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The prelamin A pre-peptide induces cardiac and skeletal myoblast differentiation

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Abstract

Prelamin A processing is unique amongst mammalian proteins and results in the production of a farnesylated and carboxymethylated peptide. We examined the effect of pathogenic *LMNA* mutations on prelamin A processing, and of the covalently modified peptide on cardiac and skeletal myoblast differentiation. Here we report a mutation associated with dilated cardiomyopathy prevents prelamin A peptide production. In addition, topical application of the covalently modified C-terminal peptide to proliferating skeletal and cardiac myoblasts induced myotube and striated tissue formation, respectively. Western blot analysis revealed that skeletal and cardiac myoblasts are the first cell lines examined to contain unprocessed prelamin A, and immunostaining of peptide-treated cells revealed a previously unidentified role for prelamin A in cytoskeleton formation and intercellular organization. These results demonstrate a direct role for prelamin A in myoblast differentiation and indicate the prelamin A peptide may have therapeutic potential.

Keywords: Lamin; Cytoskeleton; Differentiation; Farnesylation; Muscular dystrophy; Cardiomyopathy

Lamins are the oldest member of the intermediate filament (IF) family of proteins and are the major constituents of the nuclear lamina; a contiguous meshwork of protein filaments that underlies and is connected to the inner nuclear membrane. Lamins A and C are expressed from the *LMNA* gene by alternative splicing concomitantly with differentiation in all cell types that form tissue [1]. Lamin A is expressed as pre-protein which undergoes a sequential series of post-translational modifications leading to the farnesylation and carboxymethylation of the C-terminal cysteine residue (Supplementary Fig. 1). While the farnesylation of small G proteins such as Ras leads to membrane binding and activation, prelamin A processing is unique amongst mammalian proteins in that the final step in prelamin A processing is an endoproteolytic cleavage which results in the production of mature lamin A and the release of the farnesylated and carboxymethylated C-terminal pre-peptide. This peptide shares structural homology with the yeast **a**-factor mating pheromone, and sequence homology [2] and complementation analysis [3] indicate that prelamin A processing is evolutionarily related to **a**-factor processing in yeast (Supplementary Fig. 1).

Mutations in the *LMNA* gene have been shown to be responsible for at least 10 different human diseases (reviewed in [4]), including dilated cardiomyopathy with conduction defects (DCM) [5,6], Emery–Dreifuss muscular dystrophy [7] and limb-girdle muscular dystrophy [8], indicating a role for *LMNA* in normal cardiac and skeletal muscle growth and repair. As myoblast differentiation involves cellular events that appear to be functionally analogous to those involved in **a**-factor induced yeast mating and shares downstream regulatory

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pathways (Supplementary Fig. 2), we hypothesized that the covalently modified prelamin A C-terminal peptide is the mammalian homologue of the farnesylated and carboxymethylated **a**-factor mating pheromone and might function analogously as an extracellular signal for myoblast differentiation.

In order to test this hypothesis, we examined the effect of *LMNA* mutations associated with DCM on prelamin A expression and processing, and the ability of the chemically synthesized farnesylated and carboxymethylated prelamin A C-terminal peptide to induce cardiac and skeletal myoblast differentiation. In addition, we examined the expression and localization of endogenous prelamin A in differentiating skeletal and cardiac myoblasts. Here we describe a mutation associated with DCM that prevents prelamin A peptide production and show that the covalently modified prelamin A peptide induces cardiac and skeletal myoblast differentiation.

Materials and methods

Cloning and mutagenesis. The wild type human prelamin A cDNA was sub-cloned from pMMLA [9] into pAlter-1 (Promega) and the point mutations C178G, T254G, C585G, A608G, G266T, and G1130A were independently introduced by site-directed mutagenesis. The cDNAs were cloned in-frame with the N-terminal GFP coding sequence of pEGFP-C1 (Clontech) and sequenced in their entirety.

Cells and transfections. Cells were cultured in DMEM containing 10% (v/v) FBS (Gibco). Differentiation via serum starvation was induced by replacing the 10% FBS in the growth media with 2% horse serum. Effectene (Qiagen) was used to transfect plasmid DNA into cells following the manufacturer's instructions. Non-differentiating transfected cells were allowed to continue growing for an additional 24 h before being processed for microscopy or protein isolation.

