Nuclear lamin A inhibits adipocyte differentiation: implications for Dunnigan-type familial partial lipodystrophy

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Mutations in the LMNA gene encoding A-type lamins cause several diseases, including Emery–Dreifuss muscular dystrophy and Dunnigan-type familial partial lipodystrophy (FPLD). We analyzed differentiation of 3T3-L1 preadipocytes to adipocytes in cells overexpressing wild-type lamin A as well as lamin A with amino acid substitutions at position 482 that cause FPLD. We also examined adipogenic conversion of mouse embryonic fibroblasts lacking A-type lamins. Overexpression of both wild-type and mutant lamin A inhibited lipid accumulation, triglyceride synthesis and expression of adipogenic markers. This was associated with inhibition of expression of peroxisome-proliferator-activated receptor gamma 2 (PPARγ2) and Glut4. In contrast, embryonic fibroblasts lacking A-type lamins accumulated more intracellular lipid and exhibited elevated de novo triglyceride synthesis compared with wild-type fibroblasts. They also had increased basal phosphorylation of AKT1, a mediator of insulin signaling. We conclude that A-type lamins act as inhibitors of adipocyte differentiation, possibly by affecting PPARγ2 and insulin signaling.

INTRODUCTION

The nuclear lamina is a meshwork of intermediate filament proteins called lamins, which is primarily localized at the inner aspect of the nuclear envelope (1–5). In mammals, three genes encode seven lamin proteins that are expressed in somatic or germ cells. There are two B-type lamins in the nuclear lamina of somatic cells, lamin B1 and lamin B2. They are encoded by LMNB1 and LMNB2, respectively (6,7). Most differentiated somatic cells express three A-type lamins, lamin A, lamin C and lamin AΔ10, which are alternative splice variants of one gene, LMNA (8,9). Lamin A is synthesized as a precursor, prelamin A, which is modified by carboxymethylation and farnesylation and subsequently cleaved near its carboxyl terminus by the ZMPSTE24 endoprotease to yield mature lamin A (10,11).

The lamina has long been thought to provide structural support for the nuclear envelope (1,12,13). Nuclear lamins have also been implicated in DNA replication (14,15) and RNA polymerase-II-dependent transcription (16). In 1999, mutations in LMNA were shown to cause autosomal-dominant Emery–Dreifuss muscular dystrophy, a disorder that affects cardiac and skeletal muscle (17). This finding was followed by discoveries showing that mutations in A-type lamins cause several other diseases, some of which are relatively tissue-specific (for reviews, see 18,19).

The discoveries that different mutations in A-type lamins cause relatively tissue-specific disorders has led to the hypothesis that they may be essential for maintaining the differentiated state of post-embryonic somatic tissues (18). This has been examined experimentally by taking advantage of C2C12 cells as an in vitro model for muscle differentiation. Reorganization and remodeling of A-type lamins occurs during differentiation of C2C12 cells (20–22). Expression of lamin A mutants that cause autosomal-dominant Emery–Dreifuss muscular dystrophy in C2C12 myoblasts inhibits their in vitro differentiation into myotubes (21,23). Although human subjects with Emery–Dreifuss muscular dystrophy and Lmna null mice that develop a phenotype with similarities to this human condition are born with normally developed...
stirrated muscles, both develop cardiomyopathy and muscular
dystrophy after birth (24,25). This suggests that myoblast
differentiation may be inadequate to replace damaged or lost
tissue.

Another relatively tissue-specific disease caused by
autosomal-dominant inherited mutations in A-type lamins is
Dunnigan-type familial partial lipodystrophy (FPLD; OMIM
#151660) (26–28). FPLD appears to primarily affect adipocytes
and is characterized by progressive loss of subcutaneous
fat from upper and lower extremities usually at the onset at
puberty (29–31). Excess adipose tissue can accumulate in
the face and neck, causing a double chin or a Cushingoid
appearance. Affected individuals have high concentrations
of serum-free fatty acids and triglycerides, low serum concen-
trations of high-density lipoprotein, low serum leptin concent-
trations, glucose intolerance, insulin resistance and often
diabetes mellitus. Some affected individuals have skeletal
muscle hypertrophy (32,33). Loss of A-type lamin function
does not appear to be responsible for this condition, as
Lmna null mice do not develop partial lipodystrophy (34).

Although mutations in A-type lamins cause a disease-
characterized loss of peripheral fat, the effects of A-type
lamins on adipocyte differentiation have not been addressed.
We, therefore, took advantage of 3T3-L1 cells, an established
preadipose line that resembles fibroblasts and can be manipu-
lated to differentiate in vitro into adipocytes (35–37), to assess
the effect of lamin A overexpression on adipocyte differen-
tiation. We also examined the potential of fibroblasts lacking
A-type lamins (25) to become fat-containing cells. Our
results suggest that A-type lamins are inhibitors of adipocyte
differentiation and have implications for understanding how
mutations in these proteins cause FPLD.

