Mechanical and structural abnormalities of the cytoskeleton in A-type lamin deficient cells

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BMTE06.38
September 2006

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In theory, there is no difference between theory and practice. But, in practice, there is.

Jan van de Snepscheut (1953-1994)
### List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AcLDL</td>
<td>Acetylated low density lipoprotein</td>
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<tr>
<td>AD</td>
<td>Aqua destillata</td>
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<tr>
<td>Arg</td>
<td>Arginine</td>
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<tr>
<td>Asp</td>
<td>Aspartic acid</td>
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<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BAF</td>
<td>Barrier-to-autointegration factor</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>CD</td>
<td>Cluster of differentiation</td>
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<td>CH</td>
<td>Calponin homology</td>
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<td>CHO</td>
<td>Chinese hamster ovary</td>
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<td>DABCO</td>
<td>1,4-diazabicyclo(2,2,2)octane</td>
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<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
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<td>DMEM</td>
<td>Dulbecco’s modification of eagle’s medium</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DNase</td>
<td>Deoxyribonuclease</td>
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<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<td>EDTA</td>
<td>Ethylene diamine tetra acetic acid</td>
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<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
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<td>ER</td>
<td>Endoplasmic reticulum</td>
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<td>FCS</td>
<td>Fetal calf serum</td>
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<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<td>GAR</td>
<td>Goat anti-rabbit Ig</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>Gly</td>
<td>Glycine</td>
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<td>HCT</td>
<td>Human colon tumor</td>
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<td>HeNe</td>
<td>Helium-neon</td>
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<td>HF</td>
<td>Human fibroblast</td>
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<td>HFT</td>
<td>Haupt farb teiler (Main beam splitter, Zeiss)</td>
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<td>IF</td>
<td>Intermediate filament</td>
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<td>Ig</td>
<td>Immuno-globulin</td>
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<td>INM</td>
<td>Inner nuclear membrane</td>
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<td>KASH</td>
<td>Klarsicht Anc-1 Syne-1 homology</td>
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<tr>
<td>LAP</td>
<td>Lamin associated protein</td>
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<td>LB</td>
<td>Luria Bertani</td>
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<td>LBR</td>
<td>Lamin B receptor</td>
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<td>LEM</td>
<td>Lamina-associated polypeptide Emerin MAN1</td>
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<td>LMNA</td>
<td>Lamin A/C gene</td>
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<td>LMNB1</td>
<td>Lamin B1 gene</td>
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<td>LMNB2</td>
<td>Lamin B2 gene</td>
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<td>LP</td>
<td>Long pass filter (Zeiss)</td>
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<td>MAN</td>
<td>Mannose-6-phosphate receptor</td>
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<td>MCS</td>
<td>Multi-cloning site</td>
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<td>MEF</td>
<td>Mouse embryonic fibroblast</td>
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<td>MF</td>
<td>Microfilament</td>
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<td>MLC</td>
<td>Myosine light chain</td>
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<td>MoAb</td>
<td>Monoclonal antibody</td>
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<td>MT</td>
<td>Microtubule</td>
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<td>MTOC</td>
<td>Microtubule organizing centre</td>
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<td>NE</td>
<td>Nuclear envelope</td>
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<td>NET proteins</td>
<td>Nuclear envelope transmembrane proteins</td>
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<td>NPC</td>
<td>Nuclear pore complex</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>NT</td>
<td>Nutral density filter (Zeiss)</td>
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<td>ONM</td>
<td>Outer nuclear membrane</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<tr>
<td>PI</td>
<td>Propidium iodide</td>
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<tr>
<td>RAM</td>
<td>Rabbit anti-mouse</td>
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<tr>
<td>RHAMM</td>
<td>Receptor for hyaluronic acid mediated motility</td>
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<td>RIPA</td>
<td>Radio immuno precipitation assay</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>RNAi</td>
<td>RNA interference</td>
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<tr>
<td>RNase</td>
<td>Ribonuclease</td>
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<tr>
<td>SAD</td>
<td>Spindle pole body-associated protein</td>
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<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
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<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
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<tr>
<td>SUN</td>
<td>Sad1 UNC-84 homology</td>
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<td>SWAR</td>
<td>Swine anti-rabbit</td>
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<tr>
<td>UNC</td>
<td>Uncoordinated protein</td>
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<tr>
<td>WT</td>
<td>Wild-type</td>
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Abstract (English)

Mutations in A-type lamins give rise to a group of inherited diseases, called laminopathies. These laminopathies can afflict several tissues and exhibit various tissue specific phenotypes. The facts, that lamins make up a filamentous network in the nucleus and are also closely associated with gene regulation, gives rise to two hypotheses for the cause of these different phenotypes, neither mutually exclusive. Firstly, the gene regulating property of A-type lamins could give rise to the different phenotypes. Secondly, the muscle wasting seen in several of these diseases makes it likely that the affected tissues also suffer from nuclear fragility. Previous studies have shown that the mechanical strength of the cell relies largely on the nucleus and that it thus forms a structural component of the cytoskeleton. The association of the nucleus to the cytoskeleton is, however, still unclear, but it is believed that lamins play a crucial role in this association. The nuclear deformation in lamin deficient cells is different from wild-type cells, suggesting that the connection between the (actin) cytoskeleton and the nucleus might be impaired and responsible for this behavior.

This report focuses on the mechanical implications of A-type lamin deficiency, in particular the implications it has on the connection to the actin cytoskeleton.

In this study it has been confirmed that actin is less tightly associated to the nucleus in lamin A/C deficient cells than in wild-type cells. This conclusion was drawn by means of different analysis procedures of isolated nuclei, including confocal microscopy, flow cytometry and Western blotting. The isolation of these nuclei also revealed that lamin A/C deficient nuclei are less resistant to swelling, due to osmotic forces, and do not cope well with hypertonic shock, compared to wild-type nuclei. Furthermore it was shown that the isolated nuclei of both cell types deform isotropically during compression events. The anisotropic deformation of wild-type nuclei under cellular compression should therefore be attributed to the anchorage of the nuclei to the cytoskeleton. Compression of the isolated nuclei also showed that on several occasions lamin deficient nuclei burst, while wild-type nuclei never showed this behavior. This result combined with the observations during hypotonic nucleus isolation, are clear indications that the A-type lamins play an important role, in both cellular and nuclear mechanics.

Functional studies of the cytoskeleton, by means of wound healing assays performed on a vital microscope, revealed that the reorientation of the nucleus, in lamin A/C deficient cells, is altered. Significantly more wild-type cells reacted to the wounding by first reorienting its nucleus. Secondly the orientation of the nucleus proved to be a good indicator of migration direction in wild-type cells, while in the lamin A/C deficient cells this characteristic was absent. Considering the close relationship of the nucleus to the centrosome, a microtubule organizing center, these properties are an additional indication that the connection of the nucleus to the cytoskeleton in lamin A/C deficient cells is impaired.
Abstract (Nederlands)

A-type lamine mutaties kunnen leiden tot een groep erfelijke ziektes, die laminopathies genoemd worden. Deze laminopathies kunnen verscheidene weefsels aantasten en weefsel specifieke phenotypes vertonen. Gezien het feit dat lamines in de nucleus een filamenteus netwerk vormen en gen regulatie beïnvloeden, bestaan er twee mogelijke verklaringen voor de verscheidene phenotypes, die elkaar niet uitsluiten. De gen regulerende eigenschappen van A-type lamines kunnen namelijk aan de basis hiervan liggen, maar gezien het spierverlies in een aantal van deze ziektes, lijkt het ook waarschijnlijk dat er een mechanische grondslag is. Waarin de weefsels te lijden hebben van de mechanische kwetsbaarheid van de nucleus.

Vorige studies hebben aangetoond dat de nucleus een belangrijk onderdeel is in de mechanica van de cel en dat deze daarom ook gezien kan worden als een structurele component van het cytoskelet. Hoewel de connectie tussen het cytoskelet en de nucleus nog steeds niet geheel duidelijk is, zijn er aanwijzingen dat de lamines hierin een belangrijke rol vervullen. In 2004 is aangetoond dat de deformatie van de nucleï van lamine A/C deficiënte cellen anders is dan die van wild-type cellen. De isotrope vervorming van de nucleï van A-type lamine deficiënte cellen ten opzichte van de anisotrope vervorming van de nucleï van wild-type cellen, was aanleiding om te stellen dat de aanhechting van het cytoskelet aan de nucleus hieraan ten grondslag lag. Immunofluorescentie had ook al aangetoond dat er een verschil was in de actine distributie tussen beide celtypes.

Deze studie heeft bevestigd dat er minder actine aan geïsoleerde nucleï van A-type lamine deficiënte cellen zit, ten opzichte van de nucleï van wild-type cellen. Dit resultaat is bereikt met behulp van verschillende analyse technieken, waaronder microscopy, flow cytometry en Western blotting. De extractie van de nucleï bracht ook aan het licht dat nucleï van wild-type cellen minder zwellen in een isotone oplossing dan de lamine A/C deficiënte nucleï. Het vervolgens resuspenderen van deze nucleï in een hypertone oplossing leidde tot het verlies van alle A-type lamine deficiënte nucleï, terwijl dit niet het geval was bij de wild-type nucleï. Deze geïsoleerde nucleï werden ook gebruikt in compressie studies, waarbij opviel dat de nucleï van beide celtypes isotroop vervormden. Hierdoor kon geconcludeerd worden dat de anisotrope vervorming van de nucleus, bij het comprimeren van wild-type cellen, het gevolg was van een associatie van de nucleus aan het cytoskelet en dat de lamines hierbij een belangrijke rol spelen. Tijdens de compressie van de geïsoleerde nucleï, bleek ook dat de nucleï van A-type lamine deficiënte cellen fragieler waren, dan de geïsoleerde wild-type nucleï.

Door middel van wondgenezingsexperimenten zijn ook functionele studies uitgevoerd naar het dynamische karakter van het cytoskelet. Deze studies werden geanalyseerd met behulp van vitale microscopy, waarbij de oriëntatie van de nucleï kon worden geobserveerd. Hierbij viel op dat deze bij lamine A/C deficiënte cellen verstoord was. Significant meer wild-type cellen reageerden initieel op de verwonding met het draaien van de nucleus. Daarna was de orientatie van de wild-type nucleus een goede indicator van de migratie richting van de cellen, terwijl deze eigenschap nauwelijks aanwezig was bij de lamine deficiënte cellen. Gezien de relatie tussen de nucleus en de centrosomen, een microtubulus organiserend centrum, zijn deze eigenschappen een extra indicatie dat lamines een belangrijke rol spelen in het verankeren van de nucleus aan het cytoskelet.
Literature Essay
Introduction

Cells are dynamic structures that respond to extracellular signals, both mechanical and chemical. The cellular responses to mechanical strain have long been investigated and are still a matter of discussion. These responses can be divided into an initial response, the distribution of forces, and a later response, the reorganization of the cells architecture and function. This literature essay focuses on the former, the distribution of forces.

Over the years a lot of models have been proposed, that could explain certain aspects of the mechanical properties of the cell. Most of these were simple continuum models [Elson, 1988; Evans and Yeung, 1989; Dong et al., 1991; Fung and Liu, 1993; Schmid-Schonbein et al., 1995; Heidemann et al., 1999], depicting the cell as a viscous plasma surrounded by an elastic shell. Although these simple models were successful at explaining distinct aspects of the cells mechanical behavior, none of these could explain other aspects, like force transduction over relatively large distances. All these models have one thing in common, instead of being a mathematical formulation of a theory, they are computational models. The last decades increasingly more studies predict that the organization of the cytoskeletal filaments plays an important role in cellular mechanics. The mechanical properties of the cell are proposed to be the result of a cytoskeletal organization based on the tensegrity architecture. This architecture uses tensional integrity in stead of compressional continuity.

In the first chapter, the cytoskeleton and its individual components will be described, because of their importance in the tensegrity architecture. The mechanical properties of these components, which make them ideally suitable for distinct tasks in a tensegrity architecture, will also be discussed. In chapter 2 the tensegrity structure and its requirements will be described, together with evidence of meeting those requirements. The final chapter will focus on the role the nucleus and its nucleoskeleton plays in cellular mechanics, since nuclei have been reported to be particularly important in cellular mechanics.
1. The cytoskeleton

The cytoskeleton consists of a filamentous network that resides in the cytoplasm of all eukaryotic cells. It contains three principal types of filaments: microtubules, intermediate filaments and actin filaments (figure 1.1). These different types of filaments together make up a dynamic structure, which can react to mechanical and chemical stimuli.

**Figure 1.1 Schematic representation of the cytoskeletal filament structure (top) and general distribution (bottom). The three filament types are the A) Microtubules, B) Intermediate filaments and C) actin filaments. [Alberts et al., 1994]**

It is responsible for several cellular functions. Besides determining the cells shape and mechanical properties, it is also responsible for the spatial organization of the cells organelles, the cells division and it provides the machinery for intracellular and cellular movement.

