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A-type lamins are essential for TGF-β1 induced PP2A to dephosphorylate transcription factors

J.H. Van Berlo[1,4,†], J.W. Voncken[2,†], N. Kubben[1,2,4], J.L.V. Broers[3], R. Duisters[1,4], R.E.W. van Leeuwen[1,4], H.J.G.M. Crijns[1,4], F.C.S. Ramaekers[3], C.J. Hutchison[5] and Y.M. Pinto[1,4,*]

[1]Experimental and Molecular Cardiology, Cardiovascular Research Institute Maastricht, [2]Department of Molecular Genetics, Research Institute Growth and Development and [3]Department of Molecular Cell Biology, Cardiovascular Research Institute Maastricht, University Maastricht, Maastricht, The Netherlands and [4]Department of Cardiology, University Hospital Maastricht, Maastricht, The Netherlands and [5]School of Biological and Biomedical Sciences, The University of Durham, Durham, UK

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Diseases caused by mutations in lamins A and C (laminopathies) suggest a crucial role for A-type lamins in different cellular processes. Laminopathies mostly affect tissues of mesenchymal origin. As transforming growth factor-β1 (TGF-β1) signalling impinges on the retinoblastoma protein (pRB) and SMADs, we tested the hypothesis that lamins modulate cellular responses to TGF-β1 signalling, via the regulation of these transcription factors in mesenchymal cells. Here, we report that A-type lamins are essential for the inhibition of fibroblast proliferation by TGF-β1. TGF-β1 dephosphorylated pRB through PP2A, both of which, we show, are associated with lamin A/C. In addition, lamin A/C modulates the effect of TGF-β1 on collagen production, a marker of mesenchymal differentiation. Our findings implicate lamin A/C in control of gene activity downstream of TGF-β1, via nuclear phosphatases such as PP2A. This biological function provides a novel explanation for the observed mesenchymal dysfunction in laminopathies.

INTRODUCTION

The LMNA gene encodes different karyoskeletal proteins, lamin A, lamin Adel10, lamin C and lamin C2, which are the products of alternative splicing. These A-type lamins form a meshwork at the inner surface of the inner nuclear membrane known as the lamina. Formation of the lamina involves structural proteins such as emerin, nesprin, LAP2α and lamin B (1), which bind to lamins A and C. A multitude of other protein classes bind to the lamina, ranging from signalling proteins to chromatin and transcription factors. Among the latter are MOK2, SREBP1a/c and pRB (1). However, the functional relevance of the physical interaction between lamins and transcription factors remains unknown. Lamins are directly involved in mitosis and apoptosis, mainly because the lamina needs to disintegrate in order for mitosis or apoptosis to take place (2). In addition, lamin A/C levels correlate with the proliferative capacity of cells: lamin expression is upregulated in highly differentiated cells, whereas their expression is lower in cells with a higher proliferative capacity (3,4).

Mutations in the LMNA gene are implicated in at least nine clinically distinct diseases which affect different tissues in varying degrees (5–14). Currently, two main hypotheses regarding the pathophysiology of laminopathies have been proposed. The first hypothesis stresses a structural role for lamins and proposes that lamins are crucial for structural integrity of the nucleus and hence that mutated lamins may disrupt nuclear architecture. The second hypothesis emphasises the putative role of lamins in transcription and proposes that mutations in lamin A/C cause alterations in gene regulation.

*To whom correspondence should be addressed at: Experimental and Molecular Cardiology, Cardiovascular Research Institute Maastricht (CARIM), University Maastricht, P. Debyelaan 25, PO Box 5800, 6202 AZ Maastricht, The Netherlands. Tel: +31 433877097; Fax: +31 433871055; Email: y.pinto@cardio.azm.nl
†The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

Despite the large variation in diseases caused by lamin A/C mutations, most tissues affected are from mesenchymal origin and increased collagen and tissue fibrosis are common to most of these diseases (6,15–18). On the basis of this notion, we explored the hypothesis that lamins A and C are involved in the regulation of proliferation and differentiation of mesenchymal cells through the modulation of critical transcription factors.

RESULTS

Lamin A/C is important for regulation of proliferation

We first studied cell cycle parameters in mouse embryonal fibroblasts (MEFs) derived from lamin A/C null mutant mice and wild-type controls. Lamin deficient MEFs ($LMNA^{-/-}$ MEFs) showed increased proliferation when compared with wild-type control cells ($LMNA^{+/+}$ MEFs) determined by MTS assay, which directly measures changes in cell numbers (Fig. 1A). To assess which phase of the cell cycle was affected by loss of lamins A and C, cells were pulse labelled with BrdU. BrdU incorporation data corroborate the notion of an increased proliferative rate: S-phase progression of $LMNA^{-/-}$ MEFs was significantly shortened (Fig. 1B). S-phase progression is regulated by progressive phosphorylation of pRB (19). We next examined LMNA wild-type and deficient cells for altered pRB-phosphorylation: (serum starved) G1-arrested cells revealed increased phosphorylation of the retinoblastoma protein in $LMNA^{-/-}$ MEFs compared with similarly treated control $LMNA^{+/+}$ MEFs (Fig. 1C). As phosphorylation of pRB is functionally linked to G1/S transition (20), these findings are consistent with a more rapid transit through S-phase and suggested a loss of normal cell cycle control in cells lacking lamins A and C.

The aforementioned cell lines were used before to address the role of lamins in cell biology (21,22) but were independently immortalized, which may affect cell cycle characteristics of these cells differently. To address the role of LMNA in cell cycle regulation in an unbiased fashion, we therefore generated a genetically matched cell model: tagged-lamins A and C were stably re-introduced in $LMNA^{-/-}$ cells using retroviral expression vectors. Re-expression of lamins A and C, either alone or in combination (latter data not shown), resulted in the expected nuclear localization of the tagged lamins (Fig. 1D and E). We next studied DNA synthesis in the lamin A and/or C-reconstituted $LMNA^{-/-}$ MEFs as a measure for proliferation. Re-expression of lamin A or C, either alone or in combination, significantly reduced proliferation rates to $<80\%$ of their empty vector-infected controls (Fig. 1F). Taken together, the previous findings support an important involvement of lamin A/C in control of fibroblast proliferation.

Lamins A and C modulate TGF-β1 signalling

Having established an effect of lamin A/C expression levels on cell cycle regulation, and given the observation that laminopathies (i.e. LMNA dysfunction) affect mainly tissues from mesenchymal origin, we next focussed on transforming growth factor-β1 (TGF-β1) signalling. The cell cycle regulatory effects of TGF-β1 on cells depend on differentiation-state and origin of the target cell (23): TGF-β1 stimulates cell proliferation in undifferentiated cells, whereas it blocks the cell cycle progression in highly differentiated cells (24). Independently, it is known that the level of lamin A/C expression is highly related to the degree of differentiation (3,4). The analogy between these observations suggested a possible functional relationship between TGF-β1 signalling and the presence of A-type lamins: TGF-β1 may negatively regulate cell cycle progression in the presence of lamins A and C, whereas may fail to do so or stimulate cell proliferation in the absence of lamins A and C. To study lamin dependency of TGF-β1 signalling in relation to cell cycle control, we used the genetically matched $LMNA^{-/-}$ MEF model with or without reconstituted lamin A and/or C expression. DNA synthesis was quantified by measuring [^{3}H]-Thymidine incorporation during the first 6 h after release and again between 18 and 24 h after release, so that an incorporation ratio (late over early interval) of larger than one indicates net DNA synthesis. In empty vector-infected $LMNA^{-/-}$ MEFs, TGF-β1 does not reduce DNA synthesis (Fig. 2A), whereas addition of TGF-β1 strongly reduced DNA synthesis in lamin reconstituted cells. In cells re-expressing lamin C or lamins A + C, the effect of TGF-β1 addition was more pronounced than that in lamin A only reconstituted cells (Fig. 2A). In conjunction with our earlier observation that lack of lamin C or A+C affects MEF proliferation more than that of lamin A only (Fig. 1F), the previous data suggest that the loss of lamin C in our model systems is more critical than loss of lamin A, although lamin A contributes to the modulation of TGF-β1 induced signalling. The combined presence of both lamins was most effective in the inhibition of the cell proliferation in response to TGF-β1.