RESULTS

Generation of preadipocyte cell lines overexpressing lamin A

To investigate the effect of lamin A overexpression on adipo-
genesis, we generated mouse 3T3-L1 cell lines stably expres-
sing wild-type lamin A or lamin A with arginine at position
482 changed to glutamine (R482Q) or tryptophan (R482W).
These amino acid substitutions are found in human subjects
with FPLD. In their undifferentiated state, 3T3-L1 cells
resemble fibroblasts but are capable of differentiating into
lipid-containing adipocytes upon treatment of a confluent
monolayer with insulin, dexamethasone and isobutylmethyl-
xanthine (IBMX).

We made three clonal lines each of 3T3-L1 cells transfected
with plasmid vector alone and with plasmids encoding flag-
tagged wild-type human lamin A, flag-tagged human lamin
A R482Q and flag-tagged human lamin A R482W. We also
made three clonal lines of cells transfected with plasmid
encoding flag-tagged human emerin, a control inner nuclear
membrane protein. Antibiotic-resistant clones were selected
and checked for expression of flag-tagged lamin A (Fig. 1A)
and flag-tagged emerin (Fig. 1B) mRNAs by reverse transcric-
tase–polymerase chain reaction (RT–PCR). Relative expression of flag-tagged lamin and emerin mRNAs in
different clones was similar (Fig. 1A and B). Flag-tagged
wild-type and mutant lamin A as well as emerin were targeted
to the nuclear periphery, where they colocalized with
endogenous lamin B1, as seen by double immunofluorescence
microscopy with anti-lamin B1 and anti-flag antibodies
(Fig. 1C).

Figure 1. 3T3-L1 preadipocytes overexpressing human lamin A and emerin. (A and B) Ethidium-bromide-stained agarose gels with products of RT–
PCR reactions performed to detect endogenous mouse lamin A and emerin
mRNAs as well as flag-tagged human lamin A and emerin mRNAs. (A) Top row shows products of RT–PCRs that detect endogenous plus human
flag-tagged lamin A mRNAs (Total Lamin A). Bottom row shows products
of RT–PCRs that detect human flag-tagged lamin A mRNA (Flag Lamin
A). Lanes marked Vector represent RT–PCR products from clones transfected
with vector alone. Lanes marked Lamin A, Lamin A R482Q and Lamin A
R482W represent RT–PCR products from clones transfected with plasmid
that encodes either flag-tagged wild-type lamin A, lamin A with R482Q
mutation or lamin A with R482W mutation, respectively. RNAs from three
different clones were used for each (lanes 1–3). (B) Top panel shows products
of RT–PCRs that detect endogenous plus flag-tagged emerin mRNAs (Total
Emerin). Bottom panel shows products of RT–PCRs that detect human flag-
tagged emerin mRNA (Flag Emerin). First lane (V) shows RT–PCR products
from cells transfected with vector. Next three lanes (Emerin) show RT–PCR
products from cell lines transfected with plasmid encoding flag-tagged human
emerin. (C) Immunofluorescence micrographs of cells stably transfected with
vector alone or plasmids that encode flag-tagged wild-type and mutant lamin
A and flag-tagged emerin. Cells were grown on cover slides, fixed and stained
with antibodies. Panels on the left (Flag) show cells stained with mouse
primary antibody and anti-mouse rhodamine-conjugated secondary
antibody (red). The middle panels (Lamin B1) show the same cells stained
with rabbit anti-lamin B1 primary antibody and anti-rabbit fluorescein
isothiocyanate-conjugated secondary antibody (green). Panels on the right
show the merge of anti-flag and anti-lamin B1 labeling (yellow). Bar: 5 μm.
3T3-L1 preadipocytes that overexpress wild-type and mutant lamin A are unable to accumulate intracellular lipid

To examine the ability of stably transfected cell lines to undergo adipogenic conversion, we checked accumulation of intracellular lipid after treatment with insulin, dexamethasone and IBMX. Intracellular lipid was visualized by staining fixed cells with Oil Red-O. Figure 2 shows that on day 7 after treatment with insulin, dexamethasone and IBMX, cells transfected with vector alone and those expressing flag-tagged emerin accumulated lipid. Cells that expressed flag-tagged wild-type and mutant lamin A were unable to accumulate significant quantities of lipid (Fig. 2). For each condition, three independent clonal lines differentiated (vector and emerin) or did not differentiate (wild-type and mutant lamin A) into lipid-containing cells. These results show that overexpression of wild-type or mutant lamin A inhibits in vitro differentiation of 3T3-L1 preadipocytes to adipocytes.