1.1 Microtubules

The microtubules are long, hollow cylinders made up of globular tubulin molecules, consisting of a heterodimer of $\alpha$ and $\beta$-tubulin. With an outer diameter of about 25 nm they form the thickest filaments. These polar filaments have two different ends, a slow-growing minus end and a faster-growing plus end [Allen and Borisy, 1974; Dentler et al., 1974; Margolis and Wilson, 1981]. The minus ends of these filaments are stabilized in microtubule organizing centers (MTOC’s), of which the centrosome is one of the most important [Euteneuer and McIntosh, 1981; Soltys and Borisy, 1985; McNiven and Porter, 1986]. Centrosomes are typically situated near the nucleus, the centre of the cell, while the plus ends are dispersed through the cytoplasm and linked to the cell cortex (figure 1.2). The polar character of these filaments enables cells to spatially organize its organelles, because motor proteins can direct the movement of these organelles towards the plus or minus end. In fact in animal cells, the spatial organization of organelles depends predominantly on the microtubule cytoskeleton. However, there are some exceptions to this general rule. In several instances the spatial organization relies on the actions of myosin, which moves along the actin cytoskeleton. The endoplasmic reticulum (ER) for instance generally aligns with the microtubules by means of a motor protein directed towards the plus ends. However, in some instances it can also bind to a motor protein directed to the minus end [Lane and Allan, 1999] and associate with the actin-based mechanism [Kuznetsov et al., 1994; Sturmer et al., 1995]. Whereas the Golgi apparatus in most metazoan cells is located near the centrosome mediated by means of a motor protein directed towards the minus ends. It has, however, been shown that motor proteins conventionally directed towards the plus ends also co-localize with the Golgi apparatus [Marks et al., 1994; Johnson et al., 1996]. This suggests that the morphology of the Golgi apparatus may depend on opposing forces, enforced by motor proteins directed towards opposite ends.
1.2 Intermediate Filaments

Intermediate filaments are expressed in eukaryotic cells in a tissue specific manner. With a diameter of 8-10 nm, they are intermediate in diameter between actin filaments and microtubules. Instead of being made up of polarized globular molecules like microtubules and actin filaments, they are made up of highly elongated fibrous monomers. Most of the intermediate filaments can be subdivided into five groups. The monomers of these five types of intermediate filaments (keratin I, II, vimentin, neurofilament proteins and nuclear lamins) share a common ultrastructural appearance [Henderson et al., 1982; Milam and Erickson, 1982]: an amino-terminal head, a carboxyl-terminal tail and a central rod domain. This central rod domain has an α-helical region consisting of heptad repeats which promote the formation of coiled-coil dimers [Steinert et al., 1978; Renner et al., 1981] and in this case a symmetrical tetrameric subunit [Geisler and Weber, 1982] (figure 1.3). The configuration of this tetrameric subunit renders it a non-polarized structure. Because these subunits are non-polarized, they pack together in a different manner than the microtubule and actin molecules. It is believed that this ropelike array is responsible for the ability of these filaments to withstand large stresses and strains without rupture [Jänne et al., 1991] (figure 1.4a). This property makes them ideally suitable for a specific mechanical function and this might also be the reason that they are predominantly present in the cytosol of animal cells which are subject to mechanical stress [Galou et al., 1997].

The nuclear lamina mainly consists of a special class (type V) of intermediate filaments, called lamins. Unlike the other intermediate filaments, they are situated in the nucleus and form a network on the nuclear face of the inner nuclear membrane (INM) [Dwyer and Blobel, 1976; Gerace et al., 1978]. There are two principal types of lamins in mammalian cells, which are classified as A-type and B-type lamins. The A-type lamins encompass 4 proteins, lamin A, AΔ10, C and C2, which all arise from splice variants of the LMNA gene [Fisher et al., 1986; McKeon et al., 1986; Riedel and Werner, 1989; Lin and Worman, 1993; Machiels et al., 1996]. The three B-type lamins, B1, B2 and B3 are encoded by two genes, LMNB1 (B1) [Hoger et al., 1988] and LMNB2 (B2 and B3) [Hoger et al., 1990; Furukawa and Hotta, 1993]. They differ

![Figure 1.2 Diagram of the general arrangement of microtubules (green), ER (blue) and Golgi apparatus (yellow). The nucleus is shown in brown and the centrosome in light green. [Alberts et al., 1994]](image)

![Figure 1.3 Current model of the assembly of monomeric subunits in the intermediate filament polymer. [Alberts et al., 1994] (based on data from Murray Stewart)](image)
from the cytoplasmic intermediate filament proteins in at least 5 ways: 1) Instead of forming a 3-D network, they probably form a 2-D sheetlike lattice. 2) The carboxyl-terminal domain harbors a nuclear localization signal sequence. 3) The central rod domain contains an extra six heptad repeats. 4) The meshwork is unusually dynamic and disassembles and reassembles rapidly at the start and the end of mitosis respectively, due to phosphorylation and dephosphorylation of several serine residues [Gerace and Blobel, 1980]. 5) Lamins are subjected to extensive processing, preceding the incorporation into the nuclear membrane [Broers et al., 2006].

1.3 Actin Filaments

Actin is the most abundant protein in many eukaryotic cells. There are at least 6 types, which can be categorized into three classes, α, β and γ. Alpha actin can be found in muscle cells, while β and γ actin can typically be found in non-muscle cells [Whalen et al., 1976]. All actin filaments, like microtubules, form a polar structure with a slow-growing minus end and a fast-growing plus end [Smith et al., 1983]. They are however, thinner, more flexible and usually shorter than microtubules [Janmey, 1991]. Another difference is that microtubules generally function as individual fibers, while actin filaments are mostly found in networks of cross-linked aggregates and bundles, such as stress fibers [Lazarides and Weber, 1974; Lazarides, 1976a; Lazarides, 1976b]. Beneath the plasma membrane this cross-linked network forms a structure called the cellular cortex. This cortex determines the cell polarity and is responsible for the cell-surface movements. It can form outward protrusions like microspikes [Taylor, 1966] and filopodia [Trelstad et al., 1967] or sheetlike lamellipodia [Abercrombie et al., 1970a; Abercrombie et al., 1970b; Abercrombie et al., 1970c] at the leading edge of a crawling cell, but it can also pull the plasma membrane inward in case of cell division. These functions are mainly determined by the organization of these filaments (figure 1.4b). In the outward protrusions, the filaments are aligned in a parallel manner, while in the contractile bundles, called stress fibers, they are aligned in an anti-parallel manner. The third possibility is a gel-like network, in which the filaments are positioned in a cross-like array [Sheterline, 1993].

![Figure 1.4](image_url)

**Figure 1.4** A) Stress/strain behavior of cytoskeletal filaments. [Alberts et al., 1994] (based on figure from Janmey et al., 1991). B) Schematic representation of cytoskeletal actin organization alternatives, arrows indicate plus end. [Alberts et al., 1994]
2. Cellular Mechanics

As mentioned before, the cytoskeleton is responsible for the mechanical properties of the cell. However, studies using several techniques, such as magnetic twisting cytometry [Wang et al., 1993; Wang and Ingber, 1994; Wang and Ingber, 1995; Hu et al., 2003; Hu et al., 2004], magnetic tweezers [Bausch et al., 1998; Alenghat et al., 2000; Lammerding et al., 2004; Lammerding et al., 2005], optical tweezers [Schmidt et al., 1993; Choquet et al., 1997], fluid shear stress [Helmke et al., 2001], cell compression devices [Guilak, 1995; Broers et al., 2004a] and micropipette manipulation [Maniotis et al., 1997; Dahl et al., 2004; Dahl et al., 2005] show that the mechanical properties can not be attributed to the individual parts of the cytoskeleton, which governs this static and dynamic behavior. The static and dynamic behavior of cells supports the idea that a more intricate organization is responsible for these properties.

2.1 Tensegrity Model

The tensegrity architecture was first described by Buckminster Fuller in 1961 and came to the attention of Donald Ingber in 1975, because its mechanical properties mimicked the behavior of living cells. Cells round up when they are enzymatically removed from their extracellular matrix (ECM) [Revel et al., 1974] and flatten when they are anchored to highly adhesive plastic dishes [Folkman and Moscona, 1978]. They can also physically pull flexible substrata into wrinkles [Harris et al., 1980] and contract pliable ECM gels (figure 2.1) [Emerman and Pitelka, 1977]. The tensegrity model consists of a structure, in which the mechanical properties are more than a sum of the mechanical properties of its individual components. It is comprised of several components that have distinct individual properties (figure 2.1). Firstly, there is a discontinuous series of components that can withstand compression, they act as struts. Secondly, there is a continuous series of components that can generate/withstand tension and have elastic properties. In the cytoskeleton the microtubules act as struts, the actin filaments as tension components and the intermediate filaments as elastic components. In the tensegrity architecture, all these components are interconnected in a geodesic arrangement. However, only cellular tensegrity is considered in this report, tensegrity structures are present in numerous biological processes and natural phenomena. They occur at different size scales, from the molecular level to the whole organism. For example, the muscles and bones in our body can be viewed as a tensegrity structure, in which the bones act as struts and the muscles as tensional components.

![Figure 2.1 Examples of a simple tensegrity structure. A) A high magnification view of a Snelson sculpture. B) Schematic diagram of cellular tensegrity, with microtubules (MTs) as compression elements, microfilaments (MFs) as tension generating components and intermediate filaments (IFs). C) Schematic diagram of tension generated after microtubule disruption. [Ingber, 2003]](image)
2.2 Linkage between cytoskeleton and ECM

As pointed out, the first indications, that the cytoskeleton is organized as a tensegrity architecture, originate from observations involving the ECM. These imply that a physical connection has to be present between the cytoskeleton and the ECM. The connection of the cytoskeleton to the ECM is, however not only of importance in the mechanics of the cell. In order to facilitate cell motility, the attachment of the actin filaments to the ECM is vital. This attachment is mediated by a group of proteins arranged in so-called focal adhesions. This group of proteins consists of a transmembrane protein, like integrin [Hynes, 1987], and several intracellular proteins, like talin, paxillin, vinculin and α-actinin (figure 2.2). Transmembrane proteins are responsible for the attachment of cells to the ECM glycoproteins containing the Arg-Gly-Asp (RGD) sequence, a sequence commonly shared by ECM proteins. Once the integrin binds to the ECM, the intracellular proteins will arrange to form focal adhesions, which bind actin stress fibers. Studies have shown that beads which are attached to integrins can no longer be displaced, by means of optical tweezers, once focal adhesions have been formed [Schmidt et al., 1993; Choquet et al., 1997]. These findings provide evidence that these contact sites provide a rigid anchorage of the cell to the ECM. Thereby they do not only facilitate cell motility, but also provide a means of relaying signals from the exterior of the cell to the interior.

Cadherins form another set of transmembrane proteins. In a calcium-dependent manner they accumulate to form homophilic cell-cell interactions, like tight junctions, adherens junctions and desmosomes [Adams and Nelson, 1998], which in addition form numerous interactions with the actin cytoskeleton [Kovacs et al., 2002a]. A study confirmed that forces are transduced via the cadherin junctions in comparable way as integrins do [Ganz et al., 2006]. Besides the interaction with the actin cytoskeleton, cadherins interact with many signaling molecules, suggesting they are adhesion-activated-signaling receptors [Pece and Gutkind, 2000; Noren et al., 2001; Braga, 2002; Kovacs et al., 2002b].

There are several other transmembrane proteins with similar properties. Immunoglobulin superfamily [Suter et al., 1998], selectins [Pavalko et al., 1995], dystrophin-glycoprotein-complexes [Ervasti and Campbell, 1993], CD44 [Lacy and Underhill, 1987], RHAMM [Turley et al., 1990; Hardwick et al., 1992] and tetraspanin proteins [Lagaudriere-Gesbert et al., 1998] also physically link the actin cytoskeleton to the ECM.

