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Disturbed nuclear orientation and cellular migration in A-type lamin deficient cells

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ABSTRACT

The nuclear lamina and the cytoskeleton form an integrated structure that warrants proper mechanical functioning of cells. We have studied the correlation between structural alterations and migrational behaviour in fibroblasts with and without A-type lamins. We show that loss of A-type lamins causes loss of emerin and nesprin-3 from the nuclear envelope, concurring with a disturbance in the connection between the nucleus and the cytoskeleton in A-type lamin-deficient (*lmna* −/−) cells. In these cells functional migration assays during *in vitro* wound healing revealed a delayed reorientation of the nucleus and the microtubule-organizing center during migration, as well as a loss of nuclear oscillatory rotation. These observations in fibroblasts isolated from *lmna* knockout mice were confirmed in a 3T3 cell line with stable reduction of *lmna* expression due to RNAi approach. Our results indicate that A-type lamins play a key role in maintaining directional movement governed by the cytoskeleton, and that the loss of these karyoskeletal proteins has important consequences for functioning of the cell as a mechanical entity.

1. Introduction

The nuclear lamina is a network of type-V intermediate filaments located at the inner nuclear membrane of eukaryotic cells, with the major protein constituents comprising A- and B-type lamins. Lamins play an important role in maintaining the structural integrity of the cell, by forming a complex network in the nucleus and by the formation of a bridge between the nucleus and the cellular membrane via the cytoskeleton [1]. Lamins have also been suggested to modulate gene expression during e.g. cell differentiation [2,3] and mechanotransduction [4].

The lamin A/C gene (*LMNA* in humans and *lmna* in mice) encodes for at least four different splice variants i.e. lamin A, lamin AΔ10, lamin C and lamin C2 (for a recent review see [5]). Several proteins, located in the outer and inner nuclear membrane, mediate the linkage of the nuclear membrane and lamina to the different cytoskeletal components. Most nesprin isoforms are associated with the outer nuclear membrane and are linked to the nuclear lamina through SUN-proteins, which are located in the perinuclear space, forming a complex called linker of nucleoskeleton and cytoskeleton (LINC) [6]. In cells lacking A-type lamins (*lmna* −/− cells) aberrations in the organization of the cytoskeleton can be found which indicate that the link between the cytoskeleton and the nucleus is A-type lamin-dependent [7]. The absence of A-type lamins has a severe impact on the mechanical properties of the nucleus and the cell as a whole, which becomes apparent upon mechanical compression [8], and mechanical stretch [7,9]. While upon compression nuclei of normal fibroblasts deform anisotropically, nuclei of *lmna* −/− fibroblasts deform isotropically, indicating a disturbed nucleo-cytoskeletal interaction [7,9]. This is supported by the finding that disconnecting the wild type (wt) nucleus from the mechanical tension of the cytoskeleton scaffold results also in isotropic nuclear deformation as in the *lmna* −/− cells [9].

Since an intact cytoskeleton organization is critical for cell migration we now examined whether or not the disturbed nucleo-cytoskeletal interaction in *lmna* −/− cells interferes with the *in vitro* migratory behaviour of fibroblasts in wound healing experiments. To this end we compared wt fibroblasts with lamin A/C knockout and lamin A/C knockdown fibroblasts. Normally, during this process the cytoskeleton is reorganized to establish spatial polarity and to form membrane protrusions facing the direction of migration [10]. Actin rearrangements are essential for cellular migration and are shown to be mediated by Rho GTPases [11]. When cells are activated to migrate, the membrane at the leading edge shows ruffling with microfilaments containing lamellipodia and filopodia [11]. Microfilaments are linked to lamins through the LINC-complex, composed of nesprin-1 and -2 and SUN-proteins [6,12–17].