Overexpression of lamin A inhibits \textit{de novo} triglyceride synthesis

Because the absence of lipid droplets could be due to a defect in lipogenesis or overactive lipolysis, we assessed the ability of cell lines that express flag-tagged wild-type and mutant lamin A to carry out \textit{de novo} lipogenesis. Oleate is used as a substrate for synthesis of several cellular lipids, including phospholipids and triglycerides, and the quantity of oleate incorporated into triglycerides has been shown to increase as preadipocytes differentiate into fat-containing cells (38). Cell lines stably transfected with vector alone and those expressing flag-tagged wild-type or mutant lamin A were treated with insulin, dexamethasone and IBMX for 2 days and then with insulin for 5 days. Cells were then incubated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with [\textsuperscript{3}H]oleate for 4 h. Total cellular lipids were extracted and separated by thin-layer chromatography (TLC) and the amount of incorporated tritium was counted in the different lipid fractions and normalized against total cellular protein. Cells stably transfected with vector alone were able to synthesize triglycerides from oleate, as shown by the ample amount of tritium incorporated into the triglyceride fraction of extracted lipids (Fig. 3A). However, \textit{de novo} triglyceride synthesis was hindered in cells expressing flag-tagged wild-type and mutant lamin A protein, as shown by the small amount of tritium incorporated into the triglyceride fraction of extracted cellular lipids (Fig. 3A). Incorporation of tritium into the phospholipid fraction was not altered in any of the examined cell lines, implying that general oleate metabolism is not affected by lamin A overexpression (Fig. 3B). From these results, we conclude that cell lines stably expressing flag-tagged human lamin A are defective in \textit{de novo} triglyceride synthesis, which is responsible for their inability to accumulate intracellular lipid upon treatment with insulin, dexamethasone and IBMX, we wanted to determine if they expressed adipogenic markers. Several factors are temporally expressed during differentiation of 3T3-L1 preadipocytes into adipocytes, including PPAR\textsubscript{g}2, sterol response element-binding protein 1C (SREBP-1C) and Glut4 (39). 3T3-L1 cells transfected with vector or those expressing flag-tagged human wild-type or mutant lamin A were treated with insulin, dexamethasone and IBMX. Total RNA was extracted and expression of PPAR\textsubscript{g}2, SREBP-1C and Glut4 mRNAs was analyzed by RT–PCR. Expression of Glut4 was only detected in cells transfected with vector alone and expression of PPAR\textsubscript{g}2 was markedly reduced in cell lines expressing flag-tagged human wild-type and mutant lamin A proteins (Fig. 4). In contrast, SREBP-1C mRNA was detected in all examined cell lines (Fig. 4). \textbeta-Actin mRNA, a positive control, was detected at similar levels in all tested cell lines. Our results show that overexpression of lamin A is associated with decreased PPAR\textsubscript{g}2 and Glut4 mRNA levels but normal expression of SREBP-1C mRNA after treatment with insulin, dexamethasone and IBMX.

Figure 2. Overexpression of lamin A inhibits lipid accumulation in 3T3-L1 cells. Cells were grown to confluency and treated with insulin, dexamethasone and IBMX for 2 days and then with insulin for 5 days. They were then fixed and stained with Oil Red-O. Shown are scanned images of dishes of stained cells. Top three panels show three clones of cells transfected with vector only (Vector). Second row of panels shows three clones of cells that express human flag-tagged emerin (Emerin). Bottom three rows of panels show three clones each of cells that express human flag-tagged wild-type lamin A (Lamin A), human flag-tagged lamin A with R482Q mutation (Lamin A R482Q) and human flag-tagged lamin A with R482W mutation (Lamin A R482W), respectively. Bar: 0.5 cm.
Lamin A overexpression in non-clonal 3T3-L1 populations inhibits adipogenesis

To make sure that the inability of 3T3-L1 preadipocytes to differentiate into adipocytes is a consequence of lamin A overexpression and not merely stable transfection and selection of cells containing certain protein-expressing plasmids, we infected populations of 3T3-L1 cells with retroviral constructs encoding flag-tagged human wild-type or mutant lamin A. Bicistronic retroviruses were constructed in which one internal ribosomal entry site drives expression of either green fluorescent protein (GFP) alone or GFP and flag-tagged human wild-type or mutant lamin A. GFP-expressing cells were collected by fluorescence-activated cell sorting and grown without antibiotic selection. The presence of human lamin A mRNA was detected by RT–PCR (Fig. 5A). The nuclear rim localization of the flag-tagged laminas was confirmed by immunofluorescence microscopy with antibodies against the flag epitope (Fig. 5B). We then assessed the ability of cells expressing flag-tagged laminas to accumulate lipid upon treatment with insulin, dexamethasone and IBMX. Figure 5C shows that lipid accumulation was apparent after treatment in cells that expressed GFP alone but not cells that expressed GFP together with wild-type or mutant lamin A. Hence, overexpression of lamin A protein in non-clonal populations of 3T3-L1 cells inhibits lipid accumulation. To be sure that addition of the flag epitope to lamin A is not responsible for blocking adipogenic conversion of 3T3-L1 cells, we expressed untagged human lamin A using the same methods and these cells also did not differentiate (data not shown).