Connexins are transmembrane proteins that form gap junctions. They, unlike the previously described transmembrane proteins, have been shown to bind directly to α- and β-tubulin [Giepmans et al., 2001a]. In addition Giepmans et al. found that gap junctions co-localize with microtubules at the cell-periphery [Giepmans et al., 2001b]. Although, there is evidence that suggests actin is able to associate with gap junctions as well, because of the association with the zona occludens-1 protein also found in tight and adherens junctions [Giepmans, 2004].
2.3 Interaction between distinct filament types

The viability of the hypothesis, that the cytoskeleton is arranged as a tensegrity structure, depends on several important components. One of the most important is that the three distinct filament types need to be interconnected [Wang et al., 1993] and therefore span the cytoplasm. This interconnectedness therefore also implies that stress applied to the cytoskeleton should be transmitted throughout the cytoplasm to several structures connected to the cytoskeleton. A lot of studies on cellular mechanics and responses to local deformations make use of beads bound to specific transmembrane proteins like integrins. These proteins anchor the internal cytoskeletal lattice to the ECM [Ingber, 1991; Schiro et al., 1991; Schmidt et al., 1993; Scott-Burden, 1994]. The studies using this technique have shown that stress applied to these specific transmembrane proteins on the apical cell surface [Hynes, 1987; Ingber], is transferred to discrete and distant sites across the cytoplasm, like other focal adhesions at the cell base [Hu et al., 2003], the nuclear envelope (NE) [Maniotis et al., 1997] and mitochondria [Wang et al., 2001]. The transfer of stress from integrins, which are connected through microfilaments, to the mitochondria, which in turn are connected to microtubules, suggests that the filament systems are interconnected. Studies have also shown that each individual filament system is able to transmit local stress applied to the cell surface to a distinct site of the NE, but combined the effect is much larger [Guilak, 1995; Maniotis et al., 1997]. Another observation by Maniotis et al. was that the nuclear shape and nucleolar spacing changed even after triton isolation. This means that the deformation can not be a result of osmotic forces, surface tension or an ATP driven mechanism. This response can only be explained if the force is directly transmitted form the cytoskeleton to the nucleus. Other studies have shown that beads bound to transmembrane proteins, like $\beta$1-integrins, result in a proportional increase in cell stiffness as a response to stress, while disruption of microfilaments, microtubules or intermediate filaments partially impair cell stiffening and disruption of all three filament systems completely prevents cell stiffening [Wang et al., 1993]. The coupling of beads to other transmembrane proteins that do not couple to the internal cytoskeletal lattice, like acetylated-low density lipoprotein (AcLDL), results in virtually no mechanical resistance to cellular deformation [Wang et al., 1993; Yoshida et al.], displacement of mitochondria [Wang et al.], change in nuclear shape or nucleolar spacing [Maniotis et al., 1997]. The data of these and several other studies [Amos and Amos, 1991] combined give strong evidence of an interconnected network of all three discrete filament types that act together to transmit mechanical stress throughout the cytoplasm.

This in contrast to the classic continuum models of cell mechanics, in which the applied stress will always quickly dissipate and therefore will not be able to transmit any signals to its surroundings [Hu et al., 2003], let alone result in a local deformation. Recent studies describing the anisotropic nature of cell mechanics [Hu et al., 2003; Broers et al., 2004a; Hu et al., 2004] also point towards a different organization than the classic continuum model, which is isotropic. Therefore the tensegrity model holds a means by which cells can be mechanically coupled and transfer mechanical signals in a directed manner.

2.4 Tensional Component: The Actomyosin Apparatus

Another very important property of a tensegrity structure is prestress. Like the interconnectedness of the three distinct filament types, prestress is also essential for the viability of the tensegrity model. It stabilizes the structure and without it the structure would collapse under its own weight. This property can be attributed to both active and passive components. The actomyosin apparatus is responsible for the active component, while the additional passive contributions come from other cells, the ECM, osmotic forces and forces exerted by filament polymerization.

As mentioned before, the earliest indications of cellular prestress date from the 1970’s when it was shown that cells round up when disconnected from their substrata [Revel et al., 1974] and flatten when they attached [Folkman and Moscona, 1978]. In this same period it was also shown that cells can actively generate tension by plating them on flexible substrata [Emerman and Pitelka, 1977; Harris et al., 1980]. This idea became well established in the 1990’s, when these
forces generated by cells were quantified [Kolodney and Wysolmerski, 1992; Lee et al., 1994; Oliver et al., 1995; Pelham and Wang, 1997; Balaban et al., 2001; Wang et al., 2001]. There are several studies supporting the hypothesis that the actomyosin complex is responsible for a major part of the tensile prestress exerted on the cells cytoskeleton. The rounding and retraction of permeabilized cells to ATP in a dose dependent manner, shows that this response is due to an active process rather than passive elements like osmotic forces. Furthermore, this effect can be inhibited by the addition of a synthetic myosin peptide which inhibits actomyosin filament sliding [Sims et al., 1992]. Another study showed that contraction could be stimulated by the addition of thrombin, which causes major rearrangements of filamentous actin from a circumferential rim to stress fibers. However, the disruption of these actin filaments, by means of adding cytochalasin D, resulted in a quick dissipation of applied stress [Kolodney and Wysolmerski, 1992; Maniotis et al., 1997]. This effect has also been seen in other studies, where the cellular stiffness could be increased by the addition of a contractile agonist, while it could be decreased in the presence of several other drugs interfering with the actomyosin apparatus [Hubmayr et al., 1996]. The loss of stress focusing and anisotropy of the cells mechanics in response to actin filament disrupting agents, like cytochalasin D, latrunculin A and caldesmon, also shows the importance of prestress for intracellular and extracellular force transduction [Hu et al., 2003; Hu et al., 2004].

2.5 Compressional Component: The Microtubules

As mentioned before, in a prestressed tensegrity structure it is necessary to have components that can withstand the compressional forces exerted by the tensile components. For a cellular tensegrity structure several components, like the ECM or osmotic forces, are partially responsible for resisting these forces. However, if for instance the ECM would be the only component resisting these forces, cells plated on a planar adhesion substrate would look like fried eggs, which is not the case [Ingber, 2003]. It can be concluded from the studies mentioned previously, which used permeabilized cells, that osmotic forces are also only partially responsible for resisting these forces. Therefore the microtubule filament system, residing in the cells cytoplasm, has been proposed to act as a compression resisting component in a way similar to the one depicted in figure 2.1, because of its mechanical properties [Mizushima-Sugano et al., 1983]. The disruption of these filaments, in a prestressed tensegrity structure, should lead to an increase in tension exerted on the ECM, because the load is shifted from the interior to the exterior side of the cell. Several studies have shown that this is indeed the case. For instance, fluorescence microscopy of fibroblast cell lines seeded on a silicone rubber substratum has shown that these cells contract the substratum when exposed to microtubule-depolymerizing drugs, like colcemid, nocodazole and vinblastine. This effect could be prevented by the addition of taxol, which prevents microtubule depolymerization [Danowski, 1989]. Force measurements using isometric force transducers attached to a collagen matrix, on which fibroblasts were seeded, confirmed these results. The force measured, i.e. the force exerted on the ECM, increased after microtubule depolymerization with nocodazole. This effect could again be blocked by the microtubule stabilizing drug, called taxol [Kolodney and Wysolmerski, 1992; Kolodney and Elson, 1995]. Another more direct measuring method using a glass micropipette showed the same increase in tension after microtubule disruption with nocodazole [Dennerll et al., 1988; Maniotis et al., 1997]. This effect has been previously attributed to an increase in myosin light chain (MLC) phosphorylation or an increase of the intracellular Ca^{2+} concentration. However, maximally stimulated cells using histamine still showed an increase in tension after microtubule depolymerization [Wang et al., 2001]. Western blotting also showed that there was no additional increase in MLC phosphorylation when microtubules were disrupted after histamine addition above saturation levels [Wang et al., 2001]. It has also been shown that the intracellular Ca^{2+} concentration does not increase after microtubule depolymerization [Platts et al., 1999].

Direct observations of microtubules under compressional stress, either due to drugs like thrombin [Wang et al., 2001] or the migration of cells [Waterman-Storer and Salmon, 1997], have shown the buckling of these filaments. Subsequently releasing the tensile stress, in the
thrombin activated cells, by the addition of cytochalasin D showed straightening of the microtubules and even their protrusion of the cell surface. The tensegrity model has also been used to explain the elongating processes in neurites [Heidemann and Buxbaum, 1990]. This elongating process in cells like neurites and the stability of the axonal form depends on microtubules acting as compression struts. The disruption of microtubules and subsequent generation of tension by the microfilaments induces cell retraction [Solomon and Magendantz, 1981; Joshi et al., 1985]

2.6 Elastic Component: The Intermediate Filaments

Cytoplasmic intermediate filaments, like vimentin, have very different mechanical properties from actin or tubulin. They exhibit a viscoelastic stiffening behavior at high strains and are much less prone to breaking [Janmey, 1991; Janmey et al., 1991]. It has been shown that they play a role in several skin blistering diseases, in which a point mutation in keratins, the most diverse group of intermediate filaments, results in the loss of mechanical properties of the skin [Coulombe et al., 1992; Fuchs and Coulombre, 1992]. Therefore, the cytoplasmic intermediate filaments are thought to have a specific mechanical role in maintaining cell integrity [Fuchs, 1994; Fuchs et al., 1994] and cell shape [Goldman et al., 1996]. They have been suggested to act as guy-wires in the cellular tensegrity model, distributing the forces throughout the cytoplasm. The discovery that cytoplasmic intermediate filaments can couple directly or through plectin sidearms to microtubules and actin filaments certainly underlines this hypothesis [Green et al., 1986; Hollenbeck et al., 1989; Hisanaga et al., 1993; Cary et al., 1994; Svitkina et al., 1996]. They are, however, not required for the viability of eukaryotic cells in vitro [Paulin-Levasseur et al., 1988] or in vivo [Dellagi et al., 1983; Colucci-Guyon et al., 1994]. Mice lacking intermediate filaments do not even display an obvious phenotype [Colucci-Guyon et al., 1994]. This can be attributed to the fact that the cytoskeleton is a system, which has a lot of redundancy. Magnetic twisting cytometry revealed that wild-type (WT) and vimentin deficient fibroblasts exhibited the same responses at low stress levels. At higher stress levels, however, the vimentin deficient fibroblasts were less stiff and exhibited an impaired stiffening behavior compared to WT fibroblasts. The disruption of the cytoplasmic intermediate filaments in the WT fibroblasts, using acrylamide, had the same result. When these fibroblasts were plated for a longer period of time, before filament disruption, they also showed this response for lower stress levels, suggesting that the spreading of these cells is needed to get the cytoskeleton, including the cytoplasmic intermediate filaments in a prestressed state [Wang and Stamenovic, 2000]. Micropipette assays have also shown that intermediate filaments indeed play an important and distinct role in cell mechanics at high strains. Cells lacking vimentin are unable to support mechanical distension at high strains, resulting in cytoplasmic tearing [Maniotis et al., 1997; Eckes et al., 1998]. In wound healing and chemotaxis experiments it became apparent that these vimentin deficient cells exhibit reduced motility, impaired directional migration and collagen contraction [Eckes et al., 1998]. This again shows that the filament systems act together, since these properties are primarily dependent on actin filaments and microtubules. There are also several morphological differences that need to be considered. Vimentin deficient cells have a flatter morphology, disturbed actin and focal adhesion organization compared to their WT counterparts [Goldman et al., 1996; Eckes et al., 1998]. In addition there is evidence that the intermediate filaments interact with the nucleus, by anchoring it and stabilizing its form [Goldman et al., 1985; Goldman et al., 1986; Sarria et al., 1994; Maniotis et al., 1997] via the recently discovered protein Nesprin-3 [Wilhelmsen et al., 2005].
3. Nuclear Mechanics

The nucleus (figure 3.1) is an organelle surrounded by a double membrane system consisting of the INM and the outer nuclear membrane (ONM). The space enclosed by these two membrane systems is called the perinuclear space (PNS), which forms a common compartment with the endoplasmic reticulum (ER). The INM and ONM connect at the periphery of the nuclear pore complexes (NPCs). These complexes are multi protein channels, which selectively mediate signal-dependent trafficking into and out of the nucleus.

Several studies have demonstrated the physical connection between the cytoskeleton and several organelles, like the nucleus [Sarria et al., 1994; Guilak, 1995; Maniotis et al., 1997]. The nuclear mechanics and shape are therefore dependent on the cellular mechanics and shape and vice versa, as nuclei have been shown to be much stiffer and more viscous than the cytoplasm [Guilak et al., 2000; Caille et al., 2002]. The nucleus on its own also has a (nucleo-) skeleton with specific mechanical properties. This nucleoskeleton is formed by a fibrous network of nuclear lamins and is situated at the nucleoplasmic side of the INM (figure 3.2).

![Figure 3.1 Schematic representation of the nuclear envelope of a eukaryotic interphase cell. The nuclear lamins can be seen at the inner nuclear membrane as a meshwork, called the nuclear lamina. Also indicated are the ONM, the PNS, the nuclear core complexes and the ER.. [Stuurman et al., 1998]](image1)

![Figure 3.2 Electron micrograph of the nuclear lamina taken from frog oocytes. Sample was prepared by freeze drying and metal shadowing. [Aebi et al., 1986]](image2)
It serves a mechanical function, e.g. determining nuclear shape and size [Liu et al., 2000], and is responsible for the distribution and anchorage of most of the INM proteins, like emerin, nesprin and the NPCs [Lenz-Bohme et al., 1997; Raharjo et al., 2001; Hutchison, 2002; Gruenbaum et al., 2003; Holt et al., 2003].