To independently prove that the earlier described differences in proliferation are indeed caused by lamins, we generated genetically matched 3T3 fibroblasts in which lamin A/C protein levels were significantly reduced (knocked-down) by stable RNA interference through retroviral expression of short hairpin RNAs (shRNA) (Fig. 2B). Consistent with the MEF data (Figs 1 and 2A), DNA synthesis was increased in 3T3 fibroblasts in which lamin expression was knocked-down (lamin A/C-KD): upon stimulation of (serum starved) G1-arrested fibroblasts with 10% fetal bovine serum (FBS), lamin A/C-KD fibroblasts show significantly higher basal DNA synthesis level compared with empty vector-infected fibroblasts (Fig. 2C).

In addition, the inhibitory response to TGF-β1 was also dependent on lamins in the 3T3 model system: consistent with our observations in the genetically matched MEF model, indeed, TGF-β1 blocks DNA synthesis in LMNA wild-type 3T3 cells, whereas in the lamin A/C-KD 3T3 cells, TGF-β1 induced a 58% increase in DNA synthesis ($P < 0.01$ for difference in response, Fig. 2D). These data provide independent genetic proof that cell cycle progression is altered in lamin deficient mesenchymal cell types.

Phosphorylation of pRB is altered in absence of lamin A/C

It is known that pRB is essential for TGF-β1-induced growth inhibition (25). Prompted by our finding that the effect of TGF-β1 on the cell proliferation depends on the presence of

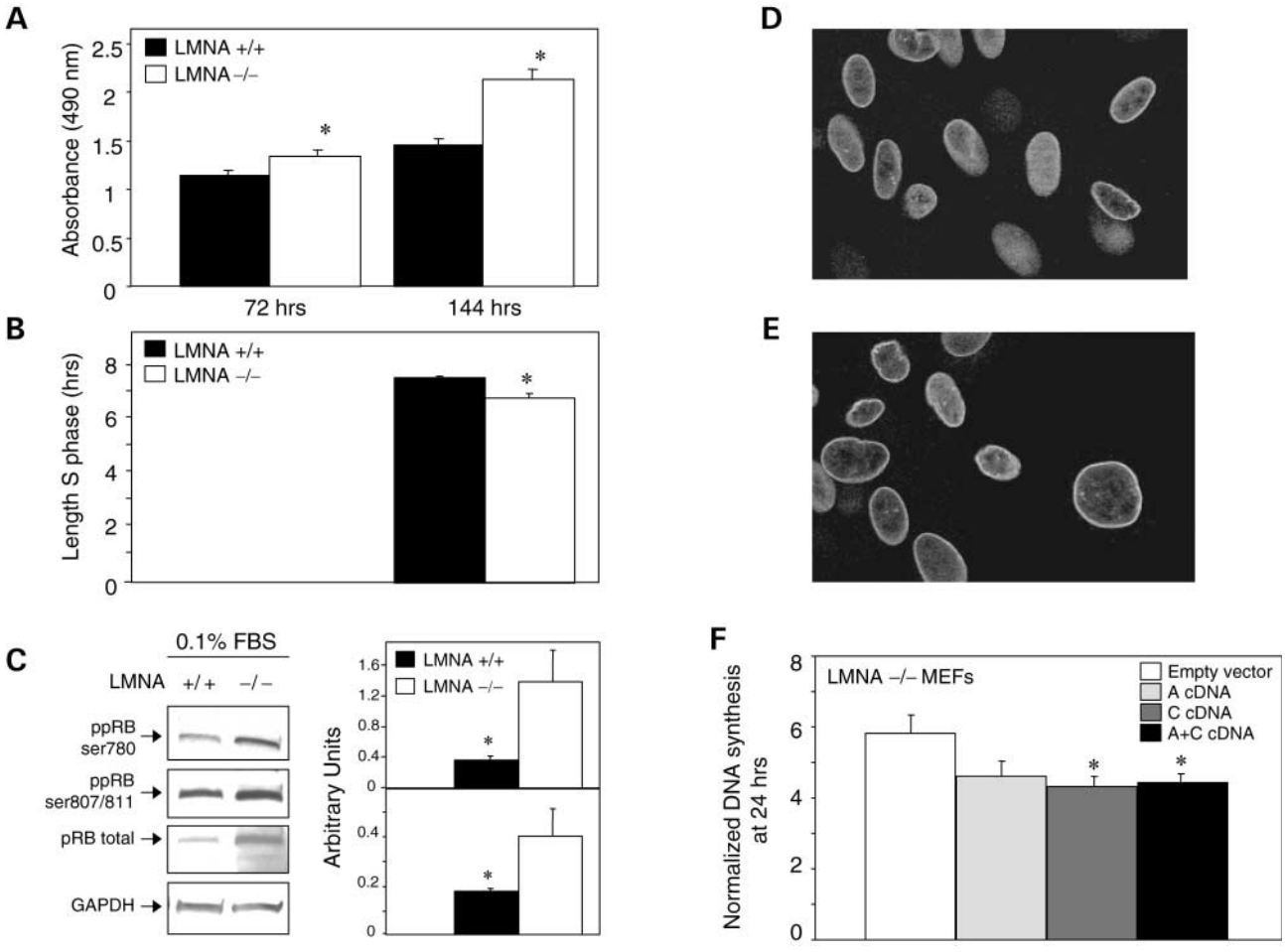

Figure 1. Loss of lamin A/C increases cell proliferation. (**A**) Proliferation of LMNA$^{-/-}$ (null) MEFs is significantly increased when compared with LMNA$^{+/+}$ (wild-type) MEF controls [n=8 for each bar, asterisk indicates $P < 0.01$ (Student's t-test)]. Cell proliferation was determined 72 and 144 h after plating of cells by MTS assay (see Materials and Methods) of non-synchronized cells in medium supplemented with 10% FBS. (**B**) Pulse chasing with BrdU reveals a significantly shortened S-phase (10%) in LMNA$^{-/-}$ MEFs when compared with LMNA$^{+/+}$ MEFs [$n = 2$, 6, respectively, asterisk indicates $P < 0.05$ (Student's t-test)]. (**C**) Synchronized (serum starved: 0.1 % FBS in figure) LMNA$^{-/-}$ MEFs show increased pRB phosphorylation when compared with synchronized LMNA$^{+/+}$ MEFs. In LMNA$^{-/-}$ MEFs, ppRBser780 and ppRBser807/811 were both significantly higher when normalized for total pRB and GAPDH as indicated by the two right panels (upper: ppRBser780/total pRB, $n = 4$; lower: ppRBser807 + 811/total pRB, $n = 3$). Antisera used—immunodetection: α-ppRB ser780, α-ppRB ser807/811, α-total-pRB (all polyclonal), α-GAPDH 6C5; asterisk indicates $P < 0.05$. Reconstitution of (HA-tagged) lamins A (**D**) and C (**E**) expressions in LMNA$^{-/-}$ MEFs. Immunostaining with an α-HA antibody reveals normal nuclear localization of both recombinant lamins A/C (magnification 400×). (**F**) Lamin A/C-reconstituted genetically matched MEFs show a significantly decreased cellular proliferation rate. Genetically matched, serum starved cells (LMNA-KO plus empty vector, A, C or A+C MEFs) were incubated with 10% FBS for 24 h. Cell proliferation was determined by measuring [^{3}H]-Thymidine incorporation during the first 6 h (t0–t6) of 10% FBS incubation after serum starvation and again during the last 6 h (t18–t24). Incorporation rates during (t18–t24) were normalized to those during (t0–t6); (Empty vector, empty vector-infected LMNA$^{-/-}$ MEFs; A/C cDNA, LMNA$^{-/-}$ MEFs with re-expression of lamin A and/or C; n=6 for all cell types, asterisks indicate $P < 0.05$).