Embryonic fibroblasts from Lmna−/− mice differentiate into fat-containing cells more readily than embryonic fibroblasts from wild-type mice

As overexpression of lamin A inhibited adipogenic conversion, we hypothesized that fibroblasts lacking lamins A and C would more readily become fat-containing cells than their wild-type counterparts. Primary embryonic fibroblasts from Lmna+/+ and Lmna−/− mice were grown to confluency and were either left untreated or treated with medium containing insulin, dexamethasone, IBMX and troglitazone for 2 days and then transferred to medium supplemented with insulin and troglitazone. Accumulated intracellular lipids were visualized by Oil Red-O. After 9 days of treatment, embryonic fibroblast lacking A-type lamins became fat-containing cells more readily compared with fibroblasts with A-type lamins (Fig. 6A). Untreated Lmna−/− and Lmna+/+ accumulated very little intracellular lipid (Fig. 6A). Semi-quantitative analysis showed that amount of Oil Red-O extracted from Lmna+/+ fibroblasts incubated with insulin, dexamethasone, IBMX and troglitazone was approximately 3-fold higher than that extracted from similarly treated Lmna−/− cells or untreated fibroblasts (Fig. 6B). The amount of Oil Red-O extracted from treated Lmna+/+ cells was higher than that extracted from untreated Lmna+/+ cells, but the difference was not statistically significant (Fig. 6B). To evaluate basal de novo lipogenesis of wild-type and Lmna−/− fibroblasts, we examined the ability of these cells to synthesize triglycerides using [3H]oleate as substrate. Untreated Lmna−/− fibroblasts as well as Lmna−/− fibroblasts that have been treated for 9 days synthesized more triglycerides compared with
similarly treated wild-type embryonic fibroblasts (Fig. 6C). De novo phospholipid synthesis remained unchanged between wild-type mouse embryonic fibroblasts and those lacking lamins A and C (Fig. 6D). Our results show that embryonic fibroblasts that lack A-type lamins synthesized more intracellular lipid upon prolonged treatment with insulin, dexamethasone, IBMX and troglitazone.

As insulin plays an important role in lipogenesis, we investigated response to insulin in Lmna+/+ and Lmna−/− embryonic fibroblasts. Serum-starved cells were stimulated with insulin for 15 min, lysed and the amount of phosphorylated AKT1/PKBα (Ser473), a key component of the insulin-signaling pathway, was analyzed by immunoblotting. Serum-starved wild-type embryonic fibroblasts had virtually no AKT1 that was phosphorylated on Ser473 (Fig. 7A). Incubation of these cells with insulin resulted in phosphorylation of AKT1 on Ser473 (Fig. 7A). In contrast, serum-starved Lmna−/− fibroblasts showed a significant amount of phosphorylated AKT1 before addition of insulin, which further increased after stimulation with insulin (Fig. 7A). The amount of phosphorylated AKT1 was similar in both cell types following insulin stimulation. Quantification of these results showed that serum-starved Lmna−/− fibroblasts contained four times phosphorylated AKT1, compared with similarly treated Lmna+/+ fibroblasts (Fig. 7B). After stimulation of Lmna−/− and Lmna+/+ cells with insulin, this difference in amount of phosphorylated AKT1 protein disappeared. It is, therefore, possible that elevated levels of basal AKT1 phosphorylation are responsible for increased basal de novo triglyceride synthesis in Lmna−/− cells.

**DISCUSSION**

We have shown that lamin A is an inhibitor of adipocyte differentiation. Overexpression of lamin A inhibits in vitro differentiation of 3T3-L1 cells into fat-containing cells. Embryonic fibroblasts without A-type lamins accumulate more lipids and synthesize more triglycerides compared with wild-type fibroblasts. These findings have implications for the role of lamin A in maintaining the differentiated state of somatic tissues and for the pathogenesis of FPLD.

**Possible mechanism of adipogenic inhibition**

Our results plus those of others (21,23) suggest that A-type lamins are inhibitors of myogenic and adipogenic differentiation. However, they may function differently in muscles than in adipose tissues. Stable overexpression of lamin A with an Emery–Dreifuss muscular dystrophy-causing mutation (R453W) in C2C12 myoblasts inhibits their differentiation into myotubes, but overexpression of a wild-type lamin A or lamin A with an FPLD-causing mutation (R482W) had almost no effect on differentiation of C2C12 cells (23). Transient overexpression of lamin A with another Emery–Dreifuss muscular dystrophy-causing mutation (W520S) in C2C12