3.1 Proteins associated with nuclear membrane

Like the proteins mentioned, there are several transmembrane proteins associated with the nuclear membrane, called nuclear envelope transmembrane (NET) proteins. They either link the cytoskeleton or the nuclear lamins to the nuclear membrane. This may provide a means of coupling the cytoskeletal mechanics to the nuclear mechanics. The nuclear lamins and their associated INM proteins form a structure called the nuclear lamina. Most of these proteins interact through large nucleoplasmic domain with lamins and/or chromatin. Amongst these is a group of proteins using a conserved nucleoplasmic residue motif, called the Lamina-associated-polypeptide Emerin MAN1 (LEM) domain. This group comprises Lap2 (Lamin associated polypeptide) [Berger et al., 1996; Dechat et al., 2000; Schoft et al., 2003], emerin and MAN1 [Lin et al., 2000]. Additional proteins such as Lem3 [Lee et al., 2000], as well as the drosophil-specific proteins often [Ashery-Padan et al., 1997a; Ashery-Padan et al., 1997b] and bocksbeutel [Wagner et al., 2000] and several yet uncharacterized human proteins named LEM2, LEM3, LEM4 and LEM5 [Lee and Wilson, 2004; Mansharamani and Wilson, 2005] have also been identified to share this LEM domain. It is known, this LEM domain, is able to interact with the barrier-to-autointegration factor (BAF), which is a protein associated with chromatin [Zheng et al., 2000]. As mentioned before some of these are able to bind directly to A or B type lamins, these include members of the LAP superfamily [Gant et al., 1999; Dechat et al., 2000], emerin [Vaughan et al., 2001] and MAN1 [Liu et al., 2003]. Besides these, LEM domain containing proteins, there are other INM proteins that bind lamins. The lamin B receptor (LBR) for instance binds B type lamin [Moir et al., 1995] and is thought to play a role in anchoring chromatin to the NE [Makatsori et al., 2004].

The proteins just mentioned are all situated at the INM. However, for a connection to the cytoskeleton, proteins that link the cytoskeleton to the NE are needed. Their existence has long been suggested, yet their exact nature and structure are still matter of discussion. Starr and Han (2003) and Lee et al. (2002) have suggested that a group of proteins, containing the SUN domain (SAD1/UNC-84 homologue), most likely located at the INM [Starr and Han, 2003; Hodzic et al., 2004] and a group of KASH domain containing proteins, located at the ONM [Apel et al., 2000; Zhang et al., 2001; Mislow et al., 2002; Zhen et al., 2002; Padmakumar et al., 2004], collectively called nesprins, fulfill this role of connecting the nuclear lamina to the cytoskeleton and thus the cellular membrane (figure 3.3).

In humans, the group of SUN domain containing proteins for now consists of SUN1 and SUN2 [Malone et al., 1999].and the group of KASH domain containing proteins consists of a variety of alternatively spliced transcripts from the nesprin-1, nesprin-2 and nesprin-3 genes [Zhang et al., 2001; Wilhelmren et al., 2005]. It is known that the SUN domain of the SUN1 and SUN2 proteins bind directly to lamins [Crisp et al., 2006] and that the ∼120 residue C-terminal motif, the SUN domain, localizes to the PNS [Hodzic et al., 2004; Crisp et al., 2006]. In addition nesprin-1 and -2, which also reside in the PNS, have been shown to co-localize at the ONM with SUN domain containing proteins [Starr and Han, 2002]. The conserved C-terminal amino acids PPPX of nesprin-1 and -2 are essential for the physical interaction with a C-terminal region in SUN1 [Padmakumar et al., 2005].

Furthermore the actin binding of the KASH containing proteins is mediated through two N-terminal calponin homology (CH) domains [Korenbaum and Rivero, 2002]. Except for the coupling of the actin cytoskeleton, a SUN containing protein and its binding partners have been proposed to mechanically link the nucleus to microtubule dependent motor proteins and thereby assist in the positioning of the nucleus inside the cell [Starr et al., 2001; Lee et al., 2002]. In addition, a recent study by Wilhelmren at al. (2005) showed the binding of nesprin-3 to the nuclear membrane on one side and via plectin to intermediate filaments on the other side.
Other lamin associated proteins have been shown to bind to chromatin [Burke and Stewart, 2002]. It has also been shown that lamins can bind directly to chromatin [Moir et al., 1995]. Because of this close relationship between the nuclear lamina and the genetic information it seems logical that they are also, at least in part, responsible for the cells reaction to mechanical stimuli. Furthermore it has been shown that indeed the nuclear lamins interact with transcription regulators. In a study using osteogenic cells, differentiation speed and expression levels could be altered by changing cellular and thus nuclear shape [Thomas et al., 2002]. This physical connection therefore provides a means for the cell to regulate gene expression and protein synthesis on the basis of extracellular mechanical signals. The large number of binding partners shown in figure 3.4 also is an indication for this broad spectrum of functions the nuclear lamins fulfill.

Figure 3.3 Schematic representation of predicted lamin - actin binding by Starr and Han (2003). The lamina binds to the UNC-84/SUN (yellow) protein, which is linked through an unknown interaction (gray circle) to the KASH domain (light blue) of syne/ANC-1. This protein in turn binds directly to actin filaments (green) in the actin cytoskeleton through its calponin domain (red). [Starr and Han, 2003]
However, this dual character is not merely a result of the many binding partners of lamins. The binding of lamins to chromatin for instance, also provides structural support for the nuclear lamins and thus the nucleus. In a study using nuclei, isolated from epithelial cells, it was shown that these nuclei were unable to shrink when exposed to osmotic forces. While treating these nuclei with DNase or RNase resulted in larger nuclei [Dahl et al., 2005]. It is believed that these properties are partly a result of the chromatin acting as a load bearing element. Therefore, to be able to determine the mechanical properties of the nuclear skeleton, it is important to study them in a state in which the contributions of the chromatin is minimal. This can be done by allowing the nuclei to swell as a result of osmotic forces. It has been shown that the chromatin does not swell accordingly, but the nuclear lamina does [Dahl et al., 2004]. In the same study the isolated nuclei were subjected to micropipette aspiration, which resulted in wrinkling of the membrane. This result indicates the nuclear membrane, associated with the nuclear lamina, behaves as a solid-like network, as a fluid membrane would smooth to relax any strain. The reversible nature of the deformation (figure 3.5), as a result of swelling and aspiration, indicates that the deformation of these nuclei is elastic. This fact in combination with the large range of elastic deformation, points to a tensegrity like organization of the nucleus, in which osmotic forces, cytoskeletal forces and chromatin anchorage put the nuclear lamina in a prestressed state. In fact it has been shown that isolated nuclei get a rough and wrinkled surface comparable to the state when cytoskeletal intermediate filaments are disrupted [Sarria et al., 1994; Dahl et al., 2005].

**Figure 3.4** Diagram of the proteins known to interact with lamins. [Gruenbaum et al., 2005]
3.2 The Role of A-type Laminopathies in impaired cell and nuclear mechanics

Lamins, as described previously, belong to the group of intermediate filaments and form a meshwork called the nuclear lamina. It has been shown that the absence of B-type lamins results in apoptosis of mammalian cells and cells from C. elegans [Harborth et al., 2001; Cohen et al., 2002]. Their role in vital processes, like the elongation phase during DNA synthesis, stresses their importance for the viability of most eukaryotic cells [Moir et al., 2000]. Unlike the B-type lamins, A-type lamins are not essential and their absence results in viable cells. Their expression is reduced or even absent in several cells, e.g. early embryonic cells, cells of a low degree of differentiation or proliferating cells [Rober et al., 1989; Broers et al., 1997]. However, along with their binding partners, A-type lamins have been associated with various genetic disorders (see table 3.1), called laminopathies. These laminopathies can be categorized into three different classes. Firstly there are those affecting striated muscle (cardiac and skeletal muscle), secondly those affecting adipose tissue and thirdly those affecting the peripheral nervous system. All laminopathies share one common aspect. They are all related to very rare ageing syndromes [Worman and Courvalin, 2004]. The most striking thing about these laminopathies is that, although A-type lamins are expressed in nearly all differentiated somatic cells, these diseases exhibit different phenotypes and some of them are tissue specific. This can be attributed to the dual function of nuclear lamins, gene regulation and mechanical support. It is, however, unclear which one of these is responsible for the phenotypes associated with these disorders. Although the presence of several muscle tissue associated phenotypes do point in the direction of impaired mechanics for some of these diseases. Lamin A/C deficient cells derived from mice have been shown to have a distorted nuclear shape and abnormal distribution of not only several proteins, including B-type lamins and emerin, but also chromatin [Sullivan et al., 1999; Raharjo et al., 2001; Nikolova et al., 2004]. Transfection of these cells with a point mutated lamin A/C gene, corresponding to a laminopathy, results in several observable defects, including an abnormal nuclear shape, in most of these cells [Raharjo et al., 2001]. Except for these nuclear defects, also the distribution of actin seems to be altered in lamin deficient cells (figure 3.6). When picture 3.6 was published by Lammerding et al., it was noted that there was no observable defect in its organization [Lammerding et al., 2004]. One can, however, clearly see the differences in the actin organization of these two pictures [Broers et al., 2004a].
In the same study Lammerding et al. investigated the differences in nuclear strain of WT and lamin deficient cells when they were subjected to biaxial strain. As can be clearly seen in figure 3.7a, the applied strain resulted in a much greater increase in nuclear size for the lamin A/C
deficient cells compared to the WT cells. This is a clear indication of a lower nuclear stiffness in these lamin deficient cells. In the same experiments it was observed that the lamin A/C deficient nuclei were also much more susceptible to tearing. The same cells were used in magnetic bead microrheology experiments. The beads were attached to integrin receptors to ensure the force could be directly applied to the cytoskeleton. These experiments revealed that the lamin A/C deficient cells on the whole were not as stiff as the WT cells (figure 3.7b). The nucleus therefore is a determinant in the overall cell mechanics. Compressional studies by Broers et al. (2004a) showed comparable results. Here individual cells were compressed by a cellular compression device [Peeters et al., 2003]. A-type lamin deficient cells had a significant reduction in stiffness compared to the WT cells and were much more susceptible to bursting. Another observation was that, after cellular integrity was compromised, large strands of DNA were protruding into the cytoplasm of the lamin A/C deficient cells, while the WT nuclei remained morphologically intact, indicating nuclear bursting in addition to cellular disruption. Some but not all mechanical characteristics could be restored by rescuing the a-type lamin deficient cells with lamin A/C. This can be attributed to the fact that emerin did not relocalize to the nucleus and the lamin organization was distorted. The rescued cells also showed another interesting characteristic. The bursting of their nuclei always occurred in places where there was an obvious lower concentration of lamin filaments. As mentioned before several studies have reported anisotropic mechanical behavior in cells. Broers et al. also showed that the deformation of the nuclei in WT cells was anisotropic, while in lamin A/C deficient or rescued cells the deformation was mainly isotropic.

Because of the close relationship between lamin A/C and emerin localization to the nucleus, Lammerding et al. investigated the differences in nuclear shape, mechanics and mechanotransduction between WT and both lamin deficient and emerin deficient cells [Lammerding et al., 2005]. As can be seen in figure 3.8a, emerin deficient cells had a higher percentage of irregularly shaped nuclei and nuclei exhibiting membrane blebbing, compared to WT cells; however, not to the same extent as lamin A/C deficient cells. When comparing the contour ratio (figure 3.8b) and area (figure 3.8c) of the nuclei it can be concluded that emerin deficient cells did exhibit all the same morphological differences as lamin deficient cells, although the latter always exhibited the more severe morphological disturbance. This could, however, not be stated for the nuclear mechanics. When subjected to biaxial strain the emerin deficient cells showed comparable nuclear strain to the WT cells, while as mentioned before the strain in lamin A/C deficient nuclei was much larger. These apparently normal nuclear mechanics for emerin deficient cells and abnormal nuclear mechanics for lamin A/C deficient cells were confirmed by the microinjection of dextran, which can not cross the intact NE, into the nuclei. The emerin deficient nuclei, just as the WT nuclei, remained intact at certain injection pressures, while in lamin A/C deficient cells the dextran leaked into the cytoplasm, indicating rupture of the NE.
In a more recent study by Lammerding et al. (2006) the contributions of the different lamin subtypes to the mechanical stiffness of the nuclei were assessed. Cells lacking both lamin A and C, cells lacking only lamin A, cells lacking WT lamin B1 and WT cells were subjected to uniform biaxial strain and the ratio of cellular to nuclear strain was assessed. This showed that the contribution of lamin A to nuclear stiffness was greater than the contribution of lamin C and that the lamin B1 only contributes to nuclear integrity but not stiffness [Lammerding et al., 2006].

