Remodelling of the nuclear lamina and nucleoskeleton is required for skeletal muscle differentiation in vitro

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Summary

Changes in the expression and distribution of nuclear lamins were investigated during C2C12 myoblast differentiation. The expression of most lamins was unchanged during myogenesis. By contrast, lamin-B_2 expression increased and LAP2α expression decreased twofold. These changes were correlated with reduced solubility and redistribution of A-type lamins. When C2C12 myoblasts were transfected with a lamin-A mutant that causes autosomal dominant Emery-Dreifuss muscular dystrophy (AD-EDMD), the mutant protein accumulated in the nucleoplasm and exerted dominant influences over endogenous lamins. Myoblasts transfected with wild-type lamins differentiated, albeit more slowly, whereas myoblasts transfected with mutant lamins failed to differentiate. Myoblast differentiation requires dephosphorylation of the retinoblastoma protein Rb. During myogenesis, Rb was rapidly and progressively dephosphorylated. Underphosphorylated Rb formed complexes with LAP2α in proliferating myoblasts and postmitotic myoblasts. In myoblasts transfected with the mutant lamins, this complex was disrupted. These data suggest that remodelling of the nucleoskeleton is necessary for skeletal-muscle differentiation and for correct regulation of Rb pathways.

Key words: Lamins, Laminopathy, Myogenesis, LAP2α, Nucleoskeleton

Introduction

The nuclear lamina is a filamentous lattice that underlies the inner membrane of the nuclear envelope (NE) and provides essential mechanical support for the nucleus. In metazoan organisms, the lamina is primarily composed of type-V intermediate-filament proteins, the lamins. In higher vertebrates, lamins are divided into two subfamilies, representing seven proteins (for review, see Hutchison, 2002). The B-type lamins are housekeeping proteins that are essential for cell survival (Harborth et al., 2001). The three vertebrate B-type lamins (B_1, B_2 and B_3) are encoded by two genes located on humen chromosomes 5q (lamin B_1) (Wydner et al., 1996) and humen 19p13.3 (lamins B_2 and B_3) (Biamonti et al., 1992). One or more B-type lamins are expressed in all cells and tissues, and these lamins have roles in determining the size and shape of the cell nucleus (Furakawa and Hotta, 1993; Schirmer et al., 2001), and its mechanical properties (Newport et al., 1990), in DNA replication (Moir et al., 2000), and in transcription (Spann et al., 2002). The four vertebrate A-type lamins (lamins A, AΔ10, C and C_2) are encoded by the gene *LMNA*, located at chromosome 1q21.2 (Fisher et al., 1986). Lamin C_2 is expressed only in spermatocytes (Furukawa et al., 1994), whereas lamin AΔ10 is found predominantly in tumours (Machiels et al., 1996). Lamins A and C are expressed in all differentiated cells and normally first appear at the time of organogenesis (Röber et al., 1989).

Recent interest in *LMNA* has centred around the finding that mutations in this gene give rise to eight distinct genetic diseases, all related to premature ageing syndromes. Collectively these diseases are called laminopathies (for review, see Burke and Stewart, 2002). The most severe diseases are Hutchinson-Gilford progeroid syndrome, which results from mutations in exon 11 (Eriksson et al., 2003; De Sandre-Giovannoli et al., 2003), and atypical Werner's syndrome, which arises from mutations in conserved residues in the coiled-coil domain (Chen et al., 2003). Three of the diseases principally affect striated muscle and include autosomal dominant Emery-Dreifuss muscular dystrophy (AD-EDMD) (Bonne et al., 1999), limb-girdle muscular dystrophy type 1B (LGMD1B) (Muchir et al., 2000) and dilated cardiomyopathy with conduction defect (DCMCD) (Fatkin et al., 1999; Brodsky et al., 2000). In addition to these diseases, mice lacking A-type lamins or deficient in lamin-A processing develop lethal skeletal- and cardiac-muscle pathologies soon after birth, indicating the importance of lamins in maintenance of skeletal muscle (Sullivan et al., 1999; Pendas et al., 2002).

Two hypotheses have been proposed to explain how mutations in *LMNA* cause inherited diseases. (1) In the structural hypothesis (Sullivan et al., 1999; Hutchison et al., 2001), *LMNA* mutations are proposed to cause structural weakness in the lamina, which is then less able to resist stress within a cell. In tissue exposed to mechanical stress (e.g. striated muscle), this results in damage to the NE and eventually cell death. Indeed evidence for this type of damage has been reported in cells and in muscle sections from EDMD patients (Markiewicz et al., 2002a; Fidzianska and

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Hausmaowa-Petruciewicz, 2003). (2) The gene-expression hypothesis proposes that a major function of lamins A and/or C is regulating gene expression in differentiating cells. This function is disrupted by laminopathy mutations, resulting in loss of a differentiated phenotype (Cohen et al., 2001). This hypothesis is supported by several observations. Several studies have revealed that lamins A and C, and their binding partners associate with transcription factors including the retinoblastoma protein Rb (Ozaki et al., 1994), MOK2 (Dreuillet et al., 2002) and SREBP1 (Lloyd et al., 2002). In the case of Rb, lamins A and C, and LAP2α have been shown to tether an underphosphorylated form of the protein in the nucleus (Markiewicz et al., 2002b). In addition, the segmental nature of mutations associated with some laminopathies, suggests that lamins A and C have distinct functional domains that interact with different transcription regulators in different tissues (for review, see Hutchison, 2002).

The differentiation of skeletal muscle in vitro is a well-characterized developmental model. Myogenesis involves the differentiation and fusion of mononucleate myoblasts into multinucleate myotubes and is regulated by combinatorial associations between basic helix-loop-helix transcription factors (Massari and Murre, 2000) and the myocyte-specific enhancer-binding protein MEF2 (Naya and Olson, 1999). Proliferating myoblasts express the helix-loop-helix proteins MyoD and Myf5, which form a repressor complex through association with MEF2 and histone deacetylase 1 (HDAC1) (McKinsey et al., 2002). Upon withdrawal of mitogens, MyoD is derepressed, leading to upregulation of a third helix-loop-helix protein, myogenin, as well as the cell-cycle regulators p21, cyclin D3 and Rb. The cell-cycle regulators are required for entry into a postmitotic state which is a prerequisite for differentiation and that involves p21-mediated Rb dephosphorylation (Novitch et al., 1999; Cenciarelli et al., 1999). Rb is also involved in MyoD-regulated expression of late genes in myotubes through regulated interaction with HDAC1 (Puri et al., 2001).

Although EDMD, LGMB1B and DCMCD are thought to arise predominantly through structural weakness of the lamina (Sullivan et al., 1999; Fidzianska and Hausmanowa-Petrucewicz, 2002; Muchir et al., 2003), there is reason to suspect that defects in gene regulation might also be involved. In C2C12 myoblasts a subset of lamins A and C is organized in intranuclear speckles. During differentiation, these lamins become antigenically masked without apparent reorganization of peripheral lamins and it has been proposed that this reflects changes in association of lamins with splicing factors (Muralikrishna et al., 2001). In a separate series of experiments, ectopic expression of lamin A in myoblasts caused upregulation of some muscle-specific genes (Lourim and Lin, 1992). Finally, in a recent report, expression of a lamin-A mutant causing AD-EDMD (R453W) in C2C12 myoblasts inhibited differentiation and promoted apoptosis, leading to the suggestion that this mutant lamin fails to build a functional scaffold required for differentiation (Favreau et al., 2004).

We wished to test the hypothesis that building a functional lamin-based scaffold is necessary for skeletal-muscle cell differentiation (Favreau et al., 2004). Therefore, we investigated the influence of ectopically expressed lamin A harbouring mutations that cause AD-EDMD on the differentiation of C2C12 myoblasts. During normal differentiation, we observed a dramatic reorganization of A-type lamins and their binding partners from the nucleoplasm to the NE. This reorganisation was correlated with increased expression of lamin B_2 and decreased expression of LAP2α, and resulted in decreased solubility of A-type lamins, particularly of lamin C. When mutant lamin A was transfected into C2C12 myoblasts, it accumulated in the nucleoplasm. When C2C12 myoblasts were induced to differentiate, the mutant lamins remained in the nucleoplasm and exerted a dominant effect over endogenous lamins. Cells transfected with mutant lamins failed to differentiate, whereas cells transfected with wild-type lamin A still differentiated (albeit more slowly). In common with previous reports, we observed progressive dephosphorylation of Rb during myogenesis. Using antibodies that detect forms of Rb phosphorylated at serine 780 (RbS780) we found that multiple phosphorylated forms of Rb were maintained until myoblasts differentiated but disappeared in cultures containing myotubes. In mitotic myoblasts and postmitotic myoblasts, LAP2α colocalized with and bound to the fastest migrating, most underphosphorylated form of Rb. In C2C12 myoblasts expressing mutant lamins, LAP2α and RbS780 were both undetectable after withdrawal of mitogens. Thus, the expression of mutant lamins in C2C12 myoblasts prevents endogenous lamins from relocating from the nucleoplasm to nuclear lamina, disrupts LAP2α organization and leads to loss of expression of Rb isoforms. Therefore, we propose that remodelling of the lamin-A/C–LAP2α nucleoskeleton is required for Rb function in C2C12 myoblast differentiation.