Next to the microfilaments also microtubules are essential in the process of wound healing [10], as they are selectively stabilized near the leading edge [18–20], allowing the formation of membrane protrusions at this side of the cell [21,22]. During the migration

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process microtubules also play a role in the rotation and translocation of the nucleus and the reorientation of the microtubule organizing centre (MTOC) [23,24]. In *C. elegans* microtubules and the nuclear lamina interconnect through the UNC-83 SUN family protein [25–28], while the Hook family protein ZYG-12 links the nucleus to the MTOC [29,30]. In *Drosophila* the MTOC and the microtubules are linked to the nuclear lamina through the nesprin-like Klarsicht protein [31]. Although so far no vertebrate homologues of these proteins have been described, the data from *C. elegans* and *Drosophila* suggest a tight binding of the MTOC and the microtubules to the nucleus via lamins (for a review see [32]). Wilhelmsen et al. [33] hypothesized that also nesprin-3 may link microtubules to the vertebrate nucleus.

While the structure of both microfilaments and microtubules shows abnormalities in *lmna* −/− cells, even more pronounced abnormalities have been described for the intermediate filament organization [7,34] [9]. Intermediate filaments are linked to the nuclear envelope via plectin and the recently discovered nesprin-3 [35]. During cell migration intermediate filaments play an essential role in interactions through (hemi-)desmosomes between adjacent epithelial cells and with the extracellular matrix [36]. Therefore, abnormalities in the structure of intermediate filaments, due to the absence of A-type lamins, could lead to a disturbance in cellular dynamics.

Through their effects on cytoskeletal filament organization lamin-mutations even appear to affect the organization of cell membrane-associated proteins. For example, in transgenic mice with a *lmna*-N195K mutation, a mislocalization of connexin-43 was detected in cardiac tissue [37], which could explain the conduction defects found in laminopathies [5].

It is thus conceivable that changes in the nuclear lamina have consequences for structural protein functioning throughout the whole cell. In the present study we show that the absence of A-type lamins indeed affects the functional organization of the cytoskeleton, leading to defects in nuclear and MTOC reorientation, resulting in an uncoordinated response to wounding.

2. Materials and methods

2.1. Cell cultures

Wild type (wt) and A-type lamin-deficient (*lmna* −/−) mouse embryonic fibroblasts (MEFs) were kind gifts from B. Burke (Department of Anatomy and Cell Biology, University of Florida, Florida, USA) and C.L. Stewart (Laboratory of Cancer and Developmental Biology, National Cancer Institute, Maryland, USA) [38]. 3T3 cells (CCL-92) were obtained from the American Type Culture Collection.

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco Life Technologies Ltd., Paisley, UK) containing 10% fetal calf serum (FCS, HyClone Laboratories, Logan, Utah, USA), 1 mM L-glutamine and 0.1% gentamycin (Eurovet, Bladel, the Netherlands) at 37 °C in a humidified 5% CO_2 incubator. Cells were passaged by splitting at 1:3 to 1:5 ratios using a 0.125% trypsin (Invitrogen Life Technologies, Breda, The Netherlands)/0.02 M EDTA/0.02% glucose solution in phosphate buffered saline (pH7.4, PBS).

2.2. RNA interference

Stable shRNA-based knockdown of lamin A/C was performed in 3T3 fibroblasts, using a lentiviral construct. Based on the successful knockdown of mouse *lmna* by Kudlow [39] we designed the following oligonucleotides:

Forward: 5′-CACCGCTTGACTTCCAGAAGAACATTTCAAGAGAATGTTCTTCTGGAAGTCAAGC-3′

Reverse: 5′-AAAAGCTTGACTTCCAGAAGAACATTCTCTTGAAATGTTCTTCTGGAAGTCAAGC-3′.

Using the Gateway cloning system (Invitrogen) the annealed oligonucleotides were ligated into the pENTR™/H1/TO vector (Invitrogen). Next, the H1/TO RNAi cassette was transferred into the pLenti4/BLOCK-iT™-DEST vector (Invitrogen) using a LR recombination reaction according to the vendor's instructions. The correct incorporation of the H1/TO *lmna* RNAi cassette was verified by sequencing. 3T3 cells were transfected using Lipofectamin-2000 (Invitrogen), and zeocin (Invitrogen, 150 μg/ml) was used to select the transfected cells. To obtain a stable reduction of A-type lamin expression cells were subcloned by limited dilution and single cell colonies with variable A-type lamin expression were selected. Two different clones, one with a partial knockdown (pkd) and the other with an almost complete knockdown (kd) of lamin A/C, were used for our experiments.