The cells used in the studies by Lammerding et al. [Lammerding et al., 2004; Lammerding et al., 2005] were also subjected to prolonged mechanical stimulation to evaluate the effect on cell survival. Total cell death and apoptosis were measured using PI staining, revealing compromised cellular integrity, and DNA content analysis by means of flow cytometry respectively. The PI staining revealed that only the strained lamin A/C deficient cells suffered from a significant increase in cell death compared to WT cells and unstrained lamin A/C deficient cells. DNA content analysis showed that in both emerin and lamin A/C deficient cells the percentage of apoptotic cells increased significantly compared to the controls and that the baseline apoptotic value was only elevated for the lamin A/C deficient cells compared to the WT controls. An explanation for the increased percentage of apoptotic cells could be the result of an impaired cellular signaling response. In WT cells several genes will be up regulated as a response to mechanical stimuli, amongst these is the mechanosensitive gene egr-1 and the stress induced anti/pro (dependant on cell type and signaling pathway) apoptotic gene iex-1 [Sadoshima et al., 1992; Morawietz et al., 1999; Ohki et al., 2002; Shen et al., 2006]. The expression of these genes, in the mechanically stimulated emerin deficient and lamin A/C deficient cell lines, was attenuated. Cytokine stimulation, however, revealed a different signaling pathway is responsible for the attenuation in both cell-types, which could also be an explanation for the differences in baseline cell apoptosis.

Figure 3.8 Differences in nuclear shape between WT, emerin and lamin deficient cells. A) Abnormal nuclear shape and nuclear blebs were shown in a significantly larger fraction of the lamin and emerin deficient cells, with the emerin deficient cells displaying the milder phenotype. B) Contour ratios show that lamin deficient cells have the least round nuclei followed by the emerin deficient nuclei. C) The nuclei of lamin deficient and emerin deficient cells have a significantly increased cross-sectional area compared with WT cells. [Lammerding et al., 2005]
Discussion

Classic models depicted the cell as a viscous plasma surrounded by an elastic membrane. These classical models could, however, not explain the relaying mechanical signals and the distribution of forces observed in living cells. A tensegrity based model on the other hand, could better explain these displayed properties. In this literature essay it was shown that the fundamental requirements for this type of arrangement are met in cell culture systems. Firstly, it was shown that there are interactions between the distinct filament types. Secondly, it was shown that the cytoskeleton is made up of filament types with distinct properties that are required for tensegrity: 1) microtubules can withstand compression, 2) intermediate filaments are ideally suited for distributing forces and 3) the microfilaments can generate tension. Furthermore it has become clear that the nucleus plays an important in cellular mechanics. It is therefore necessary to further explore the precise nature of the connection between the nucleus and the cytoskeleton in order to get a better understanding of cellular mechanics. Lamins and in particular A-type lamins seem to play an important role in this connection and the nuclear mechanics.
Master Thesis
Introduction

Lamins are a group of proteins belonging to the family of intermediate filament (IF) proteins and are situated in the nucleus of the cell. They are believed to be the evolutionary progenitors of the cytoskeletal IF proteins. In the nucleus they form a cage-like network called the lamina. The lamina is situated on the nuclear side of the inner nuclear membrane (INM) [Dwyer and Blobel, 1976; Gerace et al., 1978]. There are two types of lamins in mammalian cells; the A-type lamins and the B-type lamins. The A-type lamins are encoded by a single gene (LMNA). Alternative splicing of the mRNA products of the LMNA gene yields at least four different A-type lamins, i.e. lamin A, lamin AΔ10, lamin C and lamin C2 [Fisher et al., 1986; McKeon et al., 1986; Riedel and Werner, 1989; Lin and Worman, 1993; Machiels et al., 1996]. The B-type lamins are encoded by two genes, i.e. the LMNB1 gene encoding lamin B1 [Hoger et al., 1988] and the LMNB2 gene encoding both lamin B2 and lamin B3 [Hoger et al., 1990; Furukawa and Hotta, 1993]. Unlike the B-type lamins, whose presence is required for the viability of most eukaryotic cells [Moir et al., 2000], the A-type lamins are differentially expressed and therefore not essential. Nevertheless, several diseases have been linked to mutations in the gene coding for A-type lamins. These diseases, called laminopathies, affect striated muscle, adipose tissue and the peripheral nervous system. Although A-type lamins are expressed in nearly all differentiated somatic cells, they exhibit different phenotypes and some of them are tissue specific. This can be attributed to the dual function of these proteins. Firstly, they form a network that has a mechanical function. Secondly, the association with transcription regulators also renders them important in regulating the expression levels of certain genes. However, it is unknown to what extent each of these properties contribute to the expressed phenotypes. The association of certain laminopathies with mechanically stressed tissue points towards a mechanical dysfunction in the affected tissues. Cells lacking lamin A/C have been shown to have a distorted nuclear shape and abnormal distribution of chromatin and several proteins, including B-type lamins and emerin [Sullivan et al., 1999; Raharjo et al., 2001; Nikolova et al., 2004]. Transfection of these cells with a point mutated lamin A/C gene, corresponding to a laminopathy, results in several observable defects, including an abnormal nuclear shape, in most of the cells [Raharjo et al., 2001]. Except for these nucleus associated abnormalities, the cytoskeletal actin distribution in cells, deficient in lamin A/C, has been reported to be abnormal as well [Broers et al., 2004a]. Besides these more indirect indications of altered mechanical properties of the nucleus, studies have also shown that the nuclei of WT cells are more resistant to deformation than the nuclei from lamin deficient cells [Lammerding et al., 2004]. Several studies also clearly indicate the importance of nuclear lamins in the resistance to mechanical strain of the whole cell [Broers et al., 2004a; Lammerding et al., 2004; Lammerding and Lee, 2005].
The emphasis in this report is therefore on the mechanical properties of lamin deficient cells and nuclei in particular. Broers et al. (2004a) demonstrated that the nuclei of lamin deficient cells deform isotropically during compression, while nuclei from WT cells deform anisotropically perpendicular to the long axis in cells with a prevalent growth orientation [Broers et al., 2004a] (figure 1). The attachment of the nucleus to the cytoskeleton was opted as a possible explanation for the observed difference. This, in light of the tensegrity structure, proposed by Donald Ingber to be the fundamental organization of the cytoskeleton, is a straightforward suggestion. Consequently, first an attempt was made to isolate nuclei from both WT and lamin deficient mouse embryonic fibroblasts (MEF+/+ and MEF-/- respectively) and determine if any difference in attached actin could be detected. This developed isolation procedure can then also be used to prepare nuclei, that can be subjected to mechanical strain applied by a cellular compression device [Peeters et al., 2003; Broers et al., 2004a]. For future studies of A-type lamin deficiency, a lentiviral system was tested for lamin A/C silencing, by means of a tetracycline mediated promoter, in transduced WT cells. 

In view of the mechanical abnormalities, observed in lamin deficient compared to WT cells, they were subjected to wound healing assays to test whether the functionality of the cytoskeleton is still intact.

Figure 1 Confocal images of compression events of a A) MEF +/+ and B) MEF -/- cell. The nucleus of the MEF +/+ cell shows anisotropic deformation (hatched ovals), compared to the long axis (straight line). This in contrast to the MEF -/- nucleus, which shows isotropic deformation. Images adapted from Broers et al., 2004b.
Materials and methods

Cell culture
Wild-type and LMNA knockout mouse embryonic fibroblasts (MEF+/+ and MEF-/- cells respectively) were obtained from Dr. Colin Stewart [Sullivan et al., 1999]. Human fibroblasts, wild-type cells (HF+/+) and LMNA deficient fibroblasts (HF-/-) were obtained from a patient with a homozygous nonsense LMNA mutation [Muchir et al., 2003]. These cells were maintained in Dulbecco’s Modification of Eagle’s Medium (DMEM) (ICN Biomedicals BV, Zoetermeer, The Netherlands) containing 10% FCS (Gibco Life Technologies Ltd., Paisley, UK), 2 mM L-Glutamine and 0.05 mg/ml Gentamicine. Chinese Hamster Ovary (CHO-K1) cells and HCT-116 human colon carcinoma cells were maintained in Ham’s F12 (ICN Biomedicals BV) and McCoy’s 5A (ICN Biomedicals BV) respectively, containing 10% FCS (Gibco Life Technologies Ltd), 2 mM L-Glutamine and 0.01% 50 ug/ul Gentamicine. Cells were passaged by splitting 1:3 to 1:6 ratios using a 0.125% trypsin/0.02 M EDTA/0.02% glucose solution in PBS.

Isolation of nuclei
Nuclei were isolated either with hypotonic/isotonic Triton X-100 (BDH, Poole, UK) solutions or mechanically using a Potter S homogenizer (B. Braun Biotech International, Melsungen, Germany). First, cells were washed with PBS and harvested using a 0.125% trypsin/0.02 M EDTA/0.02% glucose solution in PBS. Subsequently, cells were resuspended in the appropriate medium, centrifuged for 5 minutes at 300 xg and 4°C, supernatant was removed and nuclei were isolated for 10 minutes at 4°C in either a 0.07% Triton X-100, 3% BSA in AD solution or a 0.5% Triton X-100, 3% BSA in an isotonic (0.16 M) PBS solution. Alternatively, the cells were resuspended in DMEM and homogenized by means of applying 10 strokes at 900 RPM and 4°C in a 5 ml vessel with a vessel-plunger clearance of 45-65 µm. The nuclei were centrifuged for 5 minutes at 300 xg and 4°C, supernatant was removed and nuclei were resuspended in a solution of 3% BSA in PBS

Immunofluorescence studies
Unless otherwise indicated, cells were plated on glass coverslips, grown for 16 hours and fixed using a 4% formaldehyde solution in PBS for 15 minutes, followed by permeabilization in 0.1% Triton X-100 for 10 minutes at room temperature. Alternatively, cells were fixed in methanol (-20°C) for 5 minutes. The following primary antibodies were used and applied onto the cells for 60 minutes.

- Mouse monoclonal antibody (MoAb) BV1118 directed against vimentin (IgG1, dilution 1:10; kindly supplied by Dr C. Viebahn, Germany).
- Mouse polyclonal antibody Jol2 directed against lamin A/C (IgG1, dilution 1:50; kindly provided by Prof. C. Hutchison, Durham, UK).
- Affinity-purified rabbit polyclonal antiserum directed against lamin B1 (dilution 1:200; kindly provided by Dr. J.C. Courvalin, INSERM, Paris, France).
- Mouse MoAb E7 directed against tubulin (IgG1, dilution 1:10; Developmental Studies Hybridoma Bank, Iowa City, USA).
- Mouse MoAb AC-15 directed against β-actin (IgG1, dilution 1:1000; Sigma-Aldrich, St Louis, USA).
- Mouse MoAb 610153 directed against β-catenin (IgG1, dilution 1:500; BD Transduction Laboratories, Lexington, USA).
- Mouse MoAb C-1821 directed against pan-cadherin (IgG1, dilution 1:500; Sigma-Aldrich).

Secondary antibodies were applied for 60 minutes at room temperature after repeated (3x) washings in PBS. The secondary antibodies used were FITC-conjugated rabbit anti-mouse Ig (RAM-FITC) (1:100, DAKO, Glostrup, DK), FITC conjugated goat anti-rabbit Ig (GAR-FITC)
(1:100, SBA/ITK, Birmingham, USA) and FITC conjugated swine anti-rabbit Ig (SWAR-FITC) (1:100, DAKO). After washing the glass coverslips in the PBS, they were mounted in 90% glycerol, 0.02 M Tris-HCL pH 8.0, 0.8% NaNo₃ and 2% 1,4-di-azobicyclo-(2,2,2)-octane (DABCO; Merck, Darmstadt, Germany) containing 0.5 µg/ml diamidino-2-phenylindole (DAPI, Sigma) or 1 µg/ml PI and 0.1 mg/ml RNase for DNA staining. Alternatively, cells cultured on coverslips were fixed and permeabilized as described earlier and incubated with Texas Red-conjugated Phalloidin (dilution 1:50; Molecular Probes, Leiden, The Netherlands). In some instances nuclei were counterstained for 15 minutes with Syto-13 (1 µM; Molecular Probes), recognizing nucleic acids.

**Confocal laser scanning microscopy**

Confocal images were collected as described by Broers et al. [Broers et al., 1999] using a Bio-Rad MRC600 confocal unit (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK), equipped with an air-cooled Argon-Krypton mixed gas laser mounted onto a an Axiophote microscope (Zeiss, Jena, Germany), using oil-immersion objectives (40x and 63x). The laser scanning microscope was used in the dual parameter set-up, according to manufacturer’s specifications, using excitation at 488 nm and 568 nm. Emission spectra were separated by standard sets of dichroic mirrors and barrier filters. Optical sections were recorded in the Kalman filtering mode using 4-6 scans for each picture. Z-series were generated by collecting a stack consisting of 20-30 optical sections, using a step size of 0.18-0.36 mm in z-direction.