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**Figure 5.** Overexpression of lamin A in non-clonal populations of 3T3-L1 cells inhibits adipogenesis. (A) Shown are ethidium-bromide-stained agarose gels with products of RT–PCRs performed to detect mouse endogenous lamin A and human flag-tagged exogenous lamin A. Top panel shows products of RT–PCRs that detect endogenous plus flag-tagged lamin A mRNAs (Total Lamin A). Bottom panel shows products of RT–PCRs that detect flag-tagged human lamin A mRNA (Hu Lamin A). Lanes 1 are results for cells infected with control virus, lanes 2 for cells infected with virus that drives expression of flag-tagged wild-type lamin A and lanes 3 for cells infected with virus that drives expression of flag-tagged lamin A with R482Q mutation. (B) Intracellular localization of flag-tagged human lamin A is shown. Cells infected with control virus (pMIG), virus that drives expression of flag-tagged wild-type lamin A (pMIG Lamin A) and of flag-tagged lamin A with R482Q mutation (pMIG Lamin A R482Q) were grown to confluency, and were either left untreated (panels on left (Flag)) show immuno-fluorescence micrographs of cells stained with mouse anti-flag primary antibody and anti-mouse rhodamine-conjugated secondary antibody (red). Middle panels (Lamin B1) show the same cells stained with rabbit anti-lamin B1 primary antibody and fluorescein isothiocyanate-conjugated secondary antibody (green). Panels on the right show the merge of anti-flag and anti-lamin B1 labeling (yellow). Bar: 10 μm. (C) Lipid accumulation is shown by Oil Red-O staining. 3T3-L1 preadipocytes infected with control virus (pMIG), virus that drives expression of flag-tagged wild-type lamin A (pMIG Lamin A) and of flag-tagged lamin A with R482Q mutation (pMIG Lamin A R482Q) were grown to confluency, and were either left untreated (panels on left (−)) or treated with insulin, dexamethasone (Dex) and IBMX for 2 days and then with insulin for another 5 days (panels on right (+)). Cells were then fixed and stained with Oil Red-O. Shown are scanned images of dishes of stained cells. Bar: 0.5 cm.
myoblasts also results in their inability to differentiate into myotubes and transient overexpression of wild-type lamin A slowed down the differentiation process (21). Clues into how some lamin A mutations cause muscle- and fat-specific diseases may be provided by structural studies of the carboxyl terminal domain of A-type laminas. A part of the carboxyl terminal tail of laminas A and C forms an immunoglobulin domain type S (40,41). This domain is destabilized if lamin A/C residue 453 is mutated from arginine to tryptophan. However, the stability of this fold in A-type laminas with R482Q or R482W mutation resembles that of a wild-type lamin A/C (41). An unstable immunoglobulin fold domain may perturb the higher-order structure of the nuclear lamina and hence lead to inability of C2C12 cells to differentiate. In contrast, lamin A with a mutation at position 482 may not change the structure of nuclear lamina but may create an aberrant interaction with an unknown factor, leading to inability of 3T3-L1 cells to differentiate. The fact that overexpression of wild-type as well as mutant lamin A has an effect on adipogenic conversion suggests that mutations responsible for FPLD are ‘gain of function’ mutations and may result in higher binding affinity to a pro-adipogenic transcription factor therefore sequestering it at nuclear periphery and preventing it from activating its target genes. Overexpression of a wild-type lamin A may lead to ‘gain of function’ phenotype not because of an increased binding affinity to this protein but because of increased number of molecules with a normal binding affinity competing for it. The identity of this putative pro-adipogenic factor is not known.

Figure 6. Mouse embryonic fibroblasts lacking lamins A and C differentiate into lipid-containing cells more readily than wild-type mouse embryonic fibroblasts. Mouse embryonic fibroblasts were grown to confluency and were either left untreated or were treated with insulin, dexamethasone (Dex), IBMX and troglitazone for 2 days and then with insulin and troglitazone for another 7 days. (A) Lipid accumulation detected by Oil-Red-O staining is shown. Cells were fixed and stained with Oil Red-O. Shown are scanned images of dishes of stained cells. Top panels show fibroblasts from Lmna<sup>−/−</sup> mice (MEF<sup>−/−</sup>). Bottom panels show fibroblasts from Lmna<sup>−/+</sup> mice (MEF<sup>−/+</sup>). Panels on the left represent cells that were left untreated and panels on the right represent cells that were treated for 9 days as described above. Bar: 0.5 cm. (B) Graph shows semi-quantitative analysis of Oil-Red-O staining shown in (A). Oil-Red-O was extracted and the optical densities of the extracts were measured. The graph shows Oil-Red-O extracted from untreated (+ Insulin, Dex, IBMX) and treated (+ Insulin, Dex, IBMX) Lmna<sup>−/−</sup> (black, MEF<sup>−/−</sup>) and Lmna<sup>−/+</sup> (grey, MEF<sup>−/+</sup>) fibroblasts. Note that the optical density of Oil-Red-O extracted from treated Lmna<sup>−/−</sup> fibroblasts is significantly higher than that extracted from treated Lmna<sup>−/+</sup> fibroblasts or untreated Lmna<sup>−/+</sup> and Lmna<sup>−/−</sup> fibroblasts (*P < 0.005). (C) and (D) Tritium incorporation into triglyceride and phospholipid fractions of cellular lipids. Fibroblasts from Lmna<sup>−/−</sup> (MEF<sup>−/−</sup>) and Lmna<sup>−/+</sup> (MEF<sup>−/+</sup>) mice were either left untreated or treated for 9 days as described earlier. Cells were then incubated with [3H]oleate for 4 h. Extracted lipids were separated by TLC, and tritium incorporated into different lipid fractions was counted by liquid scintillation. (C) Graph shows tritium incorporation into triglycerides. (D) Graph shows tritium incorporation into phospholipids. Values are means of three independent experiments with bars showing standard deviations from the means. Asterisks (∗) indicate values with a statistically significant difference in tritium incorporation between cell lines (*P < 0.005).
Phosphorylated AKT1 without insulin addition (arrow), in contrast to Lmna serum starved (∗), in which AKT1 phosphorylation occurs only after insulin stimulation. (B) Graph shows the ratio of phosphorylated AKT1 to total AKT1 in serum-starved (− Insulin) and insulin-treated (+ Insulin) fibroblasts from Lmna+/+ (black bars) and Lmna−/− (MEF−/−, grey bars) mice. Asterisk (∗) represents statistically significant increase in AKT1 phosphorylation in serum-starved Lmna−/− fibroblasts (P < 0.005). Results are means of three independent experiments with bars representing standard deviations from the means.