2.3. Immunofluorescence microscopy

MEF and 3T3 cells grown on glass coverslips were either fixed for 10 min with methanol (−20 °C), or for 15 min with 3.7% formaldehyde in PBS at room temperature followed by permeabilization for 15 min with 0.1% Triton X-100 (BDH, Poole, UK) in PBS. Primary antibodies, diluted in PBS containing 3% bovine serum albumin (BSA) (Roche Diagnostics, Mannheim, Germany), were applied during 60 min. The following antibodies were used:

- Lamin A mouse monoclonal antibody 133A2 (IgG3, 1:100, MUbio Products B.V., Maastricht, The Netherlands).
- Lamin C rabbit polyclonal antibody RalC (1:20, MUbio Products B.V., Maastricht, The Netherlands).
- Lamin B1 rabbit polyclonal antibody LB1 (1:200; a kind gift from J.C. Courvalin, INSERM, Paris, France).
- Lamin A, AΔ10 and C mouse monoclonal antibody X-67 (IgG1, 1:250, a kind gift from G. Krohne,Würzburg, Germany).
- Emerin mouse monoclonal antibody NCL–emerin (IgG1, dilution 1:60; Novocastra, Newcastle upon Tyne, UK).
- β-actin mouse monoclonal antibody AC-15 (IgG1, 1:1500, Sigma-Aldrich, St. Louis, Missouri, USA).
- β-tubulin mouse monoclonal antibody E7 (IgG1, 1:25, Developmental Studies Hybridoma Bank, Iowa City, Iowa, USA).
- γ-tubulin mouse monoclonal antibody GTU-88 (IgG1, 1:3000, Sigma-Aldrich) for MTOC staining.
- Vimentin mouse monoclonal antibody BV-1118 (IgM, 1:10, was a kind gift from C. Viebahn, University of Göttingen, Göttingen, Germany).
- Nesprin-3 rabbit polyclonal antibody (1:100, a kind gift from A. Sonnenberg, Netherlands Cancer Institute, Amsterdam, the Netherlands).
- SERCA2 ATPase monoclonal mouse antibody IID8 (IgG1, 1:100, Affinity BioReagents, Golden, Colorado, USA).

After washing with PBS (3×5 min), secondary antibodies were applied during 60 min at room temperature. Secondary antibodies used were FITC-conjugated rabbit anti-mouse Ig (1:50, DAKO, Glostrup, Denmark), FITC-conjugated goat anti-rabbit Ig (1:50, SBA/ITK Birmingham, AL, USA), FITC-conjugated swine anti-rabbit Ig (1:50, DAKO), Texas Red-conjugated rabbit anti-mouse Ig (SBA/ITK) and Texas Red conjugated goat anti-rabbit Ig (1:50, SBA/ITK). For double labelling, we used the appropriate combination of primary and secondary antibodies labelled with FITC and Texas Red, or a combination of immunolabelling and Texas Red-conjugated Phalloidin (dilution 1:100; Molecular Probes, Leiden, The Netherlands) for the detection of actin stress fibers.

After another series of washing steps, cells were mounted in 90% glycerol, containing 20 mM Tris–HCl pH 8.0, 0.2% NaN_3, and 2% 1,4-di-azobicyclo-(2,2,2)-octane (DABCO; Merck, Darmstadt, Germany) and 0.1 mg/ml RNAse (Serva, Heidelberg, Germany). Nuclei were counterstained using diamidino-2-phenylindole (0.5 μg/ml DAPI; Sigma-Aldrich) or propidium iodide (0.5 μg/ml PI; Molecular Probes) in mounting medium. Cells were visualized using a BioRad MRC600 confocal laser scanning fluorescence microscope (BioRad Laboratories