lamins, we investigated whether pRB phosphorylation was altered in response to TGF-β1 induction. Cell cycle arrest requires hypo-phosphorylation of pRB; we, therefore, investigated the phosphorylation status of pRB in response to TGF-β1 in relation to presence or absence of lamin A/C. In lamin A/C rescued LMNA$^{-/-}$ MEFs, TGF-β1 induced dephosphorylation of pRB within 15 min, whereas this dephosphorylation was not seen in empty vector-infected LMNA$^{-/-}$ MEFs (Fig. 3A and F). Importantly, also in the 3T3 fibroblasts with knock-down of lamin A/C, we observed increased levels of ppRB795 compared with control fibroblasts in response to TGF-β1 exposure (data not shown). These findings confirmed that A/C lamins are essential for the pRB-mediated arrest of cell cycle by TGF-β1. Recently published data suggest increased degradation of pRB in absence of lamin A/C as a cause for cell cycle deregulation (22), whereas our data suggest altered pRB phosphorylation underlies the observed proliferative defect. Although both independent genetically-matched model systems described in this report, reconstituted genetically-matched LMNA$^{-/-}$ MEFs (Fig. 1C) and 3T3 LMNA-KD model (Fig. 2B), do not show any effect on pRB stability, both decreased pRB protein levels and diminished pRB phosphorylation are expected to have a similar effect on cell proliferation.

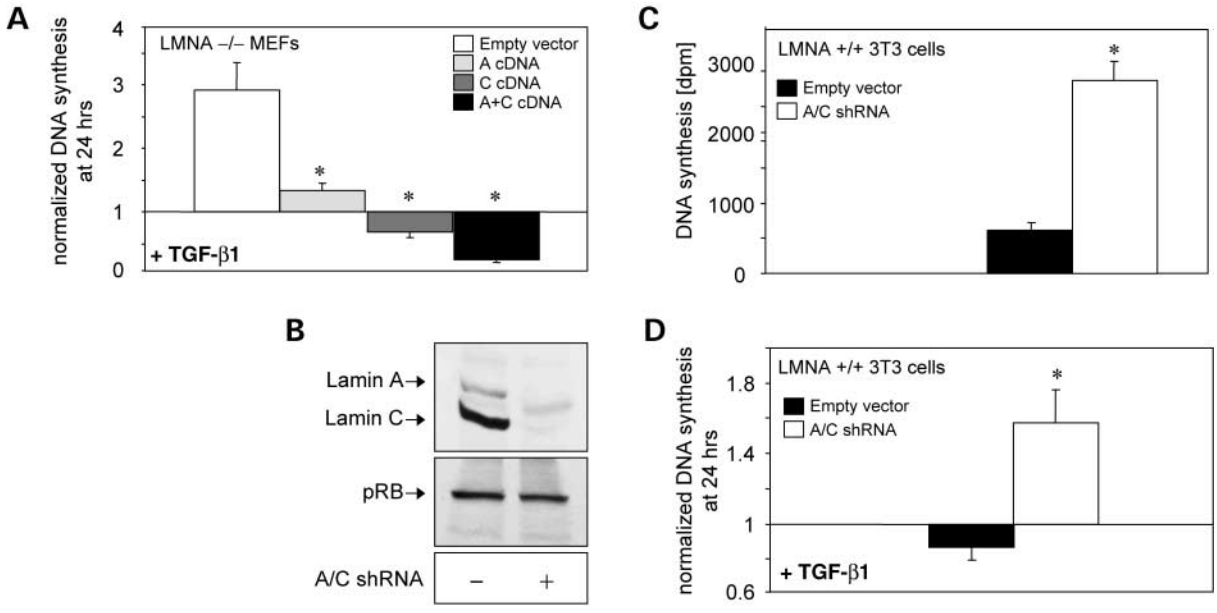

Figure 2. TGF-β1 signalling is modulated by Lamin A/C. (**A**) Lamin A/C-reconstituted $LMNA^{-/-}$ MEFs show a lamin dependent decrease in cell proliferation. Genetically matched, serum starved cells (LMNA-KO plus empty vector, A, C or A+C MEFs) were incubated with 1 ng/ml TGF-β1 for 24 h; [^{3}H]-Thymidine incorporation was determined during the first 6 h (t0–t6) and again during the last 6 h (t18–t24) of the experiment. The incorporation during (t18–t24) over that during (t0–t6) was determined; a ratio of one implies no increase in DNA synthesis (n=6 for all cell types, asterisk indicates $P < 0.01$ in comparison to LMNA). (**B**) Stable knock-down of murine lamin A/C expression in 3T3 fibroblasts. Cells were stably infected with a lamin A/C shRNA–pRetrosuper vector and selected. Upper panel: stable knock-down after 3 months of continuous culturing; antiserum used: lamin A/C (346); lower panel: total pRB levels are unaffected after 3 months of continuous culturing; antiserum used: pRB G3-245. (**C**) Knock-down of lamin A/C increased [^{3}H]-Thymidine incorporation ~4.5-fold in 3T3 cells stimulated with 10% FBS ($n = 6$, asterisk indicates $P < 0.01$). (**D**) TGF-β1 blocks proliferation in WT 3T3 cells, but not in 3T3-LMNA-KD cells ($n = 6$, asterisk indicates $P < 0.001$). [^{3}H]-Thymidine incorporation was determined as described in (A).

Protein phosphatase 2A binds to lamin A/C and is responsible for TGF-β1 induced dephosphorylation of pRB

The rapid TGF-β1-induced dephosphorylation of pRB suggested the involvement of a protein phosphatase. It is known that TGF-β1 can directly activate PP2A and thereby inhibit cell cycle progression in a post-transcriptional manner (26). Furthermore, it was recently established that PP2A directly dephosphorylates pocket protein family members, among which p107 and pRB (27). Hypo-phosphorylated pRB subsequently binds and blocks sites of DNA replication (28). Because lamin A/C co-localizes at sites of DNA replication (29), we hypothesized that PP2A is responsible for the lamin A/C-dependent dephosphorylation of pRB. This also suggested that lamins, pRB and PP2A might physically associate. We first confirmed that pRB binding to A-type lamins in $LMNA^{+/+}$ MEFs under non-restrictive (i.e. 10% FBS) growth conditions, as shown earlier (30) (Fig. 3B). In addition, we here show the novel finding that also PP2A is part of the lamin–pRB interaction complex, as it co-immunoprecipitates with lamins (Fig. 3C) and pRB (Fig. 3D). The PP2A–pRB interaction was lamin dependent in proliferating cells (Fig. 3D), because PP2A–pRB interaction was lost upon re-expression of lamins A and C (Fig. 3D, second lane). Our data show that the reduced PP2A–pRB interaction can not be explained by competitive binding of PP2A or pRB to lamin: (i) at 0.1% FBS and in the absence of TGF-β1 (Fig. 3E, first and third lanes), the PP2A–pRB interaction is clearly not dependent on the absence of lamin A/C and (ii) TGF-β1 signalling induces a rapid (within 15 min) loss of PP2A–pRB interaction in the presence of lamins under conditions of restrictive growth (0.1% FBS), concomitant with reduced pRB phosphorylation (Fig. 3E, second and fourth lanes). This suggests that PP2A–pRB interaction is at least in part determined by the phosphorylation status of the complex members. Indeed, in the absence of lamins, TGF-β1 fails to induce ppRB dephosphorylation (Fig. 3A); the sustained interaction between pRB and PP2A under conditions of limited proliferation (Fig. 3E) further supports the notion that post-translational modifications play an important role in these protein interactions. Whether the reduction in PP2A–pRB association under 10% FBS (Fig. 3D) and under 0.1% FBS + TGF-β1 (Fig. 3E) is quantitatively similar, and would therefore be the direct cause of TGF-β1 signalling, remains to be further investigated.