Materials and Methods

Cell cultures

C2C12 mouse myoblasts were established at initial seeding densities of 0.5×10^5 per 35 mm dish and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS). Cells were maintained in an incubator with 5% CO_2 at 37°C. To induce differentiation, cultures were transferred to DMEM supplemented with 2% horse serum and maintained for 24-120 hours.

Transient expression of lamin-A fusion constructs in cells

C2C12 myoblasts were grown on DMEM supplemented with 10% FCS in six-well plates at an initial density of 0.5×10^5 per well. After 24 hours, cultures were transfected with FLAG-tagged wild-type lamin A or FLAG-tagged lamin A W520S (Östlund et al., 2001). Transfection was performed using GeneJuice transfection reagent (Novagen). For transfections, a mixture of 2 μg DNA and 3 μl GeneJuice in 100 μl serum-free medium was added to 2 ml culture medium. The medium was replaced after 24 hours with either DMEM plus 10% FCS or DMEM plus 2% horse serum and fusion proteins were transiently expressed in cells for an additional 24-96 hours.

Cellular fractionation

For total protein extracts, cell pellets were directly solubilized in sodium-dodecyl-sulfate (SDS) sample buffer. For extracts of nuclei and nuclear fractions, cells were extracted with hypotonic solution containing 10 mM KCl, 10 mM HEPES-KOH, pH 7.4, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.5 mM dithiothreitol and protease inhibitors. After a 10 minute incubation at 4°C, nuclei were isolated with homogenization and samples were centrifuged for 5 minutes in an Eppendorf microcentrifuge at 10,000 ***g***. Nuclei were either

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solubilized in SDS sample buffer or extracted with buffer containing 150 mM NaCl. Samples were centrifuged for 5 minutes at 15,000 ***g*** and pellets subjected to additional extraction with buffer containing 500 mM NaCl followed by centrifugation for 5 minutes at 15,000 ***g***. Soluble low-salt and high-salt fractions, and insoluble pellets were solubilized in SDS sample buffer and prepared for gel electrophoresis and immunoblotting.

Immunoblotting and densitometry analysis

One-dimensional SDS-PAGE was performed according to Laemmli (Laemmli, 1970). For immunoblotting, proteins separated on 10% gels were electrophoretically transferred to nitrocellulose (0.2 mm; Schleicher and Schuell) in 48 mM Tris-HCl, pH 9.4, 39 mM glycine by using the Mini Transblot system (Bio-Rad). Primary antibodies were used as follows: anti-myogenin, rabbit polyclonal, 1:500, Santa Cruz Biotechnology; Jol4, anti-lamin-A, mouse monoclonal, 1:10 (Dyer et al., 1997); C-20, anti-lamin-B1, goat polyclonal, 1:500, Santa Cruz Biotechnolgy; anti-lamin-C, rabbit polyclonal, 1:500 (Venables et al., 2001); LN43, anti-lamin-B2, mouse monoclonal, 1:10 (Venables et al., 2001); LAP15, anti-LAP2α mouse monoclonal, 1:10 (Dechat et al., 2000); anti-RbS780, 1:1000, Cell Signalling. Secondary antibodies were donkey anti-mouse, donkey anti-rabbit or donkey anti-goat IgG conjugated to horseradish peroxidase (Jackson Immunoresearch). For the immunological detection of proteins, the enhanced chemiluminescence system was used.

To estimate the proportion of each fraction, densitometry was performed using UVI bandmap software (UVItec, Cambridge, UK). For whole-cell extracts, the proportion of protein in each fraction was expressed in relation to first band (0 hour time point, 100%). For nuclear fractions, the proportion of each protein represented in any one fraction was expressed as a percentage of the total of that protein at any given time point.

Immunoprecipitation

Mouse or rabbit IgG Dynabeads (Dynal Biotech) were coupled to LAP2α- or lamin-C-specific antibody by incubation for 12 hours at 4°C in the presence of 1% bovine serum albumin. At 0 hours, 72 hours and 120 hours of differentiation, C2C12 cells were extracted with hypotonic solution containing 10 mM KCl, 10 mM HEPES-KOH, pH 7.4, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.5 mM dithiothreitol and protease inhibitors. After a 10 minute incubation at 4°C, nuclei were isolated with homogenization and samples were centrifuged for 5 minutes in Eppendorf microcentrifuge at 10,000 ***g***. Nuclei were extracted with extraction with buffer containing 500 mM NaCl followed by centrifugation for 5 minutes at 15,000 ***g***. Soluble fractions were dialysed to PBS containing 0.1% Triton X-100 and processed for immunoprecipitation by using LAP2α- and lamin-C-specific antibody coupled to Dynabeads. After a 3 hour incubation at 4°C, beads were washed with PBS containing 0.1% Triton X-100 (three times in five volumes of buffer each) and processed for gel electrophoresis and immunoblotting.

Indirect immunofluorescence and confocal microscopy

C2C12 myoblasts were grown on glass coverslips. The cells were fixed for 15 minutes at room temperature in 4% formaldehyde and subsequently permeabilized for 5 minutes at 4°C with 0.5% Triton X-100. Fixed and permeabilized cells were stained with a combination of primary antibodies followed by FITC- or TRITC-conjugated donkey anti-mouse, donkey anti-goat or donkey anti-rabbit IgG. Primary antibodies were used as described above. For detection of FLAG-tagged fusion proteins, anti-FLAG FITC-conjugated antibodies were used (Sigma). Stained cells were imaged using a Bio-Rad Radiance 2000 confocal scanner attached to a Zeiss Axioskop fitted with a 40× 1.4 NA PlanNeofluor lens. Sections along the *z*-axis were collected at room temperature using a Kalman averaging program at scan speed of 150 lines per minute. Laser efficiency was adjusted to avoid saturation and therefore to allow comparative measurement of fluorescent output within a field. Midsections from selected *z*-axis series were projected and superimposed in LaserSharp 2000 to produce two-colour merged images. Surface plotting was performed on PICT images of central sections of *z*-axis series in unadjusted images using LaserPix software. Micrographs were assembled in Adobe Photoshop 5.5 using minimal greyscale level adjustment to entire micrographs.

Results

Changes in expression of lamin B_2 and LAP2α, and remodelling of the nucleoskeleton during myogenesis

To investigate the influence of lamin scaffolds on the differentiation of C2C12 myoblasts in vitro, we initially investigated the expression of all major lamin types, together with the nucleoskeleton protein LAP2α. To determine the rate of skeletal-muscle differentiation, cell extracts were prepared at daily intervals after withdrawal of mitogens and immunoblotted with antibodies against myogenin. Expression of myogenin was first detected some 48 hours after withdrawal of mitogens, indicating that myoblasts had entered a postmitotic state and continued to increase over the next 72 hours (Fig. 1a) as myotubes formed (Fig. 2). Expression of lamins A, B_1 and C did not vary during myoblast differentiation (Fig. 1b,c,e). By contrast, there was a twofold increase in the expression of lamin B_2 (Fig. 1d) and a 50% decrease in the expression of LAP2α (Fig. 1f) during myogenesis.

The increased expression of lamin B_2 and decreased expression of LAP2α in postmitotic myoblasts and myotubes had not been reported previously. Increased representation of lamin B_2 in the lamina is predicted to accommodate more of lamins A and C in this structure (Hutchison et al., 2001). In addition, LAP2α binds A-type lamins in the nucleoplasm (Dechat et al., 2000). Therefore, one consequence of increased expression of lamin B_2 and decreased expression of LAP2α might be a reduction in nucleoplasmic forms of the lamins. To test this hypothesis, myoblast cultures were induced to differentiate and then stained with an extensive and well-characterized panel of antibodies against lamins that detects both peripheral and diffuse nucleoplasmic populations of these proteins (Dyer et al., 1997; Venables et al., 2001). Confocal microscopy was used to investigate the distribution of each antibody. At the time that C2C12 myoblasts were induced to differentiate, antibodies against lamins A and C (Fig. 2a, top), lamin B_1 (Fig. 2b, top), lamin A, and lamin C (not shown), stained the NE as well as displaying diffuse staining throughout the nucleoplasm. In these cultures, none of the cells were differentiated (as judged by lack of expression of myogenin). Between 48 hours and 72 hours after cultures were induced to differentiate, increasing numbers had entered a postmitotic state, as shown by expression of myogenin (Fig. 2a,b, centre). In postmitotic myoblasts, distinct changes in the staining patterns of anti-A-type-lamin and anti-lamin-B_1 antibodies were observed (Fig. 2a,b, centre). First, the staining intensity was much greater than in surrounding undifferentiated cells. Second, staining at the lamina was greatly enhanced and nucleoplasmic staining relatively depleted. 96-120 hours after cultures were induced to differentiate, most postmitotic

myoblasts had fused to form myotubes. Anti-lamin antibodies decorated the NEs of these cells intensely in comparison to the small numbers of undifferentiated cells remaining in these cultures (Fig. 2a,b, bottom).