Expression of PPARγ2 is decreased in cells overexpressing lamin A after stimulation for conversion into adipocytes. There is extensive evidence that expression PPARγ2 is crucial for adipogenic conversion (51–59). In particular, reduction of PPARγ2 expression in 3T3-L1 cells leads to significant reduction in their adipogenic capacity (60). Expression of PPARγ is controlled by several proteins and its activation affected by cofactors, including SREBP-1C. There is evidence that SREBP-1 can bind to lamin A in vitro (61) and recently it has been shown that SREBP-1 binds to farnesylated prelamin A (62). However, we did not detect an effect of lamin A overexpression on SREBP-1C mRNA in 3T3-L1 cells that did not differentiate into adipocytes. It is, therefore, possible that lamin A overexpression affects SREBP-1C on the post-transcriptional level. It has been proposed that activation of PPARγ and SREBP-1C is accomplished by parallel pathways in adipocytes (63), which could explain the presence of SREBP-1C mRNA and the absence of PPARγ2 mRNA.

Embryonic fibroblasts lacking A-type lamins undergo adipogenesis more readily than wild-type embryonic fibroblasts. They also exhibit elevated basal levels of phosphorylated AKT1 compared with wild-type fibroblasts. It has been demonstrated that lamins A and C are important for proper function of protein phosphatase 2A (64), a phosphatase that has been shown to dephosphorylate AKT1 (65). Disregulation of protein phosphatase 2A in fibroblasts lacking A-type lamins may result in elevated levels of phosphorylated AKT1 after serum starvation. This increase may be responsible for elevated basal triglyceride synthesis in untreated Lmna−/− and Lmna−/− fibroblasts that have been previously treated to accumulated intracellular lipid. However, embryonic fibroblasts from Lmna+/+ and Lmna−/− mice have similar amounts of phosphorylated AKT1 after exposure to insulin. Hence, A-type lamins do not affect AKT1 phosphorylation in response to insulin stimulation but rather the basal level of phosphorylated AKT1 protein. Therefore, the fact that fibroblasts lacking A-type lamins accumulate more lipid upon prolonged stimulation with insulin, dexamethasone, IBMX and troglitazone compared with similarly treated wild-type fibroblasts cannot be attributed to Lmna−/− cells being more insulin sensitive. We were unable to detect any defect in AKT1 phosphorylation after insulin stimulation of 3T3-L1 cells overexpressing wild-type or mutant lamin A (data not shown). Hence, we conclude that another presently unidentified mechanism must be responsible for elevated lipid accumulation of Lmna−/− cells. Our studies examining the possible effects of A-type lamins on AKT1 phosphorylation are preliminary and require more supporting evidence to be conclusive.

Limitations of the tissue culture model and implications for human pathology

Human subjects with FPLD begin experiencing fat loss generally with onset of puberty and have normal peripheral adipose tissue until then. Therefore, the disease may be due to the inability to maintain the differentiated state of the tissue and not an inability to acquire it in the first place. In FPLD, subjects lose subcutaneous fat, but retain visceral fat (66). The difference between visceral and subcutaneous fat has been well documented (67,68) but poorly understood. Even though 3T3-L1 cell line is an accepted tissue culture model of subcutaneous fat tissue (69), it is also known that 3T3-L1 cells behave differently when injected in visceral or subcutaneous fat areas (70). Hence, our tissue culture model, as all tissue culture models of disease, lacks the context of a whole organism.

Our results reflect the results of overexpression of wild-type and mutant lamin A, which does not represent the disease state, in which affected subjects apparently have normal levels of expression of A-type lamins (71). Considering that human subjects likely express equal levels of mutant and wild-type A-type lamins in FPLD, the lamin A mutants that cause FPLD are likely ‘overactive’ in the aspect of lamin A function that negatively regulates adipocyte differentiation. In our in vitro models, we used cells that either had too much or no lamin A (and lamin C). Neither cells expressing too much wild-type lamin A nor cells expressing too much of the FPLD mutants differentiated into adipocytes. In vivo, the mutants likely have a more pronounced inhibitory effect on adipogenesis and they inhibit the process even when expressed at normal levels. Finally, as overexpression of wild-type lamin A is inhibitory to adipogenesis, it is
imaginable that subtle differences and polymorphisms in the LMNA promoter or coding sequence in humans may be partially responsible for the heterogeneity of metabolic syndrome or obesity phenotype. This intriguing hypothesis could be studied in large human populations.