The physical interaction between PP2A and pRB suggested a relevant role for PP2A in lamin-dependent TGF-β1-induced dephosphorylation of pRB. To show that PP2A is directly responsible for dephosphorylation of pRB, we measured dephosphorylation of pRB in the presence of okadaic acid (OA), a known selective PP2A inhibitor at the applied concentration (28). In lamin A/C expressing cells, OA prevented TGF-β1-induced dephosphorylation of pRB (Fig. 3F), which underscores the role for PP2A in pRB dephosphorylation in our model system. Taken together, our data provide evidence for a functional association between both A-type lamins, pRB and PP2A, and demonstrate that A-type lamins are needed for

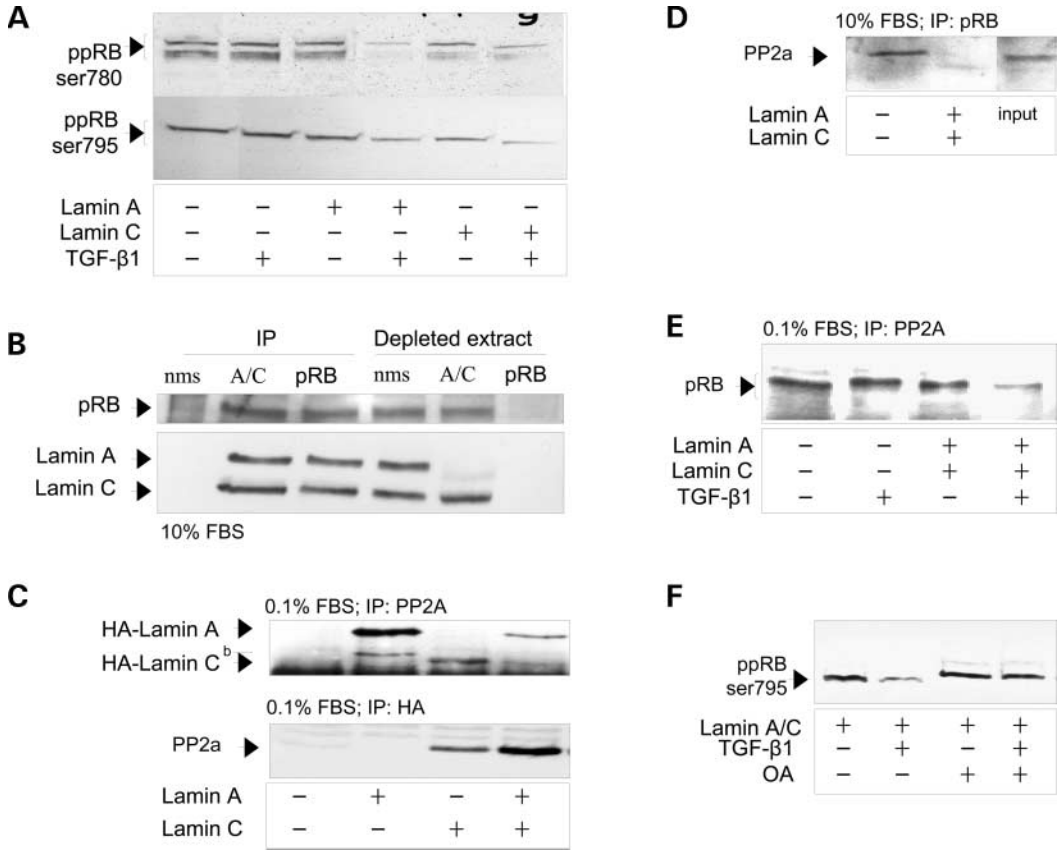

Figure 3. *In vivo* association between lamin A/C, pRB and PP2A; PP2A dephosphorylates pRB in a lamin-dependent fashion. (**A**) TGF-β1 induces rapid dephosphorylation of pRB or shows constitutively reduced phosphorylated pRB in the presence of lamin A or C. In contrast, empty vector-infected LMNA$^{-/-}$ MEFs do not show TGF-β1 induced pRB dephosphorylation. MEFs were serum starved prior to growth factor stimulation (15 min). (**B**) pRB and lamin A/C interact, as demonstrated by reciprocal co-immunoprecipitation (IP) of pRB and lamin A/C in unsynchronized LMNA$^{+/+}$ MEFs (antisera used—IP: nms (normal mouse serum), α-lamin A/C (sc-7293), α-pRB (polyclonal); immunodetection: α-pRB (polyclonal), α-lamin A/C (sc-7293). (**C**) Protein phosphatase 2A (PP2A) is a new interaction partner of lamin A/C. Co-immunoprecipitation shows that PP2A and lamin A/C interact; immunoprecipitation against PP2A co-precipitates lamins A and C (upper panel) from A-type lamin-reconstituted LMNA$^{-/-}$ MEF extracts; reciprocal immunoprecipitation confirms the specificity of the interaction of PP2A with HA-tagged lamins A/C; band indicated by '*b*' is a non-specific band (input showed similar expression of lamin A and/or C and PP2A (data not shown). Antisera used—IP: α-PP2A 5H4, α-HA-lamin A/C 12CA5; immunodetection: α-PP2A 6F9, α-HA-lamin A/C 12CA5. (**D**) In asynchronously growing cells, the interaction between PP2A and pRB is dependent on the presence of lamins A and C. PP2A co-immunoprecipitates with pRB in cycling LMNA$^{-/-}$ MEFs, but not in lamins A and C re-expressing LMNA$^{-/-}$ MEFs (antisera used—IP: α-pRB G3-245; immunodetection: α-PP2A 6F9). (**E**) In serum deprived cells, the interaction between PP2A and pRB is rapidly reduced upon TGF-β1 stimulation in lamin A/C reconstituted LMNA$^{-/-}$ MEFs, but not in LMNA$^{-/-}$ MEFs (antisera used—IP: α-PP2A 6F9; immunodetection: α-pRB G3-245) Immunoprecipitation was carried out 15 min following incubation with or without TGF-β1 (**F**) A crucial role for PP2A in mediating TGF-β1-induced pRB dephosphorylation. OA (125 nM final concentration) effectively inhibits the rapid dephosphorylation (15 min) of pRB induced by TGF-β1, in lamin A+C reconstituted LMNA$^{-/-}$ MEFs. Total pRB was equal throughout the lanes (data not shown; antisera used—immunodetection: α-ppRB ser795 polyclonal).

proper functioning of nuclear PP2A and post-translational regulation of pRB.