To confirm that the anti-lamin antibodies displayed relatively uniform staining throughout the nucleus in mitotic myoblasts but peripheral staining in postmitotic myoblasts, *z*-axis series were projected (the example shown was stained with anti-lamin-C antibodies). Midsections of mitotic myoblasts clearly displayed diffuse nucleoplasmic staining as well as staining at the nuclear envelope (Fig. 2c, asterisk). By contrast, postmitotic myoblasts only displayed NE staining (Fig. 2c, arrow). To confirm that anti-lamin antibodies stain the nuclear envelope of postmitotic myoblasts more intensely than undifferentiated myoblasts, surface plots were made of the distribution and level of fluorescence on midsections of *z*-axis series. Fig. 2d,e shows a typical result in which the postmitotic myoblast (arrow) shows intense peripheral fluorescence (red) but weak nucleoplasmic fluorescence (blue or green). By contrast, the surrounding undifferentiated cells display much weaker fluorescence in general but a more-even distribution of fluorescence throughout the nucleus. Finally, to confirm that the enhanced peripheral staining correlated with increased expression of lamin B_2, we stained cultures with both anti-lamin-C and anti-lamin-B_2 antibodies. The cells displaying intense peripheral staining with anti-lamin-C antibodies were also those displaying the highest levels of anti-lamin-B_2 fluorescence (Fig. 2f).

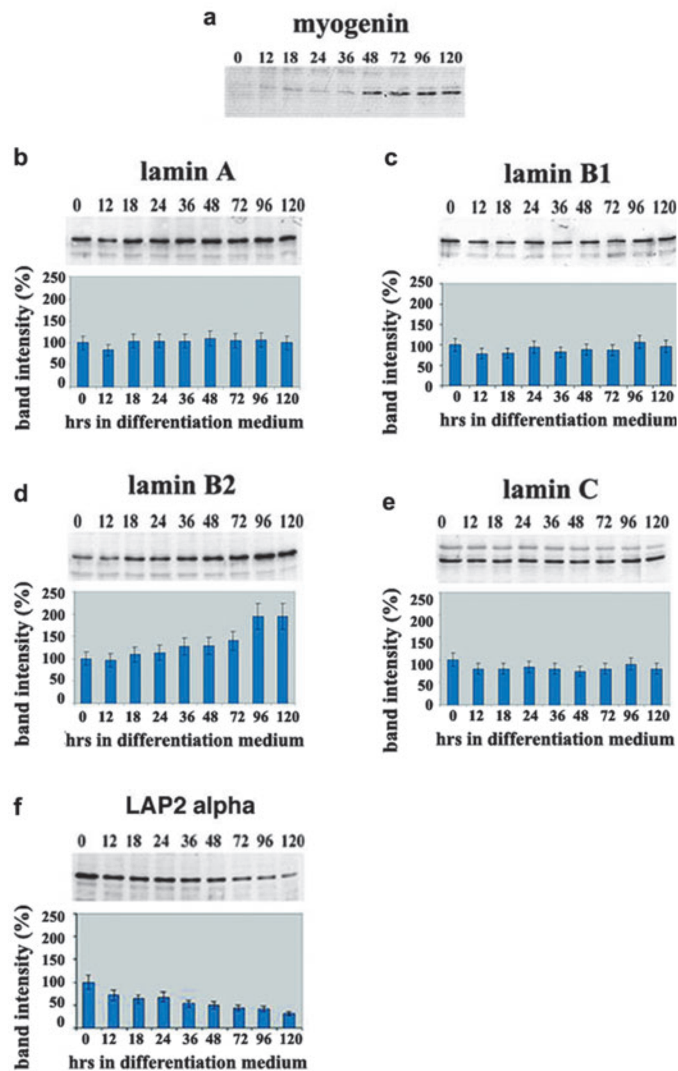

Fig. 1. Changes in expression of lamins and LAP2α during myogenesis. Exponentially growing C2C12 myoblasts were induced to differentiate with 2% horse serum. Whole cell extracts were prepared at 0, 12, 18, 24, 36, 48, 72, 96 and 120 hour time points, and quantities were standardized to equal amounts of DNA as follows. Cell extracts were prepared at various time intervals and subjected to agarose-gel electrophoresis and stained with ethidium bromide. The genomic DNA was visualized as a slowly migrating band, separated from the faster migrating RNA specimens. To assure that detection was within a linear range, the extracts were analysed in serial dilutions. The intensity of bands corresponding to genomic DNA was analysed by densitometry using UVI bandmap software and the volume of each sample adjusted so that equal amounts of DNA were added to each lane of protein gels. Levels of expression of myogenin (a), lamin A (b), lamin B1 (c), lamin B2 (d), lamin C (e) and LAP2α (f) were analysed by western blotting and the intensity of each band evaluated by densitometry. The graphs below each blot are representative of three independent experiments.

The apparent changes in staining intensity and distribution of lamins observed clearly did not arise through increased expression of lamins A/C and B_1 (Fig. 1). Often, the immunoreactivity of lamins can alter depending upon cellular behaviour, because of epitope masking (Dyer et al., 1997; Hozak et al., 1994; Muralikrishnan et al., 2001). This implies that the greatly increased staining intensity observed in differentiated cells arises through remodelling of the nucleoskeleton, which either exposes epitopes or leads to increased accumulation of lamins in a restricted position. We wished to investigate this hypothesis. We have previously shown that A-type lamins display different solubility properties depending on whether they reside at the NE or in the nucleoplasm. B-type lamins are always insoluble; by contrast, A-type lamin they are insoluble if they reside in the lamina, whereas they can be extracted with a combination of detergents and either hypotonic or hypertonic salt if they reside in the nucleoplasm (Markiewicz et al., 2002a). Therefore, we isolated nuclei from cells at various stages of differentiation and either dissolved them directly in SDS or extracted them sequentially with hypotonic or hypertonic solutions. Extracts were resolved by SDS-PAGE along with material that was resistant to extraction. Samples were blotted with antibodies against each lamin. As expected, both B-type lamins were completely insoluble throughout myogenesis in vitro (Fig. 3a,b), although the level of expression of lamin B_2 clearly increased (Fig. 3b). By contrast, the A-type lamins displayed variable solubility properties. In cultures of proliferating myoblasts, a significant proportion of lamin A (~36%) was soluble in hypertonic salt (Fig. 3c, top). Lamin C displayed even greater solubility properties, with ~17% being soluble in the presence of hypotonic salt and a further ~36% being soluble in hypertonic salt (Fig. 3d, top). By 72 hours after cultures were induced to differentiate, the solubility of each A-type lamin had reduced dramatically. In these cultures, lamin A was completely insoluble (Fig. 3c, middle) and remained insoluble in cultures containing myotubes (Fig. 3c, bottom). At the same time, ~1% of lamin C was soluble in hypotonic solution, although ~35% remained soluble in hypertonic solution (Fig. 3d, middle). In