**Flow cytometry**

Cells were collected from culture flasks by trypsin treatment. Next, nuclei were isolated as described above, and fixed in methanol, stained immunocytochemically using the beta-actin antibody AC-15, the secondary antibody RAM-FITC and RNase + PI as previously described. The labeled cells and nuclei were analyzed using a FACSort flow cytometer and Cellquest analysis software (Becton Dickinson, Synnuvale, CA). An argon laser at 488 nm was used for excitation and the emission filters used were 515-545 nm (BP) for the FITC conjugated beta-actin signal, 572-588 BP for a combination of FITC and PI signal and a 600 nm LP for the PI signal. The obtained data was processed using WinMDI v2.8 (Freeware analysis software by Joseph Trotter).

**Cell Compression**

For the compression of isolated nuclei the device described and designed by Peeters et al. (2003) was used. The cell compression device was used on an inverted microscope (Axiovert 100M, Zeiss) with a confocal laser scanning unit (LSM 510, Zeiss). Nuclei were isolated in isotonic PBS + 3% BSA, by means of a potter homogenizer, followed by staining the nuclei with 1 µg/ml PI. The stained nuclei were put in the incubating chamber of the cell compression device, which is 3 mm deep and 25 mm in diameter. The nuclei were compressed using a glass indenter, with a flat tip (Ø ~50 µm), which was placed ~15 µm above the glass coverslip, leaving little room above the nuclei. The height of the tip, relative to the glass coverslip, was determined by means of a linescan in reflection mode. For this mode of operation the HeNe laser (543 nm) was used in combination with a 80/20 neutral density filter (NT 80/20) and an oil-immersed Plan-Apochromat 40/63 x objective. Before compressing the nucleus, a z-series was recorded by collecting a stack consisting of 30-60 optical sections, using a step size of 0.2 µm in z-direction. The HeNe laser was used for this purpose in combination with a main dichroic beam splitter (HFT 543) and a high pass filter (LP 560), eliminating the reflective signal and allowing the PI signal to pass through. Subsequently the nuclei were compressed at a speed of 1 µm/sec by means of the piezoelectric actuators. These compression events were visualized using the HeNe laser with the same filter sets as used for collecting the z-series. The focal plane was just above the coverslip, enabling to collect data from the most part of the compression event.
Gel electrophoresis and immunoblotting
Approximately 15 ml of 75·10^3 cells/ml (MEF+/+ and MEF-/-) were seeded in a 75 cm^2 culture flask 18 hours prior to harvesting. For control purposes cells were also seeded at the same density in a 12 well plate. The cells seeded in the 12 well plates were counted prior to harvesting by means of a coulter counter to ensure matching concentrations of cells for the two different cell types.

Pan-Cadherin and β-Catenin
Cells in the culture flask were washed with PBS and then resuspended in 200 µl of RIPA buffer (1% NP40/0.5% sodium deoxycholate/0.1% SDS/2mM CaCl_2/2% Protease Inhibitor cocktail BD Biosciences in PBS). Cells were then scraped off the culture flask surface and vortexed 3 times after 10 minute intervals at 4°C. Sample buffer (2.3% SDS/62.5 mM Tris-HCL pH 6.8/10% Glycerol/5% β-mercaptoethanol/0.05% BPB) was then added to the cell lysate in a 1:1 ratio and denatured for 5 minutes at 100°C. Samples were loaded onto a 7.5% SDS polyacrylamide gel and run for 40 minutes at 200V. The samples were transferred onto a nitrocellulose membrane for 60 minutes at 100V. The membrane was washed in PBS and blocked with blocking buffer consisting of 1% non-fat dry milk, 1% BSA, 0.05% tween-20 in PBS for 30 minutes. The primary antibodies, pan-cadherin c-1821 (Sigma) and β-catenin 610153 (BD Transduction Laboratories), were diluted 1:5000 and 1:500 in block buffer respectively and immunoblotted for 60 minutes at room temperature.

β-actin
Cells in the culture flask were washed with PBS and harvested using a 0.125% trypsin/0.02 M EDTA/0.02% glucose solution in PBS. The cells were resuspended in culture medium and centrifuged for 5 minutes at 300 xg and 4°C. Supernatant was removed and the cells were resuspended for 10 minutes at 4°C in either a 0.07% Triton X-100, 3% BSA solution in AD or a 0.5% Triton X-100, 3% BSA solution in PBS. The nuclear/cytoskeletal isolation procedure was stopped by a 10 fold dilution of the buffers with 3% BSA in PBS. The samples were then centrifuged again at 300 xg and 4°C for 5 minutes. The supernatant was then removed and the samples were resuspended in the previously described RIPA buffer. Then the same protocol was used as described for pan-cadherin and β-catenin only with the first antibody being the β-actin from Sigma at a 1:5000 dilution.

Thereafter, the membranes were washed three times for 10 minutes in a 0.05% Tween-20 in PBS and incubated with RAMPO diluted 1:10.000 in block buffer for 60 minutes at room temperature. At the end of this incubation the membrane was washed again three times with 0.05% Tween-20 in PBS and incubated with Amersham enhanced chemiluminesence (ECL) reagents for 1 minute. Excess ECL reagent was removed and the membrane was sealed in plastic wrap and exposed to röntgen film for different time intervals ranging from 10 seconds to 5 minutes in a dark room.

Wound healing
Wound healing assays were performed on MEF+/+ and MEF-/- cells seeded at 100% confluency on coverslips. The cells were allowed to attach to the surface for at least 16 hours before wounding. The wounding was done by scratching the surface of the coverslips with either a metal needle, glass needle constructed from a Pasteur pipette or a razorblade. The samples were then analyzed over a period of up to 8 hours using fluorescence microscopy (fixation at different time intervals) or vital microscopy.

Vital microscopy
Wound healing experiments were visualized by vital imaging on an inverted automated microscope (Leica DMRBE, Mannheim, Germany) in phase contrast illumination mode. The samples (coverslips) were grown in an incubation chamber at 37 °C/ 20% CO_2 in culture medium and images were captured at 1 minute intervals for a period of 8 hours.
Fluorescence microscopy
Samples were visualized on either a Zeiss Axioskop (Zeiss) or a Leica DMRBE (Leica) at 40x magnification using appropriate filter sets.

Construction of N-terminal actin-EGFP vector
The N-terminal actin-EGFP vector (actin-EGFP-N3) was constructed using a PCR actin fragment from a previously created C-terminal actin-EGFP vector (actin-EGFP-C1). The actin fragment from the previously constructed pEGFP-C1 vector (BD Biosciences Clontech) and linearized pEGFP-N3 vector (BD Biosciences Clontech) were obtained using BamHI (Invitrogen, Carlsbad, USA) and Hind III (Invitrogen) restriction enzymes. Restriction enzyme digestion was performed for 2 hours at 37°C in a 1x react 3 (Invitrogen) buffer. Samples were loaded using orange juice into a 1.25 % agarose gel in 1x TAE buffer, followed by 30 minutes of gel electrophoresis at 100V. Bands were cut according to the desired fragment length using a 100 bp ladder. Subsequently the fragments were isolated using the Qiaquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions. The extracted actin and pEGFP-N3 (BD Biosciences Clontech) were then ligated for 16 hours at 12°C using T4 DNA ligase in 1X ligase buffer. Ligation products were then confirmed using gel electrophoresis. The vector was then transformed into DH5α cells by allowing 30 minutes of incubation at 4°C followed by a 2 minute heat shock at 42°C. Cells were then allowed to grow in Luria Bertani (LB) medium for 75 minutes at 37°C in a shaking incubator. Colonies were then grown on LB-agar plates with kanamycin added for 16 hours at 37°C. Subsequently the colonies were picked and grown in LB medium with kanamycin for 16 hours at 37°C in a shaking incubator. The amplified vector was then extracted using the Wizard Plus SV Miniprep DNA Purification System (Promega, Madison, USA) according to manufacturer’s instructions. The extracted vectors were then digested using BamHI (Invitrogen) and Hind III (Invitrogen) in 1x react 3 (Invitrogen) buffer or Dra III in NE buffer 3 (New England Biolabs Inc, Beverly, USA) and subsequently loaded with orange juice on a 1.25% agarose gel in 1X TAE buffer to confirm the correct vector was produced.

Transfection
In order to study the actin filaments displacement during compressional studies, MEF+/+, MEF-/− and CHO cells were transfected with a C- and N-terminal actin-EGFP construct and a C-terminal actin-GFP construct. The cells were transfected at 50-60% confluency using jetPEI (Qbiogene) according to manufacturer’s instructions.

Transduction
For silencing lamin A/C expression in HF+/+ and HCT-116 cells, the pLenti4-GW/H1/TO-laminshRNA of the BLOCK-iT Inducible H1 Lentiviral RNAi System (Invitrogen) was used. The lentiviral particles, both for expressing the tetracycline repressor (pLenti6/TR) and the lamin short hairpin RNA (pLenti4-GW/H1/TO-laminshRNA), were produced by transfecting the supplied human 293FT cell line according to manufacturer’s instructions in serum free DMEM (ICN Biomedicals BV). The lentiviral particles were harvested and stored according to manufacturer’s instructions. The HF+/+ cells were tested for blasticidin and zeocin resistance according to manufacturer’s instructions. The required concentrations for blasticidin (pLenti6/TR) and zeocin (pLenti4-GW/H1/TO-laminshRNA) selection were established at 4 µg/ml and 400 µg/ml for HF +/- cells respectively. Cells were transduced at 10-fold dilutions according to manufacturer’s instructions without determining the exact titer of the lentiviral stock. The titer was presumed to be about the same as indicated by the manufacturer. HF +/- cells, previously transduced with the highest concentration of lentiviral product, were transduced a second time with undiluted lentiviral stock at 2000 xg at 18°C during 90 minutes.
Results

Structural and biochemical studies

Association of the actin cytoskeleton to the nucleus

As described in the introduction, the nuclei of MEF +/+ and MEF -/- deform differently under compressional forces [Broers et al., 2004a]. The former deform anisotropically, while the latter deforms isotropically. It was hypothesized that this altered nuclear deformation could be the result of the association of actin to the nucleus, which has been shown to have an altered organization (figure 2). To further explore the difference in deformation between MEF +/+ and MEF -/- nuclei when cells were being compressed, nuclear isolation procedures for the MEF+/+ and MEF-/- cell cultures were developed. This would enable us to not only determine the mechanical properties of the nucleus on its own, but also the association of the cytoskeleton to the nucleus and in particular the role of A-type lamins. The nuclear isolation procedures were evaluated using conventional phase contrast microscopy. This resulted into two optimal isolation procedures in either an isotonic or a hypotonic environment. Thirdly a method was evaluated using a potter homogenizer to mechanically isolate the nuclei out of the cell.

Figure 2 Confocal image showing actin organization of A) MEF +/+ and B) MEF -/- cells. Cells were stained with Phalloidin-Texas Red (red) and counterstained with Syto-13 (green). Note the difference in the actin organization around the nuclei. In MEF +/+ stress fibers are uninterrupted near the nucleus, while there is a granular organization around the MEF -/- nucleus. (Courtesy of Jos Broers)

The first two isolation procedures were also visualized using vital microscopy. This revealed a difference between the two cell types and these two nuclear isolation procedures. While more MEF-/- nuclei could be isolated, as judged from visual observations, the MEF+/+ nuclei clearly had more residual cytoplasm/ cytoskeleton associated to the nuclear membrane than the MEF-/- nuclei. In addition the nuclei of the MEF-/- cells, isolated by means of a hypotonic solution, expanded to a larger volume than the MEF+/+ nuclei (figure 3).
The hypotonic isolation procedures also revealed that MEF-/- nuclei were not as rigid as the MEF+/+ nuclei. Namely, after nuclear isolation the nuclei were transferred to a PBS buffer, which resulted in the MEF-/- nuclei imploding under the stress caused by the osmotic forces while the MEF+/+ nuclei remained intact. However, for further analysis this could be avoided by allowing the nuclei to adapt more gradually to the isotonic environment.

The residual cytoplasm/cytoskeleton was analyzed by means of fluorescence microscopy, though this proved to be difficult to quantify (figure 4).

