration assay. Interestingly, we see that differences in invasion and migration observed between Lu1205 and WM35 are lost with the expression of 50LA. We suggest that the motility is so greatly attenuated with the expression of 50LA that differences between cell types and the contributions of adhesion are lost.

Expression of 50LA, which causes the premature aging disease Hutchinson Gilford progeria syndrome, is not a viable treatment option unless it can be activated in only cancer cells. We suggest that other mechanisms of nuclear stiffening may inhibit cancer cell migration: many DNA intercalating drugs are currently available to treat cancer by preventing or reducing DNA replication. These drugs may also be screened for the possibility of stiffening nuclei and reducing invasion and transmigration.

Beyond possible treatment options, we speculate that these results may suggest some rationale for the low expression of 50LA that occurs in individuals without HGPS during natural aging, albeit at a lower rate than in patients with HGPS.(22) Decreased metastasis can be statistically associated with old age,(47) but the impact of age in cancer progression is still a subject of high controversy and may depend on type of cancer.(47) Previous reviews have reported that expression of the 50LA variant in nature is a cryptic error and serves no function, and lamin A appears to be expendable to some degree.(48) However, we suggest that the nuclear stiffening that is associated with aging may serve some purpose. DNA is constantly being mutated by replication errors and mutagens, and although there are

molecular and cellular checkpoints to correct for these mutations, the probability of a cancer-causing error occurring increases with time. Thus, like other aspects of cellular aging (including telomere length decrease, senescence pathways, etc.(49)), 50LA accumulation may be part of cellular programming. We are hypothesizing that the accumulation of the 50LA splice variant with age and subsequent nuclear stiffening may be playing a role to reduce risks or severity of metastatic cancers. However, this hypothesis is extraordinarily difficult to test since accumulation of 50LA in normal individuals takes decades to accumulate,(22) which is beyond the scope of any experiment or reasonable animal model.

Materials and methods

Cell culture

Low passage WM35 cells were kindly provided by Dr. Meenhard Herlyn (Wistar Institute) (6) and were maintained in Roswell Park Memorial Institute 1640 medium (RPMI 1640; Biosource, Inc.). Lu1205 metastatic melanoma cell lines were kindly provided by Dr. Gavin Robertson (Penn State Hershey Medical Center)(50) and maintained in Dulbecco's modified Eagle's medium (Life Technologies). HUVEC (Human umbilical vein endothelial cells) were obtained from American Type Culture Collection (ATCC) and maintained in F12-K medium supplemented with 30 µg/ml of endothelial cell growth factors and 50 µg/ml heparin (Mallinckrodt Baker). All media was supplemented with 10 % fetal bovine serum (FBS; Biosource) and 100 units/ml penicillin-streptomycin (Biosource).

Transfection with cDNAs

WM35 and Lu1205 were transfected with DsRed tagged lamin cDNA: lamin A or 50LA. GFP-plasmids,(25) a kind gift of Tom Misteli (NCI NIH) were subcloned into the DsRed plasmid (Clontech) and confirmed via sequencing. Cells were transfected with Trans-It 2020 transfection reagent (Mirus) as per the manufacturer's instructions. At 48 hours post transfection, images of 20-40 transfected nuclei at 63x were obtained for adherent cells. Projected area of nuclei was compared between DsRed lamin A and DsRed 50LA to see if nuclear size was altered with expression (Supplemental Figure 1A). Nuclear area was statistically similar between DsRed lamin A and DsRed 50LA for each cell type ($p>0.1$ by Students t-test). Mean fluorescence intensity of the DsRed from DsRed lamin A or DsRed 50LA expressed in the Lu1205 or WM35 was normalized to the background (in ImageJ). Expression levels of DsRed were always at statistically similar levels between experiments ($p>0.1$ for normalized intensity by Students t-test), and expression did not vary greatly between samples (Supplemental Figure 1B). To test proper lamin localization, we imaged nuclei segments across nuclei (Supplemental Figure 1C, inset) and examined fluorescence intensity along the line for greater than 10 nuclei (Supplemental Figure 1C). All lamins localized to the nucleus, at the nuclear lamina, and there was no dramatic change in lamin localization between the samples examined (Supplemental Figure 1D).

Flow migration assay

We have previously described and characterized the flow migration assay in detail.(4; 8; 26; 27; 51) Briefly, HUVECs were grown to confluence on fibronectin-coated 6-10 µm deep polycarbonate filters (NeuroProbe). See Supplemental Figure 2 for characterization of the

monolayer. The wells on the bottom plate of the chamber were filled with HUVEC media with 2% bovine serum albumin (BSA) except for the middle 12 wells, which were coated with soluble collagen type IV (BD Bioscience antibody 354233, 100 µg/ml, optimized previously(52)). Collagen IV was found to be a specific chemoattractant to melanoma cells and, unlike other chemoattractants such as serum, does not affect the migration of endothelial cells layered on the top part of polycarbonate filters.(52) The apparatus was assembled as in Figure 1 with cells on the filter in between a flow chamber and collagen-filled wells. The assembled migration chamber was placed in a flow loop and cells in media with 2.5×10^6 melanoma cells were injected into the flow migration chamber at 0.625 dyn/cm². The flow rate was calculated to produce the desired fluid shear stress using parallel plate flow equation.(53) Cells and media were immediately perfused into the chamber, initially at a flow rate 2 mL/min, which was then increased to a desired experimental rate (0-20 mL/min). The entire chamber was placed in a 37°C, 5% CO₂ incubator.