Lamins A and C modulate TGF-β1 induced SMAD phosphorylation

A pivotal role for lamins A and C in TGF-β1-induced cell cycle control is demonstrated previously. We next asked whether A-type lamins are also important for other TGF-β1-mediated cellular processes. TGF-β1 has been implicated in tissue fibrosis in various clinical conditions. TGF-β1 increases collagen production by inducing the phosphorylation of (regulatory) rSMADs (31); phosphorylated rSMADs translocate to the nucleus, where they act as regulators of transcription. TGF-β1 receptors remain activated for a few hours, during which they continuously phosphorylate SMADs. As fibrosis is a hallmark of many laminopathies (6,15–18), we setout to investigate whether lamins are implicated in the genesis of tissue fibrosis via aberrant TGF-β1 signalling. We first studied TGF-β1-induced SMAD phosphorylation in the presence and absence of lamins. In LMNA$^{-/-}$ MEFs, TGF-β1 effectively induces phosphorylation of SMAD2 (Fig. 4A) and SMAD3 (data not shown) within 15 min, which indicated that TGF-β1 can activate downstream effector molecules in the absence of lamins. rSMAD phosphorylation kinetics are clearly altered in the absence of lamin: a more rapid and intense phosphorylation of SMAD2 and SMAD3 occurs, which also dissipates faster (Fig. 4A). Phosphorylated SMADs act as co-factors which activate collagen I and III promoters; abnormal phosphorylation of SMAD is expected

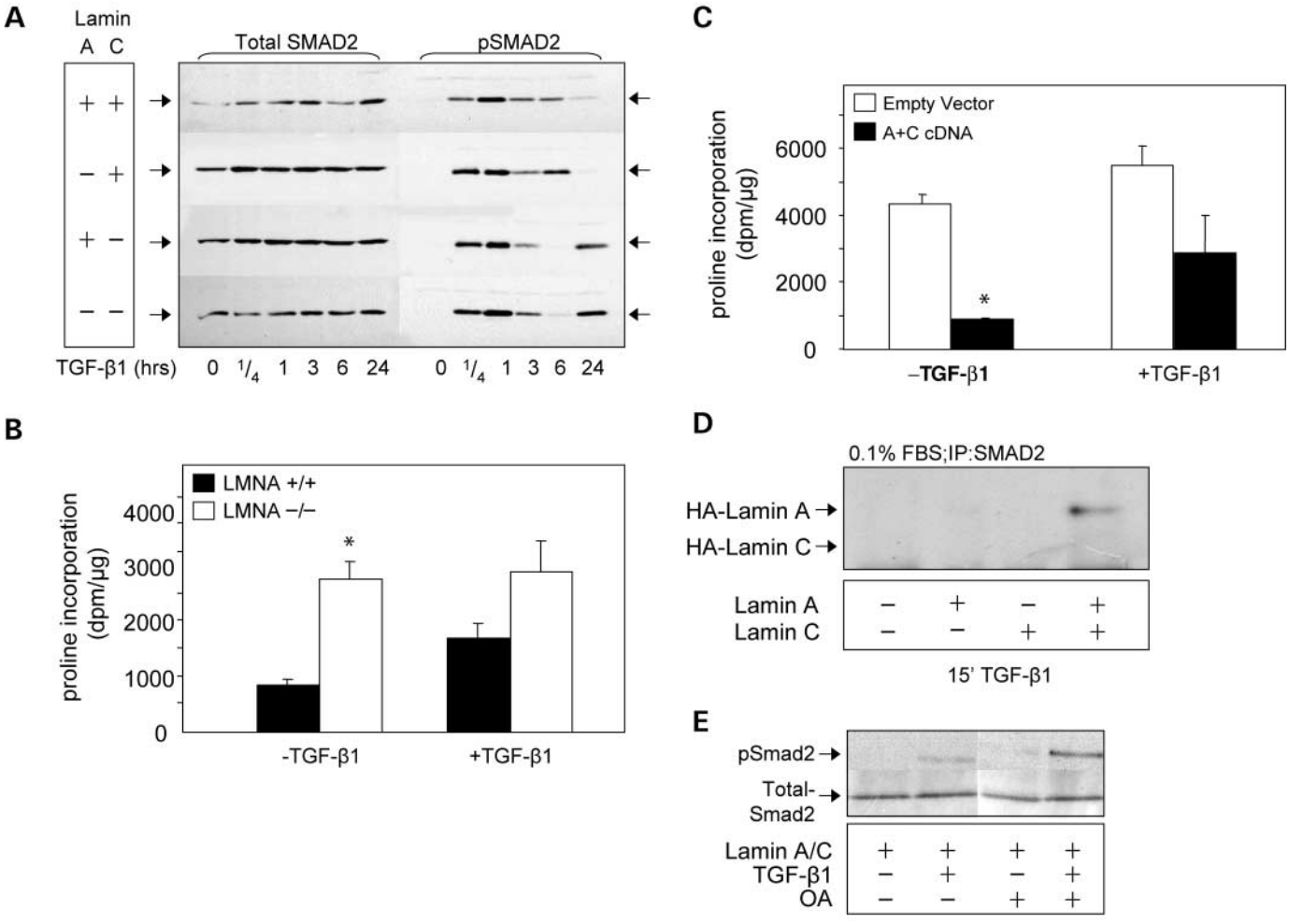

Figure 4. SMAD phosphorylation and collagen production are increased in the absence of lamin A/C. (**A**) In presence of lamins A and C, SMAD2 and SMAD3 (SMAD3 data are not shown) are phosphorylated within 15 min in response to TGF-β1 stimulation of MEFs. SMAD phosphorylation peaks after 1 h and gradually decreases to near basal levels at 24 h. In the absence of both lamins, a more rapid and intense phosphorylation of SMAD2 and SMAD3 occurs, which also dissipates faster. In lamin deficient MEFs, SMAD2 and SMAD3 phosphorylation reappears at 24 h ($n = 3$, representative blot is shown; antisera used—immunodetection: α-SMAD2 polyclonal, α-pSMAD2 polyclonal and α-GAPDH 6C5). (**B**) Increased collagen production in murine fibroblasts in the absence of lamin A/C. Wild-type MEFs show a > 2-fold induction of collagen production upon TGF-β1 induction; basal collagen production is already maximally elevated in $LMNA^{-/-}$ MEFs and is not further induced upon TGF-β1 stimulation. Collagen production was measured by [^{3}H]-Proline incorporation [no TGF-β1 stimulation—$LMNA^{+/+}$, $LMNA^{-/-}$ MEFs: $n = 7$, 9, respectively; asterisk indicates $P < 0.05$; with TGF-β1 stimulation—$LMNA^{+/+}$, $LMNA^{-/-}$ MEFs: $n = 7$, 9 respectively, ($P = 0.06$)]. (**C**) Lamin A/C-reconstituted MEFs show similar characteristics as wild-type MEFs: lamins A and C expression reverts abnormally high collagen production in $LMNA^{-/-}$ MEFs to normal (i.e. wild-type) levels (n=6 for each cell type and each condition). Reconstitution of lamin C expression only in $LMNA^{-/-}$ MEFs reduced collagen synthesis significantly (to 56% of empty vector control; $P < 0.05$) under basal conditions and further to A/C-reconstituted-levels in the presence of TGF-β1, whereas reconstitution of lamin A only did not induce a significant reduction of collagen production (data not shown). (**D**) SMAD2 associates with lamin A/C in reconstituted $LMNA^{-/-}$ MEFs. Only in the presence of both lamins A and C, an association between lamin A and SMAD2 is observed, indicating probable ternary complex formation (antisera used—IP: α-SMAD2 polyclonal; immunodetection: α-HA 12CA5). (**E**) SMAD2 dephosphorylation is inhibited by okadaic acid. Reconstituted $LMNA^{-/-}$ MEFs show a TGF-β1 induced SMAD2 phosphorylation, which is augmented by the inhibition of PP2A (antisera used—immunodetection: α-total SMAD polyclonal, α-phospho-SMAD polyclonal).

to alter collagen expression. To compare collagen production by $LMNA^{-/-}$ and $LMNA^{+/+}$ MEFs, we used Proline incorporation. This shows that collagen production by $LMNA^{-/-}$ MEFs is significantly increased compared with wild-type MEFs (Fig. 4B). TGF-β1 also increased collagen synthesis in $LMNA^{+/+}$ MEFs; basal collagen production was significantly higher in $LMNA^{-/-}$ MEFs and did not further increase in response to TGF-β1. Of relevance, re-expression of lamins A and C in $LMNA^{-/-}$ MEFs, reverted the phenotype to that observed in $LMNA^{+/+}$ MEFs (Fig. 4C). In the presence of lamins A and C, TGF-β1 induces a rapid phosphorylation of rSMAD2, which dissipates slowly between 6 and 24 h (Fig. 4A). In contrast, in the absence of lamins A and C, phosphorylation of SMAD2 peaks at 1 h and is sustained only until 3 h (Fig. 4A). Of notice, in the absence of lamin C, we observed a striking reappearance of SMAD phosphorylation at 24 h. This indicated that the kinetics of SMAD2 phosphorylation were significantly and consistently altered in the absence of lamins A and C. Comparison of SMAD2 and SMAD3 phosphorylation in single lamin reconstituted cells

suggested that lamin C plays a more prominent role in regulation of SMAD2 phosphorylation (Fig. 4A) whereas restoration of SMAD3 phosphorylation kinetics requires both lamins A and C (data not shown). This could reflect functional regulatory divergence between these rSMADs.