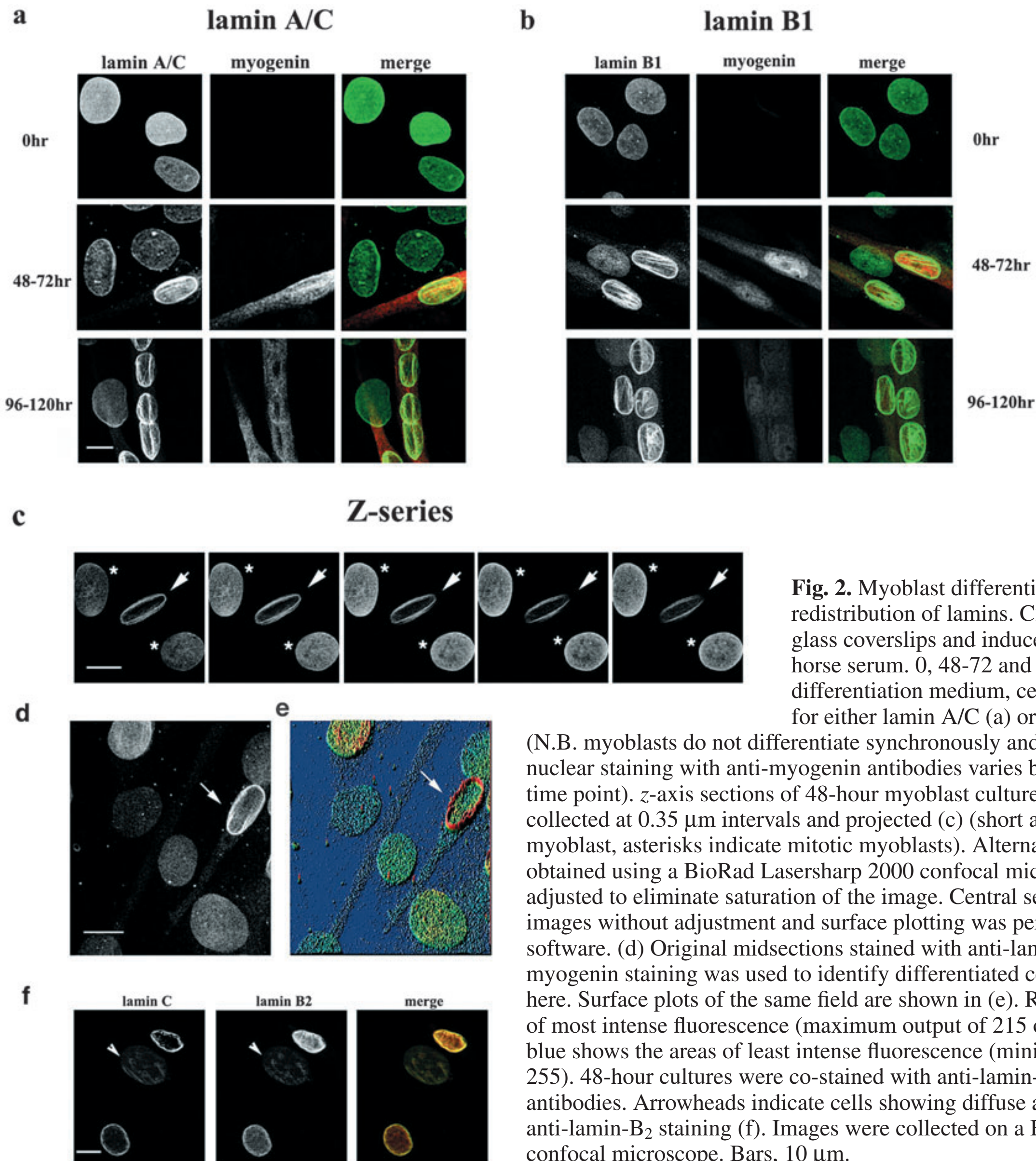

Fig. 2. Myoblast differentiation is accompanied by redistribution of lamins. C2C12 myoblasts were grown on glass coverslips and induced to differentiate with 2% horse serum. 0, 48-72 and 96-120 h after transfer to differentiation medium, cells were fixed and co-stained for either lamin A/C (a) or lamin B1(b) and myogenin (N.B. myoblasts do not differentiate synchronously and therefore the intensity of nuclear staining with anti-myogenin antibodies varies between cells within a single time point). *z*-axis sections of 48-hour myoblast cultures stained for lamin C were collected at 0.35 μm intervals and projected (c) (short arrows indicate a postmitotic myoblast, asterisks indicate mitotic myoblasts). Alternatively, *z*-axis series were obtained using a BioRad Lasersharp 2000 confocal microscope with the laser power adjusted to eliminate saturation of the image. Central sections were converted to PICT images without adjustment and surface plotting was performed using LaserPix software. (d) Original midsections stained with anti-lamin-A/C antibodies. Anti-myogenin staining was used to identify differentiated cells (arrows) but is not shown here. Surface plots of the same field are shown in (e). Red coloration shows the areas of most intense fluorescence (maximum output of 215 on a scale of 0-255), whereas blue shows the areas of least intense fluorescence (minimum output 0 on a scale of 0-255). 48-hour cultures were co-stained with anti-lamin-C and anti-lamin-B_2 antibodies. Arrowheads indicate cells showing diffuse anti-lamin-C staining and weak anti-lamin-B_2 staining (f). Images were collected on a BioRad Lasersharp 2000 confocal microscope. Bars, 10 μm.

cultures containing myotubes, ~2% of lamin C was soluble in hypertonic solution, whereas >98% was insoluble. These data support the hypothesis that A-type lamins accumulate in the lamina following myoblast differentiation.

Next, we investigated the solubility properties of LAP2α. We reasoned that, if A-type lamins did migrate from the nucleoplasm to the nuclear lamina in differentiated cells, this would influence LAP2α's solubility properties. In proliferating myoblasts, LAP2α was distributed roughly equally between hypotonic soluble, hypertonic soluble and insoluble fractions (Fig. 4a, top). By contrast, 72 hours after cultures were induced to differentiate most LAP2α (>50%) was present in the hypertonic soluble fraction (Fig. 4a, middle). 120 hours after cultures were induced to differentiate, LAP2α was barely detected, but most was now insoluble. These data imply that LAP2α organization changes dynamically during myogenesis. The monoclonal antibody used to detect LAP2α only reacts with relatively soluble forms of the protein in immunofluorescence assays (Dechat et al., 1998). If LAP2α becomes transiently more soluble in postmitotic myoblasts, this ought to be reflected by an increased signal in immunofluorescence assays. To investigate this, we performed immunostaining and confocal microscopy on C2C12 cultures throughout the process of differentiation. Proliferating myoblasts displayed uniform nucleoplasmic staining with anti-LAP2α antibodies (Fig. 4b, top). By contrast, 72 hours after cultures were induced to differentiate, postmitotic myoblasts (Fig. 4b, middle, arrows) were stained intensely compared with neighbouring undifferentiated cells (Fig. 4b, middle, arrowheads). Finally, LAP2α was only weakly detected in myotubes (Fig. 4b, bottom). Finally, to confirm that changes in LAP2α's behaviour correlates with changes in A-type-lamin distribution, we stained 48-hour myoblast cultures with both anti-lamin-C and anti-LAP2α antibodies. We found that cells displaying intense anti-LAP2α immunofluorescence were always those displaying intense perinuclear lamin-C staining

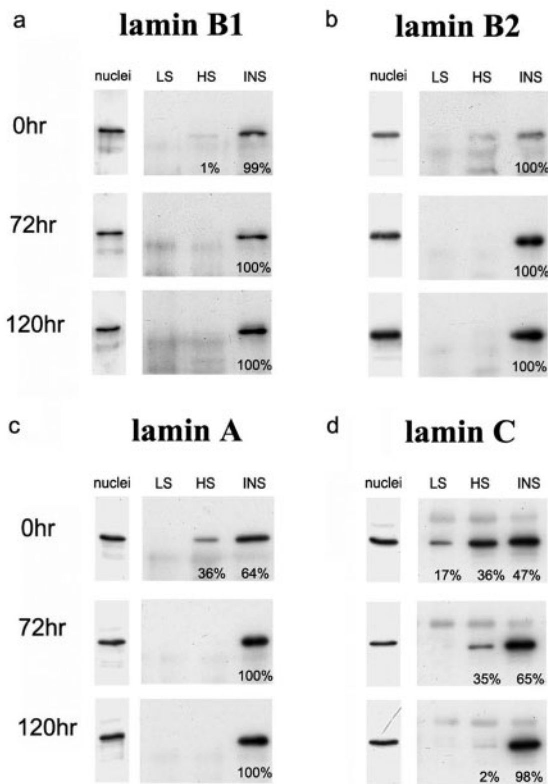

Fig. 3. Solubility properties of lamins and lamina-associated proteins during myogenesis. C2C12 myoblasts were induced to differentiate with 2% horse serum. At 0, 72 and 120 hours after transfer to differentiation medium, cells were harvested and subjected to nuclear isolation. Nuclei were either solubilized in SDS (nuclei) or sequentially extracted with hypotonic (LS) or hypertonic (HS) buffer. Samples were resolved by SDS-PAGE along with material resistant to extraction (INS), transferred to nitrocellulose and blotted with antibodies against lamin B_1 (a), lamin B_2 (b), lamin A (c) and lamin C (d), and the intensity of each band evaluated by densitometry and expressed as a proportion of each protein in whole nuclear extracts.