Therefore, cells and isolated nuclei from both MEF+/+ and MEF-/- were stained with a beta actin antibody and PI and analyzed by means of flow cytometry. Initially the yield of isolated nuclei was very low (~ 5-10 %), which interfered with determining a mean fluorescence intensity with an acceptable standard error. The low yield could be attributed to the fragility of the nuclei and their adhesive affinity for the test tubes. The addition of 3% BSA to the mixtures largely eliminated the low yield problem by raising it to 50-100 %. The procedure, however, did prove not to be reproducible and to be prone to large standard errors. This could be attributed to the high number of cells/nuclei needed for this approach in combination with immunocytochemical staining. Namely, when using these high concentrations of cells/nuclei, the antibodies would be depleted. This resulted in an unstable antibody concentration and as a consequence, a fluctuating fluorescence intensity, as a function of the yield of cells/nuclei. This effect can be observed in figure 5, a 4-fold increase in the amount of cells led to a 3 fold decrease in fluorescence intensity. It was not possible to compensate for these fluctuations by counting the cells prior to isolation and staining, because the yield fluctuated during each step.
These difficulties could, however, be overcome by Western blot analysis. Using this method the amount of nuclei could be measured prior to lysis. After isolating and lysing the nuclei, all the protein stays within the same solution, thereby eliminating the concentration issue. The Western blot analysis confirmed the hypothesis, that more actin was associated to the MEF+/- nuclei than the MEF-/- nuclei, for either the hypotonic or isotonic isolation procedure (Figure 6). It even revealed that there was more actin associated to the nuclei isolated by means of a hypotonic solution than an isotonic solution, showing the former was a more drastic isolation method. For this Western blot analysis the same amount of protein was loaded, as can be seen in the coomassie stain.
Mechanical Studies

Construction and application of EGFP-N3 actin

As mentioned before, the deformation of the nuclei of MEF+/- cells under compression is anisotropic, while the MEF-/- nuclei show an isotropic deformation. In the previous section we have shown that the cytoskeleton is not bound as tightly to the nucleus in the MEF-/- cells as in the MEF+/- cells. Although this difference could explain the deformation of the nuclei, it does not prove that this association is responsible for the difference in the deformation. To directly visualize this relationship, actin-egfp/gfp plasmid DNA constructs were used. Since these constructs, C-terminal GFP and EGFP actin vectors, did not show clear stress fibers when transfected into CHO-K1 cells (Broers, personal communication) we set out to make a construct with its EGFP tag at the opposite side of the actin molecule, a N-terminal EGFP-actin construct. For this purpose a previously constructed actin per product was cut from a pEGFP-C1 vector and ligated into the multi-cloning site (MCS) of a pEGFP-N3 vector (see Appendix). After verification of the accurateness of the new EGFP-N3 actin constructs, CHO-K1 cells were stably transfected with C-terminal tagged GFP and EGFP-actin constructs and the N-terminal tagged EGFP-actin construct. Confocal microscopy revealed that the fluorescence signal in transfected cells was too diffuse to get a detailed picture of the attachment of stress fibers to the nucleus. Also photo bleaching the unpolymerized globular actin was of no avail (figure 7). While the diffuse actin fluorescence signal disappeared, no additional stress fibers became visible. It is therefore not possible to track enough individual actin stress fibers during compression events to determine their behavior in relation to nuclear deformation.

Figure 7 Confocal images of CHO cells transfected with EGFP-C1 actin. A) Image showing diffuse signal of unpolymerized actin in cytoplasm. B) This cell was bleached subsequently, which did reveal a reduction in the diffuse signal, however the remaining signal is not detailed enough to track the displacement of individual actin stress fibers during compression events.
Involvement A-type lamins in anisotropic behavior

Although it was not possible to track the individual actin stress fibers during compression, in order to assess their contribution in the anisotropic behavior of the MEF+/+ nuclei compared to the isotropic behavior of the MEF-/- nuclei, it still needed to be validated whether this behavior was not caused by the compromised nucleoskeleton in MEF-/- cells.

For this purpose nuclei, isolated by means of a Potter homogenizer, were compressed using the cellular compression device described by Boers et al. (2004) (figure 8).

![Figure 8 A) Schematic representation and B) picture of cellular compression device. Indentor can be placed on top of a single cell or nucleus by means of the micromanipulators and then compressed by means of the piezoelectric system](image)

The compression events were visualized on a confocal microscope, which allowed the nuclear deformation to be followed. This revealed that the width/length ratio of 5 correctly compressed MEF+/+ and MEF-/- nuclei was on average 0.74 and 0.85 respectively. This ratio changed 5.2% (standard deviation 3.4%) for the MEF+/+ nuclei and 3.9% (standard deviation 3.1%) for the MEF-/- nuclei during compression. Compared to the nucleus deformations observed by Broers et al. (2004a) when compressing cells, it can be concluded that this deformation for both the MEF+/+ and MEF-/- nuclei is isotropic (figure 9). Namely, the average change of width/length ratio for nuclei in that study was reported to be 40% (anisotropic) for MEF+/+ cells and 8-11% (isotropic) for MEF-/- cells.

The anisotropic versus isotropic deformation can therefore not be attributed to the nuclear lamina directly and has to be the result of external forces acting on the nucleus in case of cellular compression, which points to a link between the nuclear lamina and the cytoskeleton. Combined with the fact more actin is associated to the MEF +/- nuclei, when they are isolated, make it very plausible the actin stress fibers are directly involved in the anisotropic behavior exhibited by MEF +/- nuclei when intact cells are compressed.
Mechanics of MEF+/+ versus MEF-/- nuclei

The mechanical behavior, of the MEF+/+ and MEF-/- nuclei under compression, was determined by compressing the nuclei, by means of the previously described cellular compression device. The stress-strain curves (figure 10) of these compression events were, however, inconclusive. This was due to several factors. In retrospect, the distance between the indenter and the coverslip needs to be determined more accurately, in order to establish the correct strain in z-direction. This can be achieved by means of using a smaller pinhole setting in reflection mode. In addition, the small amount of correctly compressed nuclei and possibly the selection that already preceded the compression event, interfered with establishing a conclusive dataset. The isolation procedure namely already selects the more rigid nuclei. This selection was noticeably more prominent for the MEF-/- nuclei, since it proved hard to locate intact nuclei in these samples and in some instances they proved to be more rigid than MEF+/+ nuclei. Therefore it is required to obtain a more substantial dataset than acquired till present.

The small amount of correctly compressed nuclei could be attributed to several different unforeseen difficulties. The first compressions were performed with a Syto 13 staining. This provided a relatively good resolution for cellular compression, although for nuclear compression this resolution was too poor to determine the correct nucleus height. Staining the nuclei with PI provided a much better resolution. Setting up the microscope for the different tasks, determining the distance of the tip to the coverslip, imaging the compression events and operating the compression device, were some additional initial difficulties.

A vibration in the set-up was another unforeseen inconvenience, which was encountered on several occasions. This vibration was pinpointed to be due to the wiring, of the compression device, carrying environmental vibrations to the set-up. This also brought to light that the set-up was not rigid, the compression device was able to move relative to the frame it was attached to. These and other difficulties interfered in the acquisition of consistent data. However, since most of these obstacles were overcome, it should be able to get a consistent data set in future.

**Figure 9** Confocal images of nuclei during compression events. A) MEF+/+ nucleus before compression and B) MEF+/+ nucleus during compression clearly shows an isotropic deformation. MEF-/- nucleus (C,D) exhibits the same behavior as MEF+/+ nucleus.
What could be observed in 2 of the 6 correctly compressed MEF-/- nuclei, was a burst of the nucleus (figure 11). This occurred at 42 µN in one instance and 47 µN in the other. This behavior could not be detected in any of the 7 correctly compressed MEF+/+ nuclei, which shows that these nuclei cope better with compressional forces. This result also shows that the nuclei seem stiffer than the MEF+/+ and MEF-/- cells, which have been shown to burst at ~6 µN and ~2 µN respectively [Broers et al., 2004a]. This considerable difference was unexpected and preliminary compression data from HF+/+ and HF-/- cells, show that the calibration of the force transducer needs to be validated. However, it should also be noted that fluids and solutes can more easily cross the nuclear membrane through the nuclear pore complexes than the cellular membrane and that the cytoskeleton tethered to the nuclear and cellular membrane might play a role in cellular rupture.

![Force versus axial strain curve of isolated MEF+/+ (red) and MEF-/- (blue) nuclei.](image1)

![Force versus axial strain curve of isolated MEF-/- nucleus showing nuclear bursting force (arrow).](image2)

**Figure 10** Force versus axial strain curve of isolated MEF+/+ (red) and MEF-/- (blue) nuclei.

**Figure 11** Force versus axial strain curve of isolated MEF-/- nucleus showing nuclear bursting force (arrow).
Functional Studies

Wound healing

Besides the mechanical behavior of cells and nuclei, which preferably only takes a statically arranged cytoskeleton into account, there are several techniques to observe the dynamic rearrangement properties of the cytoskeleton. One of these techniques is wound healing. Wound healing relies on cellular division and directed cellular movement. These actions require extensive cytoskeletal reorganization. This assay is therefore very suitable to study cytoskeletal dysfunctioning. Since the actin-nuclear interaction is disturbed in MEF-/- cells compared to MEF+/+ cells, there was reason to assume that MEF-/- cells would show a disordered wound healing. Firstly, wounding experiments were done to assess the efficiency of three different techniques. The most consistent wound area combined with least amount of damage to cells or glass lining the wound was created by means of scratching the coverslip surface with the tip of a bluntly flamed glass Pasteur pipette.

The wounds, created by means of a Pasteur pipette, were assessed at 2 hour intervals (0-6 hours) following wounding by centrosome, vimentin, tubulin and actin staining. There was, however, no apparent difference between the MEF+/+ and MEF-/- cells, both cell types show lamellipodia, filopodia and membrane ruffles (figure 12). The only dissimilarity that can be observed is an unexpected faster wound healing for the MEF-/- cells (compare figure 12b and 12d).

To further explore underlying mechanisms and find explanations for this difference, wound healing assays were live recorded on an inverted automated microscope. This allows cellular movement to be tracked at 1 minute intervals over periods of 8 hours. These recordings revealed that the faster wound healing in MEF-/- was largely the result of a higher proliferation rate, a higher cell density and a faster initial response. This higher proliferation rate of MEF-/- cells could, however, also be observed during normal cell culture and could therefore not directly be attributed to the wound healing properties of this cell line. The same can be said for the higher density, which is an artifact of this property. Compensating for the faster growth rate, by means of seeding at lower density, still showed an increased speed of wound closure in MEF-/- cells. This is due to the fact that the MEF-/- cells adhere more tightly to the neighboring cells than MEF+/+ cells, leading to an accumulation of cells lining the wound. This property of MEF-/- cells could also be observed when the cells were passaged.

Figure 12 Pictures of immunohistochemical staining of β-actin in MEF-/- cells A) 2 hours and B) 4 hours, MEF+/+ cells C) 2 hours and D) 4 hours after wounding.
Figure 13 Frames of wound healing assays performed on a vital microscope. Frames were taken at 1 minute intervals and revealed several distinct reorganization and migration types, which can be divided into 8 categories. These categories are formed following cell migration direction (yellow) and nucleus orientation (green), tracked by means of nucleoli positioning. These 8 categories are formed by:

- A, B) The nucleus rotates initially.
- C, D) The nucleus rotates in accordance with changing migration direction.
- E, F) The nucleus orientation stays the same while cell migrates unidirectionally.
- G, H) The cell spreads and nucleus is static.
- I, J) The cell spreads and the nucleus rotates.
- K, L) The cell changes migration direction and the nucleus rotates in another direction.
- M, N) The cell changes migration direction and the nucleus orientation stays the same.
- O, P) The nucleus rotates while the cell moves unidirectionally.
On the other hand, several differences between MEF+/+ and MEF−/− cells in response to wounding were observed, that can be attributed to altered dynamic behavior of the cytoskeleton. First the difference in initial response was striking. About 20% of the MEF+/+ cells lining the wound responded by turning the nuclei, supposedly a directional reorganization, while only 2% of the MEF−/− cells displayed this behavior. Additionally, 37% of the nuclei of MEF+/+ cells turned according to changes in displacement direction whereas only 9% of the MEF−/− nuclei exhibit this conduct. The different categories of cellular and nuclear rearrangements are summarized in figure 13 and the fraction of cells in which these can be observed during wound healing assays, for the two cell types, is depicted in figure 14.

The data shown in figure 14, were subdivided into two groups called: directed nuclear rearrangement and random actions. The first 4 categories in the legend (figure 14) belong to the group of directed nuclear rearrangement, while the latter 4 categories were labeled random actions. A student T-test, for independent sample surveys, revealed the null hypothesis, MEF+/+ and MEF−/− exhibit the same directed nuclear rearrangement, could be rejected with 99% certainty. The pooled data are shown in figure 15. Overall 79% of the MEF+/+ cells exhibit directed nuclear rearrangement, whereas this was true for just 32% of the MEF−/− cells.

The apparent need for the MEF+/+ cells to reorient the nucleus and possibly several other cellular components explains the slower initial migration response observed in MEF+/+ cells. This results in faster wound healing in MEF−/− cells for these relatively small wounds. Though, for larger wound areas this difference in initial response could lead to slower wound closure and even impaired wound healing.