To further explore how lamins A and C are involved in the response to TGF-β1, we tested whether SMADs associate with A-type lamins. Our data show that SMAD2 co-immunoprecipitates with lamin A/C (Fig. 4D); the absence of signal in the control immunoprecipitation ($LMNA^{-/-}$ MEFs) shows specificity of the association. Interestingly, the SMAD2–lamin A/C association is only detected in the presence of both lamins A and C, as reconstitution with either lamin type by itself does not support interaction (Fig. 4D). We identified PP2A as a novel interactor of A-type lamins (Fig. 3); this positions PP2A in close proximity to phosphorylated SMAD and potentially implicates PP2A in the regulation of SMADs as well. To test this idea, we assessed the effects of OA treatment on SMAD2 phosphorylation. OA-treated cells show both increased basal and TGF-β1-induced phosphorylated SMAD2 levels (Fig. 4E). These combined results demonstrated that, in analogy to their regulatory role in pRB functioning, A-type lamins are functionally involved in control of SMAD2 phosphorylation. It appears that A-type lamins exert their effect on these nuclear transcription regulators by altering the action of phosphatases, like PP2A, which are crucial for dephosphorylating these transcription factors.

DISCUSSION

We here report that $LMNA^{-/-}$ MEFs display defective transcription factor regulation. First, aberrant pRB phosphorylation, accelerated S-phase transition and increase in cell numbers in genetically matched, synchronized cell cultures point toward a cell cycle regulatory defect in $LMNA^{-/-}$ MEFs. In addition, we find that $LMNA^{-/-}$ MEFs lack the proper inhibitory signalling that is normally activated in response to TGF-β1. This lack of inhibitory signalling may contribute to the increased proliferation of $LMNA^{-/-}$ MEFs. Defective TGF-β1 signalling also results in the increased production of collagen. These abnormal characteristics of $LMNA^{-/-}$ MEFs are effectively corrected by stable re-introduction of both lamins A and C expressions. The observation that the phenotypic rescue is less pronounced in $LMNA^{-/-}$ MEFs re-expressing only lamin A or C may indicate the need for the combined presence of both lamins A and C.

Our findings provide the first evidence that lamins A and C modulate downstream effects of TGF-β1 signalling. Present concepts on the biological role of lamin A/C either highlight their role in maintaining integrity of nuclear structure or a thus far hypothetical role for lamin A/C in gene regulation (32). We here propose a novel hypothesis that links the 'structural' and 'gene regulation' concepts. It may help to explain why mutations in lamin A/C have such distinct clinical consequences and underscores the important role of lamins A/C in mesenchymal biology (33). Our findings also shed new light on earlier published data. For instance, in a first description of the cellular role of lamins, Lammerding *et al.* (21) reported that lamins A and C are important to maintain nuclear stability and revealed altered NFκB activity in $LMNA^{-/-}$ MEFs (21). Our data suggests a possible explanation for the altered NFκB activity in $LMNA^{-/-}$ MEFs as the lack of dephosphorylation of relA, the functional nuclear subunit of NFκB in these cells, may explain the observed altered NFκB activity.

To explain the loss of TGF-β1 modulation at the molecular level, it is conceivable that lamins function to position PP2A as a regulator of incoming TGF-β1 signalling: via PP2A-dependent dephosphorylation pRB and other transcription regulators like SMAD2, TGF-β1 signalling is quenched (see hypothetical model; Fig. 5). Absence of A-type lamins interrupts the negative regulatory action of TGF-β1. Although the exact molecular mechanism of this negative action is currently not known, it is possible that A-type lamins provide an important docking site for PP2A or one of its subunits, e.g. PP2A subunit A, B or C or alternatively ppRB. A link between failure to respond to the growth inhibitory properties of TGF-β1 and loss of A-type lamins is not without precedent. Some metastatic cancer cells show strongly reduced lamin A/C expression, which is paralleled by insensitivity to TGF-β1 induced cell cycle arrest (24). In line with this, we find that in the presence of lamins, TGF-β1 signalling rapidly diminishes the interaction between PP2A and pRB, while ppRB is concomitantly dephosphorylated. In contrast, in $LMNA^{-/-}$ cells, ppRB dephosphorylation is abrogated; the sustained PP2A–pRB interaction suggests this interaction does not lead to dephosphorylation of ppRB. Our observation that PP2A preferentially co-immunoprecipitates ppRB (Fig. 3D) and the finding that A-type lamins preferentially interact with hypo-phosphorylated pRB (34) support such a model. We propose that PP2A/lamin A/C docking is needed to restore pRB functionality (i.e. transcriptional repression and consequent cell cycle control), by rapid dephosphorylation of ppRB in response to TGF-β1. Lack or mutation of A-type lamins may leave PP2A/pRB complexes unable to inactivate relevant targets, as a result of improper sub-nuclear localization of one or both factors. It is plausible that a transient ternary complex formation between lamins, PP2A and pRB is needed for proper transcriptional repression.

Furthermore, it is known that TGF-β1 can act through SMAD-independent pathways (26). One of the SMAD-independent pathways employs the phosphatase PP2A. TGF-β1 activates a B-subunit of PP2A. This B-subunit then forms a ternary structure with the A/C dimer of PP2A. Most likely, this also happens in our model. TGF-β1 activates both SMADs and simultaneously a B-subunit of PP2A, which then activates PP2A to dephosphorylate nuclear SMAD and/or pRB. Phosphorylation of pRB most likely involves cyclins D1 and E, which activate cyclin-dependent kinases to phosphorylate pRB. However, dephosphorylation of pRB is probably, in part, dependent on TGF-β1. This will probably be regulated in a similar manner as the dephosphorylation of SMAD; TGF-β1 activates a B-subunit, which in turn activates PP2A to dephosphorylate pRB. Recently, PP2A was described as an important element in the equilibrium of pRB phosphorylation (35). Which PP2A subunit is responsible for nuclear PP2A activity is unknown. PR59 is a candidate, as it has been described to associate with pRB (27).