(Fig. 4c). These data show that changes in A-type-lamin's solubility properties and their apparent distribution is correlated with remodelling of the LAP2α nucleoskeleton.

C2C12 myoblasts transfected with dominant mutants of lamin A do not accumulate in the lamina and inhibit myoblast differentiation

A recent report has suggested that an EDMD mutant form of lamin A inhibits myoblast differentiation (Favreau et al., 2004). To test whether mutant lamins that inhibit differentiation also affect remodelling of the nucleoskeleton, we reasoned that mutant lamins of this type would fail to localize to the NE even after cultures were induced to differentiate. We therefore transfected C2C12 myoblasts with FLAG-tagged wild-type lamin A or lamin A mutated at W520S (Östlund et al., 2001). Cultures that were maintained in growth medium for 48 hours after transfection initially displayed an aggregated distribution of exogenous lamin A in the nucleoplasm (Fig. 5a,c) but later displayed distribution patterns that were similar to those of endogenous lamins (Fig. 5a, asterisk). Cultures maintained in differentiation medium for 96 hours after transfection with wild-type lamin A differentiated (as judged by expression of myogenin; Fig. 5b, top). In differentiated cells, the exogenous lamin A had clearly migrated to the NE. By contrast, cells transfected with mutant lamin A did not express myogenin, even after 96 hours in differentiation medium (surrounding untransfected cells did differentiate). Moreover, the exogenous mutant lamin was distributed fairly uniformly between the NE and the nucleoplasm (Fig. 5b, bottom). When we investigated the behaviour of endogenous lamin B_1 (Fig. 5c,d) or lamin C (Fig. 5e,f), we found that, in the presence of exogenous wild-type lamin A, both lamins displayed an intense nuclear-rim staining pattern (with undetectable nucleoplasmic staining) in postmitotic myoblasts (Fig. 5a, top). By contrast, in cells transfected with mutant lamin A, anti-lamin-B_1 staining (Fig. 5d, bottom) showed a reduced fluorescence intensity and more-uniform nuclear staining, typical of undifferentiated cells, whereas significant fractions of lamin C appeared to be trapped in the nucleoplasm (Fig. 5f, bottom).

To quantify the effects of the exogenous lamins, C2C12 myoblasts were transfected in triplicate experiments and immunostained. 200 transfected cells were counted at 24 hour intervals after transfer of cultures to differentiation medium for the accumulation of exogenous lamins either in the nucleoplasm (Fig. 6a) or at the NE (Fig. 6b), or for the expression of myogenin (Fig. 6c). Between 0 hours and 96 hours after transfer to differentiation medium, the proportion of transfected cells having mutant lamins in the nucleoplasm remained at 96-100%. Similarly, over the same time period, the proportion of cells having mutant lamins in the NE was maintained at 0-4%. By contrast, over the same 96 hour time period, the proportion of transfected cells having wild-type lamin in the nucleoplasm declined from 100% to >35%, while the proportion of cells having wild-type lamin A exclusively at NE increased from 0% to >65%.

When cells transfected with mutant lamin A were scored for expression of myogenin, between 10% and 15% were positive after 96 hours. By contrast, ~70% of cells transfected with wild-type lamin A were myogenin positive at the same time point. Taken together, these results demonstrate that mutant lamin A is unable to relocate from the nucleoplasm to the NE after transfer of cultures to differentiation medium and that this is correlated with a failure of the same cells to differentiate.

Expression of mutant lamin A in C2C12 myoblasts disrupts LAP2α organization and inhibits expression of phosphorylation isoforms of Rb

We have previously shown that, in human fibroblasts, a complex of LAP2α and A-type lamins is required for nuclear anchorage of the transcription regulator Rb (Markiewicz et al., 2002b). It is well established that Rb is required for myogenesis (Novitch et al., 1999). Therefore, we wished to investigate the effects of expressing the lamin mutant in C2C12 myoblasts on LAP2α and Rb organization or expression. Initially, we investigated Rb expression with an antibody that detects RbS780 because we have previously shown that LAP2α expression is correlated with the expression of RbS780 (Markiewicz et al., 2002b). Using immunofluorescence, we found that RbS780 was expressed in dividing myoblasts (Fig. 7a) and in postmitotic myoblasts (Fig. 7b arrows) but was either greatly reduced in or absent from myotubes (not shown).

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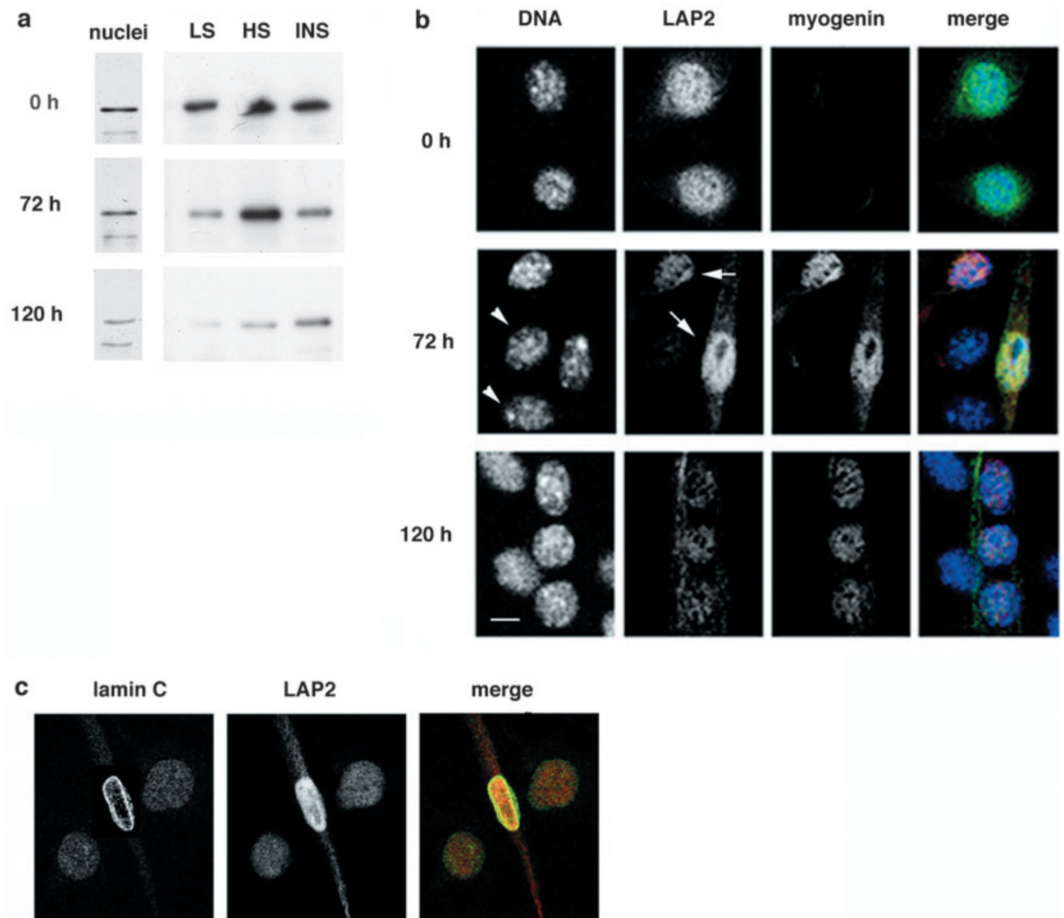

Fig. 4. Changes in solubility properties of LAP2α during myogenesis. (a) C2C12 myoblast cultures were induced to differentiate and, after 0, 72 and 120 hours, cells were harvested and subjected to nuclear isolation. Nuclei were either solubilized in SDS (nuclei) or sequentially extracted with hypotonic (LS) or hypertonic (HS) buffer. Samples were resolved by SDS-PAGE along with material resistant to extraction (INS), transferred to nitrocellulose and blotted with antibodies against LAP2α and the intensity of each band evaluated by densitometry. (b) C2C12 myoblasts were grown on glass coverslips and induced to differentiate with 2% horse serum. After 0, 48, 72, 96 and 120 hours of differentiation, cells were fixed and immunostained with anti-lamin, anti-LAP2α and anti-myogenin antibodies, and observed using a confocal microscope. Arrows show postmitotic myoblasts and arrowheads show mitotic myoblasts. (c) 48-hour cultures of differentiating myoblasts were stained with anti-lamin C and LAP15 (anti-LAP2α) antibodies. Bars, 10 μm.