In a recent paper, Kudlow et al. (72) reported that overexpression of wild-type lamin A or lamin A with FPLD-causing mutations did not inhibit 3T3-L1 differentiation, in contrast to what we have reported here. Kudlow et al. (72) also observed that prelamin A accumulation did not inhibit differentiation of 3T3-L1 preadipocytes, in contrast to that reported by Capanni et al. (62), who treated 3T3-L1 cells with mevinolin as well farnesyl transferase inhibitors to accumulate prelamin A. Kudlow et al. (72) only studied populations of 3T3-L1 cells transduced with retroviral vectors for lamin A overexpression, whereas we also carried out our experiments in multiple clonal populations of 3T3-L1 cells and obtained similar results. Our data in fibroblasts other than 3T3-L1 preadipocytes, namely embryonic fibroblasts from lamin A/C ‘knockout’ mice, also support the fact that A-type laminas are inhibitors of lipid accumulation. The reasons for the conflicting results in 3T3-L1 cells, while not readily apparent, will be clarified with additional studies and comparisons of the exact culture and differentiation methods used.

MATERIALS AND METHODS

Plasmid construction

Complementary DNAs encoding human emerin, wild-type prelamin A (lamin A) and prelamin A with R482Q (lamin A R482Q) or R482W (lamin A R482W) mutations fused to the sequence encoding the flag epitope were cloned into pSVK3 (Amersham Biosciences) as previously described (73,74). From these clones, flag-tagged lamin A cDNAs were excised using EcoRI and XhoI restriction endonucleases and ligated into pIND (Invitrogen) digested with these enzymes. Flag-tagged emerin cDNA was excised from pSVK3 with BamHI and XhoI restriction endonucleases and ligated into the same restriction sites of pIND. Complementary DNAs encoding wild-type and mutant lamin A fused to the flag epitope sequence were also cloned into pMIG (75) using BamHI and XhoI restriction endonucleases. To express proteins without flag epitopes, DNA sequences lacking the flag-coding region were excised from pSVK3 with Smal and XhoI restriction endonucleases and ligated into pMIG vector digested with HpaI and XhoI.

Cell culture, transfection and cell line selection

3T3-L1 cells (American Tissue Culture Collection) were grown in DMEM supplemented with 10% fetal bovine serum (FBS) at 10% CO2 and 37°C. For transfection, cells were grown to 70% confluency in 100 mm plates and co-transfected with pIND constructs and pVgRXR (Invitrogen) using Lipofectamine 2000 (Invitrogen) for 20 days. Surviving cells were transferred to 96-well plates at a seeding density of 0.5 cell/well and tested for expression of flag-tagged proteins by indirect immunofluorescence microscopy using an anti-flag antibody. Cells that expressed the proteins were further grown and aliquots were frozen. Although pIND and pVgRXR comprise a muristerone-A-inducible expression system, in our experiments, the system was ‘leaky’ and transcription of flag-tagged lamins as well as flag-tagged emerin occurred without addition of muristerone A.

Mouse embryonic fibroblasts from Lmna−/− and Lmna+/+ mice have been previously described (67). These fibroblasts were grown in DMEM supplemented with 10% FBS in 5% CO2 and 37°C. 293T cells were grown in DMEM supplemented with 10% FBS in 5% CO2 and 37°C.

Cell differentiation

3T3-L1 cells were grown to confluency in DMEM supplemented with 10% FBS. After 2 days, medium was switched to DMEM supplemented with 10% FBS, 0.5 mM IBMX (Sigma-Aldrich), 1 μM dexamethasone (Sigma-Aldrich) and 1 μM insulin (Sigma-Aldrich) for 48 h. In all cases, after 48 h, this medium was replaced by DMEM supplemented with 10% FBS and 1 μM insulin for additional 3 or 5 days. Geneticin and Zeocin were always present in the medium.

Mouse embryonic fibroblasts were grown to confluency in DMEM supplemented with 10% FBS. After 2 days, medium was switched to DMEM with 10% FBS, 0.5 mM IBMX, 1 μM dexamethasone, 5 μM insulin and 1 μM troglitazone (BioMall) for 48 h. In all cases, after 48 h, medium was replaced with DMEM with 10% FBS supplemented with 5 μM insulin and 1 μM troglitazone for another 7 days.

Oil Red-O staining and extraction

Intracellular lipid accumulation was monitored by Oil Red-O (Sigma-Aldrich) staining. A 0.5% (w/v) solution of Oil Red-O was prepared in 60% isopropanol. Cells were washed twice with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde for 20 min at 4°C, washed once with PBS and once with 60% isopropanol. Plates were then incubated with Oil Red-O solution at room temperature for 5 min, rinsed with 60% isopropanol and overlaid with PBS. Plates were scanned into a G3 Macintosh computer (Apple Computer) using an Expression 636 flatbed scanner (Epson) using 3× or 1× enlargement of actual size. For quantitative analysis of Oil Red-O staining, dye was extracted by incubating stained cells with 4% SDS in 60% isopropanol at room temperature for 15 min. Optical density of the extracted Oil Red-O was measured in a DU-6 spectrophotometer (Beckman) at wavelength of 520 nm.