Vital imaging revealed the MEF−/− cells show another property, not exhibited by the MEF+/+ cells. During wound healing and cellular movement a great number of vesicles appear at the periphery of the cell at the side of the wound, which travel towards the nucleus (figure 16).
Due to the greater adhesion of the MEF-/- cells to the surrounding cells, it was hypothesized that these vesicles could be used for the trafficking of adhesion molecules, such as N-cadherin, a calcium dependent cell adhesion protein. This is the most significantly expressed cadherin in MEF cells [Guo et al., 2005]. It is well established that its function depends on the association with the actin containing cytoskeleton [Nagafuchi and Takeichi, 1988; Kintner, 1992; Fujimori and Takeichi, 1993], which is mediated by α- and β- or γ-catenin. β-catenin binds to the cytoplasmic domain of cadherin and interacts with α-catenin, which in turn binds to actin either directly, or through α-actinin [Knudsen et al., 1995; Rimm et al., 1995]. This association with the actin containing cytoskeleton and the reported difference in association of actin with the nucleus for MEF+/+ and MEF-/- cells, could also explain the difference seen in nucleus reorientation seen in wound healing.

Immunohistochemical staining of these cells, with a pan-cadherin antibody, revealed no significant difference in the number of cadherin-positive vesicles between MEF+/+ and MEF-/- cells (figure 17). Nonetheless, the overall fluorescence intensity was significantly greater in MEF-/- cells, possibly explaining their greater adhesion.

**Figure 16** During vital imaging, vesicles could be observed moving from the periphery of the cell towards the nucleus (arrows). This property can almost exclusively be observed in MEF -/- cells during wound healing assays.

**Figure 17** Z-projection of confocal image sequence of A) MEF+/+ and B) MEF-/- cells stained with PI (red) and pan-cadherin (green), shows vesicles in both cell types are N-cadherin positive and that the overall N-Cadherin expression is higher in MEF-/- cells.
Western blotting analysis of total cell lysate was used to confirm the difference in protein levels. This also revealed a decrease in the cadherin protein level of the MEF+/- cells (figure 18). Also, a Western blot analysis was performed for β-catenin, because of its close relationship with cadherin. As for cadherin, the protein levels of β-catenin, is also upregulated in MEF-/- cells. Besides being involved in actin binding, β-catenin is the central and essential component in the Wnt signaling pathway [Brembeck et al., 2006]. This signaling pathway is one of the regulators of cell proliferation, motility, differentiation and also cell-cell interactions. This could therefore explain the difference in these properties, expressed by the MEF -/- cells.

**Preliminary study for future research**

* A-type lamin silencing

For examining the mechanical and other characteristics of cells and nuclei, one would ideally want to use a single cell line, in which the expression of the desired protein can be regulated. For this purpose HF+/- cells were transduced with an inducible lentiviral transduction kit, the pLenti4-GW/H1/TO-laminABRNA of the BLOCK-iT Inducible H1 Lentiviral RNAi System (Invitrogen). A lentiviral transduction approach was chosen, because regular transfection, using liposomes, will cause the fibroblasts to go into senescence.

The transduction process was performed using several concentrations of viral particles. Fluorescence microscopy, however, showed that lamin A/C expression was not downregulated, despite their zeocin resistance (data not shown). Subsequent transduction improvement steps, including centrifugation, were performed, but did not improve the lamin silencing. Because a different promoter sequence was used for the expression of laminABRNA than the zeocin resistance, the problem could possibly be traced back to an inherently low activity of the former promoter sequence in the chosen cell line. To test this hypothesis a different cell line, HCT-116, was transduced. As can be seen in figure 19 lamin A/C expression was prominently downregulated in this cell line.
Discussion and recommendations

Broers et al. (2004a) hypothesized that a connection between the A-type lamins and the actin cytoskeleton would explain the isotropic versus the anisotropic deformation of the nucleus, observed when compressing A-type lamin deficient cells and wild-type cells, respectively. Another observation was that the A-type lamin deficient cells were more fragile and less resistant to deformation than the wild-type cells. The goal of this study was to substantiate this hypothesis, by assessing whether a physical connection between the actin cytoskeleton and the nucleus exists, in particular the connection of actin to nuclear A-type lamins, and whether this connection tethers the nucleus to its surroundings.

For this purpose, nuclear isolation procedures were developed. These procedures encompass two isolation procedures, hypotonic and isotonic, involving a non-ionic detergent, used for solubilizing membranes. The other procedure utilizes a Potter homogenizer to mechanically tease the nuclei out of the cells. Phase contrast vital microscopy used for evaluating the first two procedures, did reveal there were residues attached to the nuclei of wild-type cells, while this was mostly absent in isolated lamin A/C deficient nuclei. The hypotonic isolation also showed that, due to osmotic forces, lamin A/C deficient nuclei swelled more than the wild-type nuclei. When these nuclei were subsequently put in an isotonic environment, the lamin A/C deficient nuclei collapsed and were lost, due to the inability to shrink without rupture. These first findings are the first indicators that the A-type lamins indeed give structural support to the nucleus and that they provide a connection to the surroundings of the nucleus. Characterization of the attached residues, however, proved difficult. Quantification of the attached actin, by means of immunolabeling of β-actin in combination with fluorescence microscopy, was not possible and using this procedure combined with flow cytometry did not generate reproducible data either. This was largely due to the fragility of the isolated nuclei. Most nuclei were namely lost during washing steps in between staining procedures. The adhesion of the nuclei to the plastic of the test tubes also contributed to the variation in the data. This could, however, be reduced by the addition of BSA to the isolation solution and the solutions used in the washing steps. The data, however, proved to remain irreproducible. Therefore Western blot analysis was performed, which did not have the drawbacks of washing steps and was not complicated due to the adhesive properties of the nuclei. These Western blot analyses revealed that there was indeed more actin associated to the nuclei of wild-type cells than of A-type lamin deficient cells and even that some more actin was associated to the nuclei isolated by means of the isotonic solution. This was an expected result, as the isotonic isolation is believed to be mechanically less abrasive. These results also substantiate the hypothesis, that actin association to the nucleus is dependent on the presence of A-type lamins. However, it does not prove that this connection is responsible for the anisotropic behavior of nuclei seen during compression events of wild-type cells. For this purpose a preliminary study was used, to examine if individual actin stress fibers, in cells transfected with an actin-EGFP construct, could be visualized detailed enough to track them during compression events. The fluorescent signal of actin visualized on a confocal microscope was, however, too diffuse to track individual stress fibers during compression. Reducing the diffuse actin signal in the cytoplasm, by means of bleaching the cells, did not improve the level of detail enough to be able to track the stress fibers during compression events. Nevertheless, the compression of isolated nuclei did reveal that both wild-type and lamin A/C deficient nuclei deform isotropically. Combined with previous observations, this indicates that the nucleus, of wild-type cells, in its regular state is tethered to the cytoskeleton and thus the plasma membrane in order to deform anisotropically. Unfortunately, the compression of the isolated nuclei did not reveal to what extent A-type lamins give structural support to the nucleus. Nonetheless, it did show that the nuclei of the lamin A/C deficient cells, unlike the wild-type nuclei, ruptured under compression. This finding underlines the importance of A-type lamins in nucleus mechanics and confirms the results observed during nucleus isolation, by means of a hypotonic solution. In
fact, recently Lammerding et al. (2006) has shown by other mechanical studies that indeed A-type lamins are critical for nuclear stiffness. Another part of this study, focused on the dynamic behavior of the cytoskeleton, by means of performing wound healing assays. This functional study assesses the structural rearranging properties of the cytoskeleton, which are indicative for the cellular response to stress. Initially these studies were carried out on a fluorescence microscope, by staining different cytoskeletal components like actin, tubulin and vimentin at several time intervals. This revealed that there was no obvious difference in cytoskeletal rearrangement during the wound healing of lamin A/C deficient cells compared to the wild-type cells. The most prominent difference was the faster wound healing of the lamin A/C deficient cells. Vital microscopy showed that this was a result of the difference in initial response. While the wild-type cells reacted to the wounding by rearranging their nucleus orientation, an indication of cytoskeletal rearrangement, the lamin A/C deficient cells almost instantly initiated migration and cell spreading, without this reorganization. Furthermore, the nuclear orientation was correlated to the migration direction in wild-type cells, while this property was significantly less present in lamin A/C deficient cells. These findings substantiate the previous evidence that the A-type lamins are, at least in part, responsible for the anchorage of the nucleus to the cytoskeleton.

For future research, a preliminary study was performed on the knockdown of lamin A/C in wild-type cells, by means of transduction with an inducible siRNA system. This enables regulated knockdown of A-type lamins. Thereby the effect of the presence or absence of a single protein, such as lamin a/c, can be studied in the same cell line by regulating their expression with tetracyclin. The vectors of this siRNA system are packaged in a lentivirus, which enables the transfection of the fibroblasts, without going into senescence. Fluorescence microscopy showed that the knockdown of lamin A/C in a human fibroblast cell line is not yet possible. Transfection of a different human cell line showed the validity of the system and did result in the knockdown of lamin A/C expression. For this reason it was hypothetically the promoter sequence, used in the vector coding for the short hairpin RNA (shRNA), which was not suitable for the use in the human fibroblasts.

**Recommendations**

Even though the results substantiate the hypothesis, that A-type lamins serve a purpose in linking the actin cytoskeleton to the nucleus, additional studies are required. For instance, wild-type cells can be compressed after being treated with agents, like Cytochalasin D, that break down the actin cytoskeleton. If the hypothesis is correct, this should result in isotropic deformation of the nuclei in wild-type cells. However, complications with using drugs for such a purpose, are caused by the fact that most drugs do not only effect the cytoskeleton of cells, but can also influence other functions.

Additional nucleus compression needs to be performed, in order to assess the mechanics of the nucleus and the role of the nuclear lamins in nuclear mechanics. Although most difficulties were overcome, data analysis revealed that the distance between the indenter and the coverslip needs to be determined more accurately, since this is crucial for determining the correct axial strain. This can be achieved by means of using a smaller pinhole diameter in reflection mode. Since chromatin could complicate the compressional studies, it also needs to be validated if these studies should be performed on swollen nuclei in stead of nuclei suspended in an isotonic environment.

Western blot analysis revealed that more actin is associated to wild-type nuclei, however, it also needs to be confirmed whether other cytoskeletal components are associated to these nuclei and to which extent compared to the lamin A/C deficient cells. Preferably these studies would be carried out on cell lines transduced with the inducible siRNA system. This would also enable the use of different cell lines, which can more easily be stably transfected with an EGFP labeled cytoskeletal protein, for the use in vital imaging studies, such as wound healing, shear stress, cell spreading and chemotaxis.
Appendix

A previously constructed actin pcr product was cut from a pEGFP-C1 vector (figure 20a), using restriction sites for BamH I and Hind III in the multi-cloning site (MCS) (figure 20c). The pEGFP-N3 vector was linearized using the same restriction sites. The products, corresponding to the predicted length, were then cut and isolated from the agarose gel. Subsequently the isolated constructs were double checked by running them on an agarose gel (figure 21). The resulting fragment lengths corresponded to the predicted length, pEGFP-N3 (4700 bp) and actin (1200 bp).

The two fragments, pEGFP-N3 and actin pcr product, were ligated in volume ratios 1:1 and 1:4 respectively. The products were run on an agarose gel to assess the result of the ligation process, which revealed a good ligation procedure for both the 1:1 and the 1:4 mixtures.
Subsequently, DH5α cells were transformed with pEGFP-N1 (+ control), linearized pEGFP-N3 (-control), the two products from the ligation process and grown for 45’ on LB medium prior to seeding on LB agar with kanamycin. Culturing the DH5α cells overnight resulted in several colonies for the positive control only. Therefore the DH5α cells were transformed again and the culturing without kanamycin was prolonged by 30 minutes, ensuring enough copies of the plasmid DNA were present and expression of the kanamycin resistance gene was high enough. Cells were grown on LB agar plates, pEGFP-N2 (+ control), no plasmid DNA (-control), the two products from the ligation process, with kanamycin at the prescribed concentration or at half this concentration. This resulted in colonies for all plates except the negative control. Several colonies were picked, grown overnight, followed by DNA extraction. The extracted DNA was verified by cutting with several restriction enzymes, BamHI and HindIII or DraIII. The resulting fragment lengths corresponded to the predicted fragment lengths, 1133/4691 and 180/1849/3795 respectively (figure 22 and 23).

**Figure 22** Map showing restriction sites of pEGFP-N3 actin vector, used to confirm the amplified plasmid DNA construct

**Figure 23** A) Digestion with BanH1 and HindIII resulted in correct fragment lengths. B) Fragments resulting from digestion with DraIII, also show correct plasmid was formed.
Acknowledgements

I would like to thank Jos Broers for interesting me in the field of molecular cell biology through his lectures and giving me the opportunity to expand my knowledge in this field of scientific research during my internship.

I would also like to take this opportunity to thank Mieke Henfling, Helma Kuijpers and Jorike Endert, for all the help they gave me in getting to know all the required laboratory techniques and Frederik Houben for his assistance in the compression studies.

The people accompanying me during the daily short breaks, Bert Delvoux and Lenneke Prinzen deserve my gratitude for their pleasant company, as is the case for the rest of the department of molecular cell biology (students, PhD students and staff).
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