Similar results were obtained when cell extracts were prepared for immunoblotting. In extracts from cultures containing mitotic and postmitotic myoblasts, RbS780 was readily detectable as multiple bands migrating with a molecular mass of 110-120 kDa, whereas extracts prepared from cultures containing myotubes expressed little or no RbS780 (Fig. 7d,e). In the immunofluorescence micrographs, LAP2α and RbS780 displayed considerable colocalization in both mitotic and postmitotic myoblasts (Fig. 7a,b). Therefore, we investigated whether the two proteins existed in a cellular complex with each other. To do this, we immunoprecipitated either LAP2α or lamin C from cell extracts prepared from cultures containing mitotic myoblasts, postmitotic myoblasts or myotubes. The immunoprecipitates were then blotted with antibodies against LAP2α, lamin C or RbS780. When cell extracts were immunoprecipitated with empty immunobeads, none of LAP2α, lamin C or RbS780 were pulled down (data not shown). By contrast, LAP2α was recovered efficiently from cell extracts using anti-LAP2α immunobeads (Fig. 7e) and lamin C was recovered efficiently from cell extracts using anti-lamin-C antibodies (data not shown). RbS780 immunoprecipitated with LAP2α from cell extracts prepared from mitotic and postmitotic myoblast cultures, but not from myotube cultures (Fig. 7c). In addition, bands with two different mobilities co-immunoprecipitated from myoblast cultures (Fig. 7c, * and –), whereas only the more rapidly migrating band (Fig. 7c, –) immunoprecipitated with LAP2α in postmitotic myoblast cell extracts. The fastest migrating form of RbS780 also immunoprecipitated with lamin C in mitotic myoblast extracts (Fig. 7d, –), but RbS780 did not immunoprecipitate with lamin C in postmitotic myoblast and myotube extracts (Fig. 7b). These data suggest that, as in skin fibroblasts, LAP2α and lamin C exist in a cellular complex with the least phosphorylated form of Rb in proliferating myoblasts. However, lamin C is excluded from this complex in postmitotic myoblasts and neither LAP2α nor lamin C associates with Rb in myotubes. One interpretation of these data is that the remodelling of the lamina during myogenesis effectively removes lamin C from LAP2α-Rb complexes as myoblasts enter a postmitotic state. Because dominant-negative lamin-A mutants maintain lamin C in the nucleoplasm, we investigated how this mutant affects the organization and expression of LAP2α and RbS780. We therefore transiently expressed C2C12 myoblasts with wild-type or mutant lamin A and induced them to differentiate. Undifferentiated and differentiated cultures were then stained with anti-LAP2α or anti-RbS780 antibodies. Cultures transfected with wild-type lamin A displayed expression patterns and distributions of both LAP2α and RbS780 that were indistinguishable from controls (Fig. 7f,h). Myoblasts that were transfected with mutant lamin A also displayed distributions of LAP2α and RbS780 that were indistinguishable from controls before cultures were induced to differentiate (Fig. 7g,i, top). By contrast, when cells expressing mutant lamin A were induced to differentiate, both LAP2α and RbS780 were barely detectable or absent (Fig. 7g,

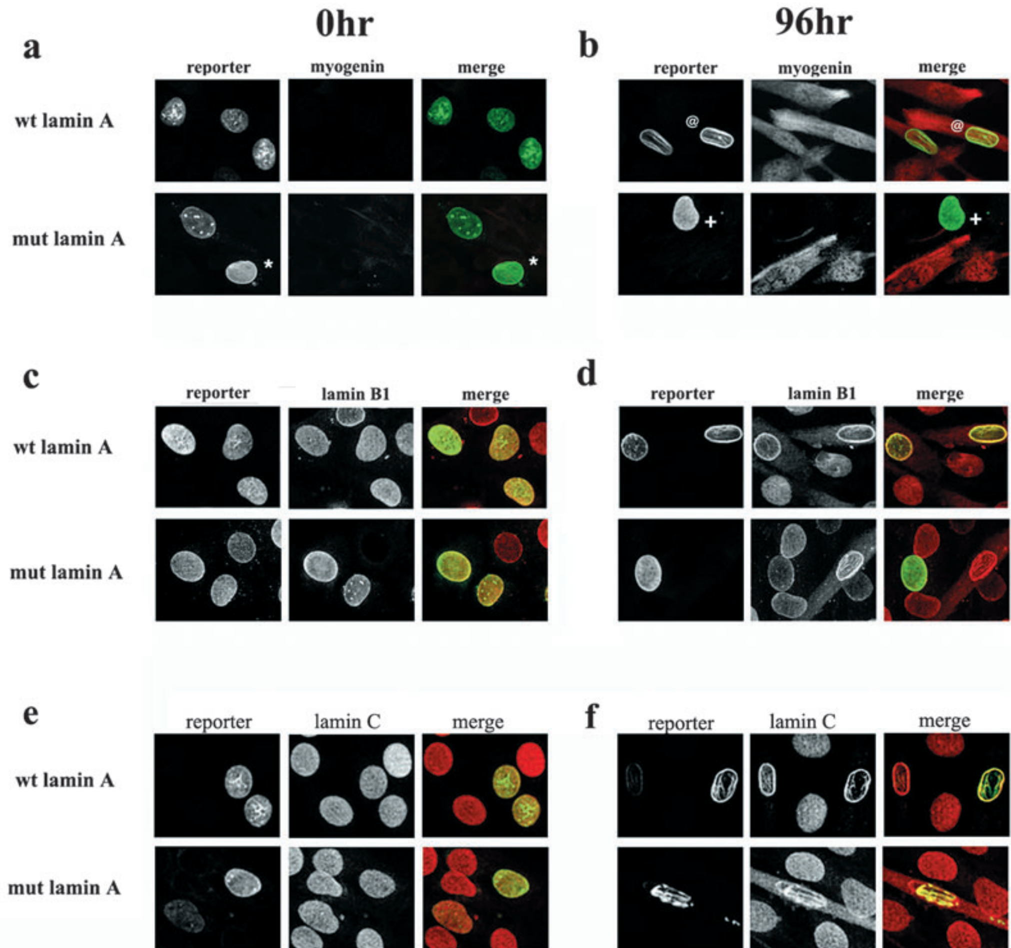

Fig. 5. Transfection of C2C12 myoblasts with mutants of lamin A prevents nuclear envelope organization and inhibits myoblast differentiation. C2C12 cells grown on glass coverslips were transiently transfected with wild-type lamin A or lamin A with point mutation W520S. 24 hours after transfection, cells were either incubated in growth medium for next 24 hours (undifferentiated, a) or induced to differentiate with 2% horse serum (differentiated, b). The cultures were fixed and co-stained for the presence of the exogenous lamin A (with anti-FLAG antibodies) and endogenous myogenin (a,b), endogenous lamin B1 (c,d) or endogenous lamin C (e,f). (a,b) Examples of nuclei scored as having nucleoplasmic staining (see Fig. 6) are indicated with * or +; examples of cells scored as having nuclear envelope staining are indicated with @. Following transfection with any FLAG vector, differentiation events are delayed by approximately 48 hours. However, cultures transfected with FLAG-W520S did not differentiate at all. Images were obtained using a BioRad laser sharp 2000 confocal microscope. Bars, 10 μm.

bottom, arrowheads; Fig. 7i, bottom, arrow,). Taken together, these data show that expression of mutant lamins that maintain lamin C in the nucleoplasm leads to loss of expression and/or disruption of LAP2α and RbS780.

Discussion

The data presented in this paper indicate that, during myogenesis in vitro, there is a dramatic remodelling of the nucleoskeleton. This remodelling involves increased expression of lamin B_2 and reorganization of lamins A and C, particularly at the lamina. The apparent consequence of this reorganization of lamins is the removal of lamin C from a complex between LAP2α and underphosphorylated Rb in postmitotic myoblasts. Expression of dominant mutants of lamin A prevents the reorganization of lamins and alters LAP2α associations. This disruption is correlated with loss of expression of Rb and inhibition of myogenesis. Taken together, these data suggest that the organization, rather than the expression, of A-type lamins within the nucleus directly influences cellular differentiation and suggests a major role for lamins and LAP2α in regulating Rb function.