After running flow migration experiments at 37°C for 4 hr, the number of melanoma cells that migrated through the endothelial layer onto the bottom of the filter was counted on an inverted fluorescent microscope (Nikon microscope and EZ Coolsnap camera). One field of view from each of the 12 wells was imaged using a 10X objective (Supplemental Figure 3). Results were averaged from the 12 wells and reported as cells per field of view; roughly cells per 0.5 mm².

Micropipette experiments and imaging

Live cells were trypsinized and resuspended in fresh media for more than 30 minutes and less than 2 hours for aspiration. Cell suspensions in coverslip-bottom dishes (Mattek) were maintained in a large heated chamber with 5% CO₂ and humidity control (PeCon and Leica) on an inverted microscope (Leica DMI6000) using a 63x (1.4 NA) oil immersion objective. 1 µg/mL each of Hoechst 33342 and propidium iodide (Life Technologies) was added to the media to label the nucleus and test for membrane integrity, respectively. Images were analyzed using the Leica AF6000 software and ImageJ.

Micropipette aspiration technique

Micropipettes were pulled from capillary tubes (ID of 1 mm from World Precision Instruments) using a micropipette puller (PMP-102 MicroData Instruments). Pipettes of inner diameter 5 – 8 µm were used with aspiration pressures of 0.2 – 2 kPa to induce different levels of strain. In every case, data was only considered in which the initial diameter of the cell was 1.5 - 2.5 times the diameter of the pipette and the length of the cell, L_c , was greater than the radius of the pipette. We were focused primarily on the relative deformability of subcellular components with respect to one another rather than the overall mechanical properties of the cell. As such, we did not record the absolute pressure. For suspended cells, there were not obvious differences in nuclear or cellular size, similar to adhered cells (Supplemental Figure 1). As we have shown previously, cell diameters above 2.5x the size of the pipette only impacts the local region; below 1.5, cells are aspirated into the pipette and do not adequately deform.(31) Variations in mechanics of nuclei,(25) cells(31) and cell aggregates(54) measured by MPA do not correlate significantly with variations of pipette size and aspiration pressures within the range used in this study.

The inside and the outside of the pipette were passivated with 1% filtered BSA. The back-filled pipette was connected via a Narishige micromanipulation system in parallel with a water-filled manometer connected to a pressure transducer (World Precision Instruments). Initially, the individual cell and pipette were aligned in the absence of pressure. Aspiration pressure was applied and held constant. Imaging was initiated immediately after setting the constant pressure. The images were acquired first every 5 s for 20–30 s and for every 10 s after that for 90–120 s. Images were acquired in brightfield and fluorescent modes for propidium iodide and Hoechst 33342. DsRed was also captured with the propidium iodide channel in the case of transfected cells. For analysis, only cells that did not contain red fluorescent DNA (propidium iodide) throughout the experiment were used; propidium iodide positive cells were rarely observed. Micropipette aspiration experiments were run by multiple investigators on different days with different passage cells.

Statistical analysis

Statistical comparisons were performed using Student's t-test for 2 comparisons or via one-way ANOVA for multiple samples. Results represent at least 3 independent experiments and are shown as averages $\pm$ SEM. Results were considered significant at $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic of experimental apparatus used for this study

(A) Migration potential was measured with a parallel plate flow chamber above a Boyden chamber with layer of endothelial cells cultured in between. Tumor cells circulate through the flow chamber, and metastatic cells are able to adhere to the endothelial cells, pass through endothelial cells, and migrate through the 8 μm pores toward the chemoattractant. Cells able to reach the bottom of the apparatus are then counted by microscopy. (B) Deformability of individual cells and subcellular structures are measured by micropipette aspiration. Suspended cells are aspirated into a glass micropipette by applied suction pressure. By imaging the cell and nucleus independently, relative deformability can be measured.

Figure 2. Imaging during micropipette aspiration of cells shows nuclear and cellular deformation

Representative overlaid images of cells (brightfield, blue nucleus and DsRed- 50LA) with increasing strain into a micropipette. Cells were aspirated at constant pressure and images from the left panel to the right were taken with 60-120 sec of additional creep. Arrows have been added to highlight boundaries. (A) Aspiration of WM35 cells shows that the cell and nucleus are able to deform into the micropipette. Cell deformation (gray arrow) is longer than nuclear deformation (blue arrow). (B) The nucleus of Lu1205 cells appears more deformable with the cell. (C) Expression of DsRed- 50LA prevents the nucleus from entering the pipette (red arrow). In WM35 cells, the cell also showed reduced deformability. (D) Lu1205 cells were able to deform, but the DsRed- 50LA-stiffened nucleus was unable to deform into the pipette. For length scale, micropipettes of 5-8 μm inner diameter were used.

Figure 4. Stiffening nuclei with $\Delta 50$ LA causes altered cellular deformation

(A) The ability of the nucleus to deform into the micropipette while cells are being aspirated is given a binary value, and percent of cells with nuclear deformability is quantified. Few cells expressing $\Delta 50$ LA show nuclear deformation into the micropipette compared with nearly 100% for untransfected control cells or cells exogenously expressing lamin A. (n is the number of cells measured per sample) (B) For cells that show a nuclear deformation into the pipette, the ratio of Ln/Lc is much lower for $\Delta 50$ LA expressing cells. (n is the number of cell with multiple measurements per cell) (C) The expression of $\Delta 50$ LA severely reduced the migration potential, calculated as the number of cells which can escape from the circulation domain through the endothelial later and through the pores of a polycarbonate filter (Figure 1A). Statistical differences from control (* is $p < 0.05$ from relevant control).