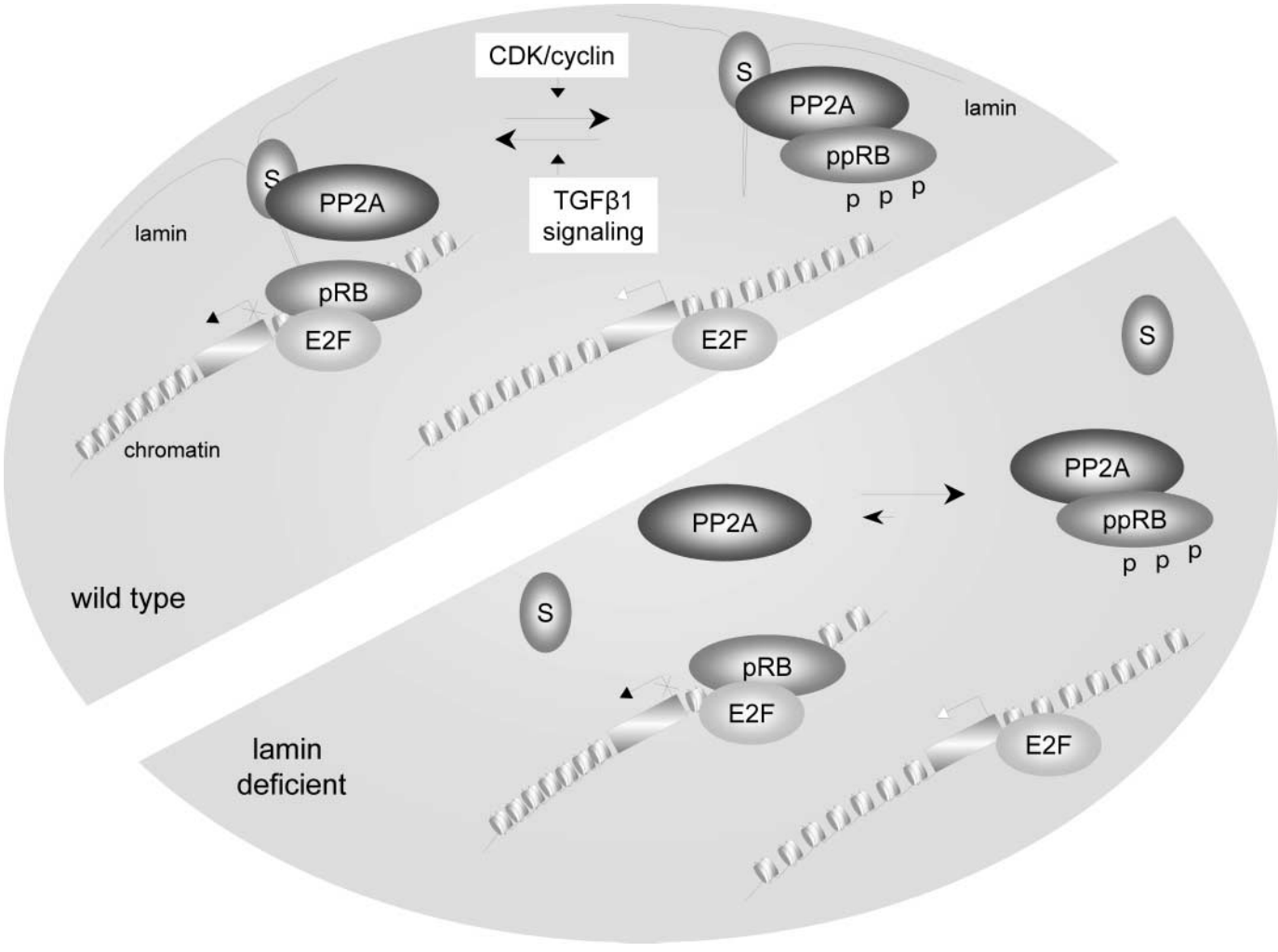

Figure 5. Hypothetical model for lamin-dependent gene regulation via PP2A and pRB. In this hypothetical model, PP2A and pRB are used to illustrate how lamins may affect transcriptional regulation. In resting wild-type cells (upper left), both PP2A and hypo-phosphorylated pRB are bound to lamin A/C; however, interaction between PP2A and pRB is low. Mitogenic signalling induces cyclin/CDK-dependent pRB phosphorylation, resulting in the release of transcriptional repression by pRB and activation of E2F-dependent gene expression, for example. TGF-β1 counteracts mitogenic signalling by stimulating PP2A activation: phospho-pRB (ppRB) and PP2A interact and PP2A rapidly dephosphorylates ppRB, thereby restoring its interaction with lamin A/C and effectively silencing E2F-dependent transcription. The mechanism by which TGF-β1 signalling induces nuclear PP2A activity remains unresolved; it may, however, depend on binding of and/or activation by crucial regulatory units (symbolized by S in the diagram), like PR5g(27). In absence of lamins (lower right), pRB may still bind E2F. However, incoming mitogenic signals that promote E2F-dependent cell cycle progression are ineffectively quenched: in lamin deficient cells, phospho-pRB is inefficiently dephosphorylated, despite increased PP2A/pRB interaction. Consequently, there may not be sufficient hypo-phosphorylated pRB available or alternatively, pRB may be mislocalized in the nucleus. It is likely that also docking of PP2A to lamin A/C is required for proper catalytic activation. Both are expected to result in failure to repress E2F-dependent gene targets, resulting in increased proliferation of lamin A/C deficient cells. This hypothetical model integrates hypotheses on the role of lamins both regarding structure and transcriptional regulation.

Recent reports suggest that MAN1, an integral protein of the inner nuclear membrane with physical connections to lamin A/C, is involved in TGF-β1 signalling through binding and possibly the regulation of SMAD2 and SMAD3 (36,37). Although whether and how MAN1 is involved in laminopathies has yet to be resolved, these reports corroborate our findings that nuclear membrane associated proteins represent an important site of regulation of transcription factor activity. Furthermore, the finding that MAN1 may regulate SMAD2 and SMAD3 could, in part, explain the observations in SMAD phosphorylation here described. In the absence of lamin C, we observed a striking reappearance of SMAD phosphorylation at 24 h. This latter somewhat surprising finding may be due to a loss of regulation of SMAD phosphorylation via factors like MAN1. MAN1 interacts with lamins. It is possible that MAN1 preferentially binds lamin C or that it needs at least lamin C to function properly. This might explain why in presence of lamin C, SMAD phosphorylation might be regulated more in accordance with the wild-type situation.

Our data suggest a novel mechanism for the pathogenesis of laminopathies. A key element of many diseases caused by mutations in lamin A/C is tissue fibrosis (6,15–18). The mechanism we describe here proposes that lamin A/C functions as a

vital regulatory factor of nuclear processes downstream of TGF-β1 signalling. Without lamin A/C, nuclear signalling is enhanced due to sustained activation (i.e. decreased dephosphorylation) of transcriptional regulators. As a consequence fibroblasts show higher proliferation rates and produce more collagen. In patients with a lamin A/C gene mutation this may result in enhanced cell turnover and increased fibrosis. Interestingly, these are also characteristic clinical features of another lamin A/C related disease: progeria. The enhanced cell turnover, accelerated ageing, and accompanying increased fibrosis, typical for progeria, suggest a common link to lamin dysfunction. In addition, a mouse model for progeria shows increased collagen deposition in the skin and increased numbers of fibrocytes in the heart (38). Evidently, this conjecture requires experimental confirmation. The important clinical implication is that if fibrosis in laminopathies is confirmed as a primary cause rather than secondary to myocyte loss, then patients might benefit from treatment aimed primarily at reduction of fibrosis.

In conclusion, our study is the first to show that lamin A/C interacts with pRB, SMAD2 and PP2A and that this interaction is crucial for proper regulation of mesenchymal cellular physiology. Loss of lamins results in a loss of dephosphorylation of pRB and SMAD2. The consequence of abnormal transcription factor phosphorylation is increased proliferation and excessive collagen production by fibroblasts. This novel pathophysiological concept provides a molecular rational for the observed mesenchymal dysfunction in laminopathies and suggests that lamins may be important for various transcriptional processes by interacting with nuclear phosphatases.

MATERIALS AND METHODS

Cell culture

$LMNA^{-/-}$ MEFs and $LMNA^{+/+}$ MEFs were kind gifts of Burke and Stewart. Cells were cultured in Dulbecco's modified Eagle's Medium (Gibco) containing 10% FBS, 1 mM L-glutamine and 0.1% gentamycine at 37°C in humidified 5% CO_2/air. Lamins A and C rescued and empty virus infected MEFs were cultured under 1 μg/ml puromycine and 37.5 μg/ml hygromycine. Proliferation experiments were performed under 10% FBS or 0.1% FBS conditions with addition of TGF-β1 as indicated.