Remodelling of the nucleoskeleton accompanies myogenesis

The reorganization of A-type lamins within the nucleus during myogenesis has been reported previously. Using a monoclonal antibody reagent LA-2H10, which detects lamin A at nuclear speckles (Jagnatheesan et al., 1999), Muralikrishna et al. (Muralikrishna et al., 2001) reported that epitope masking renders these sites undetectable in postmitotic C2C12 myoblasts. However, these authors did not find additional changes in the distribution of A-type lamins at nuclear envelope. In the previous study, a monoclonal antibody reagent was used that only detects peripheral lamin A (Muralikrishna et al., 2001). Therefore, redistribution of nucleoplasmic A-type lamins to the nuclear lamina would not have been detected. In our investigations, we used a panel of lamin antibodies, all of which detect C-terminal epitopes and all of which detect a diffuse population of lamins in the nucleoplasm (Dyer et al., 1997; Venables et al., 2001). In addition, the reorganization of A-type lamins during myogenesis is supported by dramatic changes in their solubility properties, greatly enhanced immunostaining and also by changes in expression of lamin B_2 and LAP2α. We have shown previously that A-type lamins display different solubility properties depending upon their distribution. For example, lamin C when distributed within the nucleoplasm is readily solubilized following sequential extraction with detergents and salts. By contrast, when it is located in the lamina, the same protein is resistant to extraction (Markiewicz et al., 2002a). Therefore, the changes in solubility properties that accompany C2C12 myoblast differentiation are entirely consistent with relocation of a significant proportion

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of lamins A and C from the nucleoplasm to the NE. In support of this conclusion, when a wild-type lamin-A reporter was expressed in C2C12 myoblasts, its distribution was mainly nucleoplasmic in undifferentiated cells but was almost exclusively at the NE in postmitotic myoblasts.

We have previously shown that lamin A is incorporated into the lamina through its association with B-type lamins (Dyer et al., 1999). We have also shown that A-type lamins bind to the nucleoskeleton protein LAP2α in the nucleoplasm (Dechat et al., 2000). Thus, the predicted outcome of a twofold increase in expression of lamin B_2 and a 50% decrease in the expression of LAP2α is the accommodation of more A-type lamin in the lamina and less in the nucleoplasm (Hutchison et al., 2001). In fact, we observed an almost twofold change in the combined solubility properties of lamins A and C during myogenesis, which is consistent with the observed changes in expression levels of lamin B_2 and LAP2α. One apparent consequence of the relocation of lamin A from the nucleoplasm to the nuclear lamina is that, in postmitotic myoblasts, LAP2α displays intense immunoreactivity and a temporary increase in solubility properties. The increased immunoreactivity of postmitotic myoblasts to anti-LAP2α antibodies is probably explained by its increased solubility, because the antibody used only detects relatively soluble forms of the protein in immunostaining (Dechat et al., 1999). Taken together, these data are all entirely consistent with a dramatic reorganization of the nucleoskeleton during myogenesis, which leads to the accumulation of A-type lamins at the lamina and resultant changes in LAP2α organization.

Is remodelling of the nucleoskeleton necessary for myogenesis?

Lamin A constructs harbouring mutants in either the rod or the tail domain produce proteins that localize to the nucleoplasm when expressed in C2C12 myoblasts (Östlund et al., 2001). In a recent study, a common EDMD mutation (R453W) was reported to inhibit C2C12 myoblast differentiation in vitro, leading to the hypothesis that this mutant lamin fails to build a functional scaffold that is necessary for myogenesis (Favreau et al., 2004). Here, we show that a second AD-EDMD mutant (W520S) predominantly localizes to the nucleoplasm in C2C12 myoblasts. Moreover, the mutant exerts dominant effects over endogenous lamins, which maintain a nucleoplasm distribution and/or display depressed immunostaining, as well as LAP2α whose immunoreactivity becomes almost completed suppressed. It is known that LAP2α can be associated with structures in the nucleus, which are highly resistant to salt extraction and this population of LAP2α is undetectable in immunofluorescence assays (Dechat et al., 1998). Therefore, the loss of LAP2α immunoreactivity associated with expression of mutant lamin A probably reflects increased binding of LAP2α to exogenous and endogenous lamin A because these proteins accumulate in the nucleoplasm rather than in the lamina. Therefore, dominant-negative lamin-A mutants appear to remodel the nucleoskeleton in two ways: first, by preventing the relocation of lamins from the nucleoplasm to the lamina; and, second, by altering LAP2α organization, presumably by sequestering it into insoluble complexes.

Remodelling of the nucleoskeleton by mutant lamin A is correlated with a failure of myoblasts to differentiate. This data provides direct support for the hypothesis that correctly building a functional lamin scaffold is essential for myogenesis (Favreau et al., 2004). We now propose that this remodelling

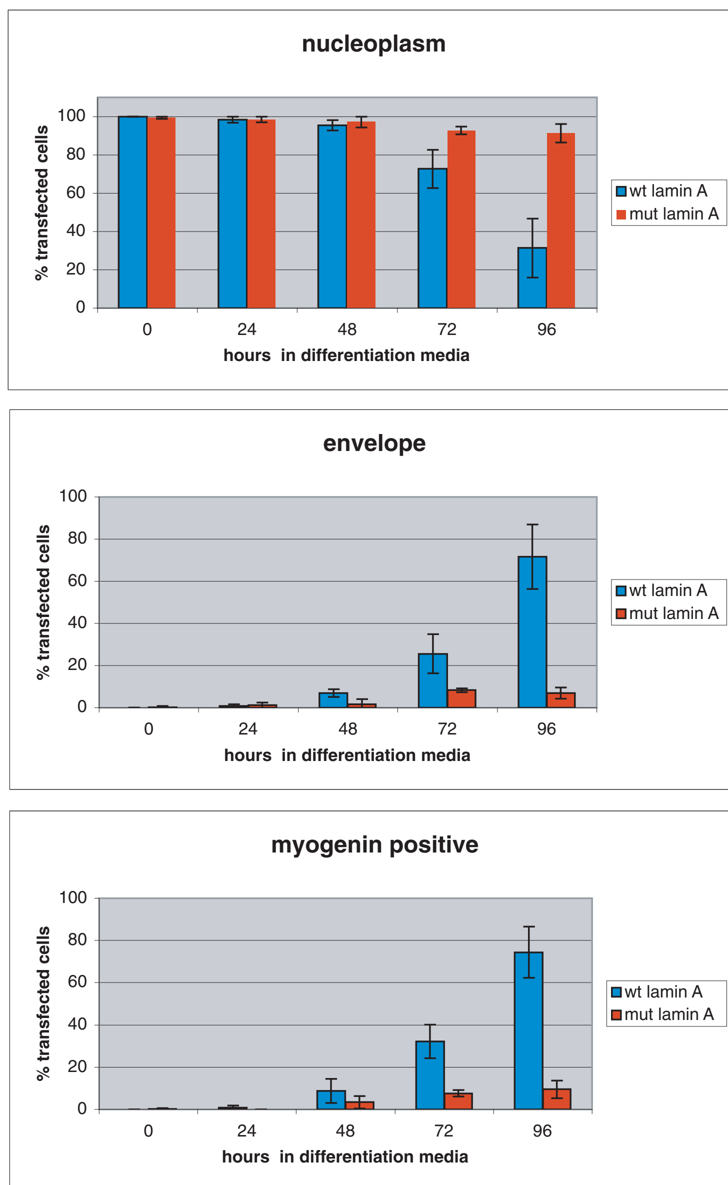

Fig. 6. Transfection of C2C12 myoblasts with dominant mutants of lamin A prevents nuclear-envelope organization and inhibits myoblast differentiation. C2C12 myoblasts were transfected in triplicate experiments with wild-type (wt) lamin A or lamin A W520S. 24 hours after transfection, cells were induced to differentiate with 2% horse serum. At 24-hour intervals, cells were fixed and stained with anti-myogenin antibody and anti-FLAG antibody. Two hundred transfected cells were counted for strong staining of exogenous lamins in nucleoplasm (a) or at the nuclear envelope (b), or for expression of myogenin (c). Results show the mean ± the standard deviation from triplicate experiments.