Western blot analysis. Cells were harvested in lysis buffer containing 1% Triton X-100 for 10 min on ice. Total protein concentration was determined using Bio-Rad Protein Assay reagent (Bio-Rad). Equal amounts of total protein were separated by SDS–PAGE, transferred to nitrocellulose membranes, and probed with a human-specific anti-lamin A/C monoclonal antibody (Novocastra), species cross-reactive lamin A/C polyclonal antibody (Santa Cruz), anti-prelamin A polyclonal antibody (Santa Cruz) or anti-myosin heavy chain antibody (Santa Cruz). HRP-conjugated secondary antibodies were used (Santa Cruz, Pierce), and secondary antibody detection was performed using Super Signal HRP (Pierce).

Peptide synthesis and treatment. The farnesylated and carboxymethylated human prelamin A C-terminal peptide N—LLGNSSPRTQSPQNC (S-Farnesyl OMe) was generated by FMOC solid phase peptide synthesis (Biosynthesis, Inc.). Peptide purity and structure were confirmed by HPLC and laser desorption mass spectrometry, and the lyophilized peptide was resuspended in DMSO at 10 mg/ml. For peptide treatment, cells were grown to 50% confluence, growth media were decanted, and cells were exposed to the peptide in complete growth media containing 10% FBS. The percentage of fused cells was calculated from multiple independent microscopy fields after immunostaining for desmin.

Microscopy and immunocytochemistry. Cells were fixed in ice cold 70% methanol/30% acetone solution for 10 min. Immunostaining was performed with anti-human lamin A/C polyclonal antibody (Novocastra), polyclonal anti-prelamin A antibody (Santa Cruz), or polyclonal anti-desmin antibody (Sigma–Aldrich). Secondary antibodies used were conjugated to Fluorescein or Texas Red (Vector) and coverslips were mounted in anti-fade media containing DAPI. Images were captured on an Olympus IX81 and IX70 microscope.

Results and discussion

Pathogenic LMNA mutations result in aberrant nuclear lamina formation in skeletal myoblasts and differentiating myotubes

In order to examine the effects of pathogenic *LMNA* mutations on prelamin A expression and processing, N-terminal GFP-prelamin A fusion protein expression constructs were generated and used to express both wild type prelamin A fusion protein and mutant variants containing the previously described R60G, L85R, N195K, E203G [5], R89L and R377H [10] mutations that cause DCM in proliferating C2C12 skeletal myoblasts.

Fluorescence microscopy revealed all of the mutant fusion proteins were expressed and localized properly to the cell nucleus (Supplementary Fig. 3). Expression of prelamin A containing the N195 K mutation and the R89L mutation resulted in the formation of intranuclear lamin aggregates (Fig. 1D and G) in almost all transfectants (Supplementary Fig. 4A). When transfected myoblasts were induced to differentiate by the standard laboratory procedure of replacing the fetal bovine serum (FBS) in the growth media with 2% horse serum, the N195K and R89L mutations continued to result in the formation of nuclear GFP-fusion protein aggregates (Fig. 1E, F, H, I and Supplementary Fig. 4A), demonstrating that aberrant lamin organization resulting from *LMNA* disease mutations persists during myoblast differentiation. In addition, expression of these mutations resulted in thickened and branched myotubes with aberrant intercellular organization (Fig. 1E, H and Supplementary Fig. 4B), demonstrating that some *LMNA* mutations that cause DCM inhibit normal skeletal myoblast differentiation. These findings are consistent with the observation that myoblasts isolated from the Bio 14.6 dystrophic hamster with mutations in the δ-sarcoglycan gene, which develops cardiac and skeletal muscle pathologies of Duchenne and limb girdle muscular dystrophies, also differentiate into myotubes which display an aberrant cell morphology [11].

A DCM mutation prevents prelamin A processing and skeletal myoblasts contain unprocessed prelamin A

Fusion protein expression was next examined by Western blot analysis of human HeLa cervical carcinoma cell transfectants with an antibody that recognizes a common epitope shared by lamins A and C. In addition to the endogenous HeLa lamins A and C proteins that were detected in all samples, a band of the predicted size corresponding to the GFP-fusion protein was detected in all samples except the untransfected control (Supplementary Fig. 5A). The fusion protein containing the R89L mutation displayed a retarded electrophoretic mobility as compared to the other mutant fusion protein constructs and the wild type control, indicating this mutation may affect prelamin A processing.