Generation of lamins A and C rescued $LMNA^{-/-}$ MEFs; shRNA expressing 3T3 fibroblasts

cDNA of lamins A and C was generated from peGFP-LaminA and pS65-LaminC by PCR of the cDNA using primers 5′-CAGTGTCGACCGAGACCCCGTCCCAGCGG-3′ for both lamins A and C and 5′-CATAGAATTCTTCTAGACAGAT TACATGATGCTGC-3′ for lamin A and 5′-CATA GAATTCTTCTAGAGGCCTCAGCGGCGGCTA-3′ for lamin C. This introduced a *Sal* I site with the deletion of the original ATG site and an *Eco* RI site after the stop-codon. The PCR product and pMT-HAX (haemaglutinin-tag; containing an extra upstream *Xho* I site) were digested with *Sal* I and *Eco* RI and the cDNAs of lamin A or C were ligated in frame into pMT-HAX after the HA-epitope. The resulting plasmid was digested with *Xho* I and *Eco* RI and HA-epitope tagged lamin A was ligated into retroviral vector pBabe–hygroX and HA-epitope tagged lamin C was ligated into retroviral vector pBabe–puroX; both babe vectors carried an additional *Xho* I cloning site. Human 293T packaging cells were transfected with pBabe–HA-lamin A, pBabe–HA-lamin C or empty vector. Supernatant was aspirated and filtered to obtain infectious virus. $LMNA^{-/-}$ MEFs were infected in the presence of 2 μg/ml polybrene. Infected cells were selected with 150 μg/ml hygromycine or 2 μg/ml puromycine, respectively, resulting in lamin A, lamin C, lamin A/C rescued $LMNA^{-/-}$ MEFs or empty vector control $LMNA^{-/-}$ MEFs.

Post-transcriptional silencing of murine lamin A/C was achieved by the shRNA sequence: gagcttgacttccagaagaacat. This sequence was used in forward (GATCCCC**GAGCTT GACTTCCAGAAGAACAT**ttcaagaga**ATGTTCTTCTGGA AGTCAAGCTC**TTTTTGGAAA) and reverse primers (AGCTTTTCCAAAAA**GAGCTTGACTTCCAGAAGAAC AT**tctcttgaa**ATGTTCTTCTGGAAGTCAAGCTC**GGG) and ligated into pRetrosuper (39). This sequence was stably introduced into 3T3 cells using the pRetrosuper system. Cells were infected either with an empty vector or the shRNA-containing vector. Infected cells were selected with 2 μg/ml puromycine.

Gel electrophoresis and immunoblotting

Cells were lysed with sample buffer (62.5 mM Tris pH 8.6 containing 2% SDS and 10% β-mercapto-ethanol). Cell lysates were sheared through 23G needle and boiled at 95°C for 10 min. Proteins were separated into 4–20% gradient gels or specific percentage acrylamide gels. Separated proteins were transferred to PVDF membrane and detected with specific antibodies. Primary antibodies were incubated with HRP-linked secondary antibodies that are visualized with enhanced chemo-luminescence. Primary antibodies were lamin A/C [Santacruz 7293 (346)], pRB, ppRBser780, ppRBser795, ppRBser807/811 [Cell Signalling Technology (CST) 9300 and BD G3-245], GAPDH (RDI 6C5), PP2A (5H4 and 6F9, Covance Research MRT-204R), HA (12CA5), total Smad2 (CST 3102), pSmad2ser465/467 (CST 3103). Secondary antibodies were rabbit anti-mouse (DAKO P0161), goat anti-rabbit (DAKO E0432) and goat anti-rat (SCBT 2003), all HRP-linked. Intensity of signals on autoradiograms was quantified by densitometry. Data were compared using the Student's *t*-test. The number of experimental repeats is indicated in the figure legends.

Immunoprecipitation

Cells were lysed on ice in ELB buffer (250 mM NaCl, 0.1% NP40, 50 mM Hepes pH 7.0, 5 mM EDTA) containing protease inhibitor tablet (Roche 1836153), 0.5 mM DTT and 0.5 mM Na_3VO_4. Cell lysates were sonicated on ice and centrifuged. Lysates were rotated for 1 h at 4°C with 1 μg primary antibody. Normal mouse and rat serums were used as negative controls. Fifty percent protein A and 50% protein G sepharose beads were washed with ELB buffer and added to cell lysates for 1 h at 4°C. Beads were pelleted and washed five times with

ELB buffer. Proteins were dissolved in sample buffer and separated by gel electrophoresis.

Proliferation assays

^{3}H-Thymidine-incorporation assay. Cells were trypsinised, counted and seeded at 6000 cells per well in 96 wells plates. After attachment cells were synchronized by serum deprivation for 48 h. TGF-β1 or 10% FBS was added as indicated. Six hours prior to endpoint 10 μl 25 nCi/μl [^{3}H]-Thymidine (Amersham) was added. Incubation was stopped by freezing at −20°C. Cells were spotted on filters, which are placed in liquid scintillation fluid (Filtercount). Scintillations were counted and represented as disintegrations per minute. DNA synthesis was depicted as dpm at a certain time point. Normalized DNA synthesis was dpm at 24 h after 6 h incorporation normalized to dpm at 6 h after 6 h incorporation. Statistical analysis was done by Student's *t*-test.

MTS-assay. Cells were trypsinised, counted and seeded at 6000 cells per well in 96 wells plates. At indicated time point 20 μl 2 mg/ml 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (Promega CellTiter 96® AQueous MTS) and 1 μl 0.92 mg/ml phenazine methosulfate (Merck) was added for 3 h. The tetrazolium is reduced by living cells into a coloured formazan product, which was measured with a colorimeter. The quantity of formazan is directly proportional to the number of cells. Absorbance was measured at 490 nm. Statistical analysis was done by Student's *t*-test.

Determination of S-phase progression rate

Cells were trypsinised, counted and seeded at 200 000 cells per well in six wells plates. After attachment cells were pulse labelled with 10 μM bromodeoxyuridine (BrdU, Sigma B5002) for 30 min and chased with 5 μM deoxythymidine (Sigma T1895). Cells were harvested at 0, 1, 2, 3, 4 and 6 h after start of chasing. Cells were fixed in methanol, washed in PBS, treated with 0.4 mg/ml pepsin in 0.1 N HCl and incubated with anti-BrdU. Primary antibodies were counterstained with FITC-conjugated secondary antibody. DNA was stained with propidium iodide. Total DNA content and BrdU status were analysed by flow cytometry. S-phase duration was calculated by extrapolating to 100% BrdU incorporation from the studied time points. Statistical analysis was done by comparing extrapolations from repeated experiments by Student's *t*-test.

Quantification of collagen production

Cells were trypsinised, counted and plated in six wells plates. After attachment cells were washed twice with PBS and synchronized in 0.1% FBS containing DMEM for 24 h. 0.15 mM L-ascorbic acid with or without 10 ng/ml TGF-β1 was added for 24 h, after which 1 μCi/ml [^{3}H]-Proline (Amersham) was added. After 48 h incubation cells were washed with PBS twice and incubated with 10% trichloroacetic acid for 30 min at 4°C. Cells were scraped and harvested, pelleted and resuspended in 10% TCA at 4°C. Cells were pelleted and resuspended in 1 N NaOH. Protein concentration was determined with Bradford assay. Protein solution is dissolved in hi-ionic scintillation fluid and disintegrations per minute are counted in a scintillation counter. Statistical analysis was done by Student's *t*-test.

Indirect immunofluorescence microscopy

Cells were trypsinised, counted and plated on cover slips. After attachment cells were fixed in 3.7% formaldehyde and permeabilized in 0.1% triton. Cells were incubated with first specific antibodies against lamin A or C (133a2 and ralC) for 1 h and with FITC-conjugated secondary antibody for 1 h. Preparations were mounted in 1,4-diazabicyclo[2.2.2]octane (DABCO, Sigma). Fluorescent images were photographed using a Nikon Eclipse E800, equipped with a Nikon DXM1200 digital camera. Images were processed using Nikon ACT-1 software.

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Conflict of Interest statement. None declared.

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