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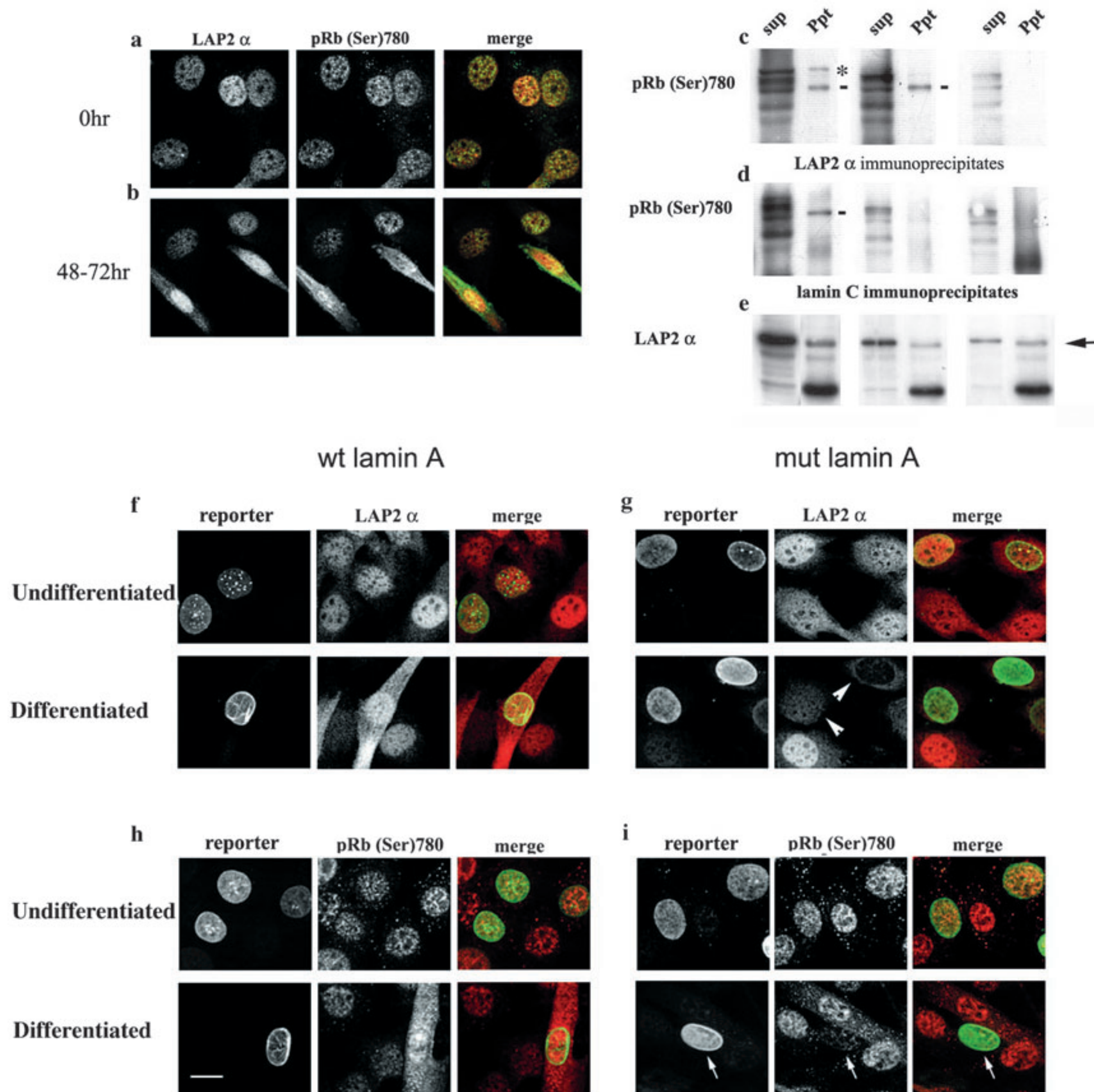

Fig. 7. Association of Rb with LAP2α and disruption of Rb-LAP2α complexes by mutant lamin A. The expression and localization of Rb and LAP2α were investigated by double immunofluorescence and confocal microscopy by co-staining undifferentiated (a) and differentiated myoblasts (b) with anti-LAP2α antibodies (left) and anti-RbS780 (centre). Micrographs are displayed as individual black and white or two-colour merged images (right). The formation of multiprotein complexes containing Rb, LAP2α and lamin C was investigated by immunoprecipitation (c-e) from cultures containing undifferentiated myoblasts (left), differentiated myoblasts (centre) and myotubes (right). Cell extracts were immunoprecipitated with anti-LAP2α antibodies (c,e) or anti-lamin C antibodies (d) and blotted with anti-RbS780 (c,d) or anti-LAP2α antibodies (e). sup, supernatant; Ppt, precipitate; *, hyperphosphorylated Rb; –, hypophosphorylated Rb (c,d); arrow in e, LAP2α. Expression of mutant (mut) lamin A disrupts LAP2α and leads to loss of expression of Rb (f-i). C2C12 myoblasts were transfected with wild-type (f,h) or mutant (g,i) lamin A constructs and fixed and stained with antibodies against LAP2α (f,g) or RbS780 (h,i) before (top) or after (bottom) cultures were induced to differentiate. Each montage shows the exogenous lamin (left), the endogenous LAP2α or Rb (middle) and two-colour merged images (right). Arrowheads (g, bottom) show transfected cells in which LAP2α is no longer detected. Arrows (i, bottom) show transfected cells in which RbS780 is no longer detected. Bars, 10 μm.

involves removal of lamin C from a LAP2α scaffold and the purpose of this remodelling is to promote Rb function.

Remodelling of the nucleoskeleton and Rb function

We have previously demonstrated that A-type lamins form a functional scaffold in the nucleoplasm together with LAP2α, which tethers forms of Rb in the nucleoplasm. Moreover, in fibroblasts, expression of LAP2α is correlated with expression of RbS780 (Markiewicz et al., 2002b). In the early stages of muscle differentiation, MyoD upregulates Rb, p21, cyclin D3 and the myogenic helix-loop-helix transcription factor myogenin in an Rb-dependent manner (Bergstrom et al., 2002; Cenciarelli et al., 1999; Novitch et al., 1999). These early events require the dephosphorylation of Rb (Zacksenhaus et al., 1996). Here, we show that dephosphorylation of Rb occurs progressively and is only complete when myotubes form. In mitotic myoblasts, LAP2α associates with two isoforms of Rb, with relative mobilities of 110 kDa and 120 kDa, both of which are phosphorylated at S780. These associations represent two

different LAP2α-Rb complexes, because lamin C is a component of one complex (with the 110 kDa isoform of Rb) but not the other. In postmitotic myoblasts hypophosphorylated nuclear Rb is readily detectable and associates with LAP2α (but not lamin C). However, in the presence of dominant lamin-A mutants, Rb is undetectable. Rb might not be detected because it is not expressed or because it is sequestered within a protein complex and is consequently epitope masked. Therefore, our data can be explained if expression of mutant lamin A inhibits myoblast differentiation because it maintains or enhances lamin-LAP2α associations, leading to the loss of availability or of expression of a form of Rb that is required for entry into a postmitotic state.

Implications for disease

We and others (Favreau et al., 2004) have now shown that two different lamin-A mutants causing AD-EDMD inhibit myogenesis when expressed in C2C12 myoblasts. Individuals harbouring these mutations develop skeletal muscle but are subject to progressive muscle weakness after childhood. Because AD-EDMD individuals do develop skeletal muscle in utero, the face-value physiological relevance of this study and that of Favreau et al. (Favreau et al., 2004) is unclear. However, this type of result is not without precedent. *MyoD1*$^{-/-}$ mice develop normally with no overt morphological or physiological skeletal-muscle abnormalities and remain viable and fertile (Rudnicki et al., 1992). By contrast, cultured myoblasts from these mice display severely impaired capacities to differentiate (Sabourin et al., 1999). Moreover, the capacity of *MyoD1*$^{-/-}$ mice to respond to crush injury by regenerating skeletal muscle is also severely impaired (Megeney et al., 1996), implying that myoblast differentiation in vitro is a more accurate model of skeletal-muscle regeneration than of skeletal-muscle development. Therefore, we propose that our results reveal a crucial role for lamins in skeletal-muscle regeneration and predict that one reason for progressive weakness and wasting in AD-EDMD patients is an impaired capacity to regenerate skeletal muscle.

Inhibition of C2C12 differentiation is correlated with a failure to remodel the nuclear lamina and/or nucleoskeleton. The idea that transcription is organized by a nucleoskeleton is well established (for review, see Jackson, 2004). However, there is little direct evidence that individual nucleoskeleton proteins influence transcriptional events. It has been reported that RNA-polymerase-II activity is dependent upon lamina integrity in *Xenopus* oocytes (Spann et al., 2002). The current report provides the first direct evidence that the precise location of lamins within the nucleoskeleton is important for cellular differentiation. This finding has significant implications for human disease. There are currently two hypothesise to explain how laminopathy mutations cause degenerative diseases: the structural hypothesis suggests that pathologies arise because a weakened lamina is susceptible to damage, which eventually leads to cell death (Sullivan et al., 1999; Hutchison et al., 2001); the gene expression hypothesis suggests that A-type lamins bind to transcription factors and influence their activity – mutant lamins presumably alter specific transcriptional processes and this leads to disease states (Cohen et al., 2001). Our new findings now suggest that these two models are convergent, because lamin-A mutants, which either fail to modify a structure (the lamina) or inappropriately maintain a structure (the LAP2α nucleoskeleton), also inhibit specific differentiation pathways. Thus, a more appropriate hypothesis for laminopathies might be that structural integrity and modification of the lamina-nucleoskeleton is essential for both cellular differentiation and survival.

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