Retroviral production and infection

293T cells were grown to 70% confluency in DMEM supplemented with 10% FBS and were co-transfected with 15 μg of pMIG constructs along with 10 μg of pVSV-G and pCL-Eco (a kind gift of Dr Jonathan Barach, Columbia University) using Lipofectamine 2000. Medium that contained
viral particles was collected 24 h after transfection, filtered through a 0.8-μm filter, supplemented with 8 μg/ml of poly-
prene and stored at 4°C. For infection, 3T3-L1 cells were
grown to 50% confluency in 6-well plates in DMEM sup-
plemented with 10% FBS. Growth medium was supplemented
with medium containing viral particles at 1:1 (v/v) ratio and
added to 3T3-L1 cells for 24–36 h. Infected cells expressing
GFP were isolated by fluorescence-activated cell sorting.

Antibodies and immunofluorescence microscopy

Rabbit polyclonal antibodies raised against lamins A and B1
were used as described previously (76). Anti-flag M5 mouse
monoclonal antibody was purchased from Sigma-Aldrich. Anti-AKT1/PKBα antibody was purchased from Santa Cruz
Biotechnology. Anti-phospho-AKT1/PKB (Ser473), clone
11E6 was purchased from Upstate Cell Signaling Solutions.
Affinity purified, secondary antibodies were purchased from
Jackson ImmunoResearch Laboratories. Cells were grown in
2-, 4- or 16-well chamber slides and fixed with 100% metha-
ol for 10 min at −20°C. Cells were then incubated with
primary antibodies for 2 h, washed and incubated with second-
ary antibodies for 1 h. Immunofluorescence microscopic
analysis was done on Zeiss LSM 510 confocal laser scanning
system attached to a Zeiss Axiovert 200 M inverted
microscope (Carl Zeiss, Inc.).

Fluorescence-activated cell-sorting analysis

Cells were grown to 80% confluency, trypsinized, washed
with PBS and resuspended in PBS at 2 × 10^5 cells/ml. Cells
expressing GFP were sorted on Becton Dickinson FACSAria
(BD Sciences) set to select highest 40% of GFP-expressing
cells. Selected cells were collected and pelleted at 2000g,
resuspended in DMEM supplemented with 10% FBS and
grown in 6-well plates for further analysis.

De novo lipogenesis assay

Cells, cultured in 6- or 12-well plates, were incubated in
DMEM supplemented with 1% free fatty-acid-free bovine
serum albumin and 5 μCi/ml of [3H]oleate for 4 h. Cells
were then washed three times with PBS. Cellular lipids were extracted three times with 1 ml of hexane:isopropanol (3:2, v/v) solution. Extracted lipids were dried under N_2 and resuspended in 0.15 ml of chloroform:methanol (2:1, v/v) and spotted onto silica-
coated TLC plates (Fisher) next to a lipid standards (Sigma-
Aldrich). Total cellular lipids were separated by TLC with
hexane:ether:acetic acid (70:30:1, v/v/v) as solvent and visual-
ized by I_2. Lanes corresponding to appropriate lipid fractions
were excised and incorporated tritium was counted in a
1409 liquid scintillation counter (Wallac). Total cellular
protein was extracted with 1 or 0.5 ml of 0.1 M sodium
hydroxide and protein concentration was determined by
Biorad Protein Assay Kit (Biorad Laboratories). Amount of
incorporated tritium was calculated by using the formula

\[
\frac{[\text{H}] \text{ incorporated (pmol/μg)}}{\text{activity (dpm)/specific activity (dpm/pmol)}} = \frac{\text{total cellular protein (μg)}}
\]

RT–PCR, primer sequences and RNA isolation

Total cellular RNA was isolated with RNeasy Mini Kit
(Qiagen) at confluence or on day 5 of differentiation. Reverse
transcription was performed using the First Strand Synthesis
Kit (Invitrogen). PCRs were performed using Platinum PCR
SuperMix (Invitrogen) or Eppendorf Hot MasterMix (Eppen-
dorf) according to the manufacturer’s instructions on
GeneAmp PCR System (Applied Biosciences). Endogenous
plus flag-tagged lamin A mRNA was detected by amplification
with primers complementary to sequences that are identical
in human and mouse lamin A cDNAs. Human flag-tagged
lamin A mRNA was detected by amplification with primers
complementary to a sequence encoding the flag epitope and
a sequence identical in human and mouse lamin A cDNA.
Also, human flag-tagged lamin A mRNA was detected
by amplification with primers complementary to sequences
specific to human lamin A cDNA. Endogenous plus flag-
tagged emerin mRNAs were detected by amplification
with primers complementary to sequences in mouse and human
emerin cDNA. Human flag-tagged emerin mRNA was detected
by amplification with primers complementary to a sequence in
the flag epitope and a sequence in emerin cDNA. Endogenous
PPAR-γ2, SREBP-1C, Glut4 and β-actin were detected by
amplification with primers complementary to sequences in
their respective cDNAs.

Insulin response assay and immunoblotting

Mouse embryonic fibroblasts were grown to 80% confluency
and serum starved for 6–8 h in DMEM. Cells were then trans-
ferred to medium supplemented with 100 nM insulin for
15 min. To prepare whole cell lysates, cells were washed
with PBS, scraped from plates and resuspended in 50 mM
Tris–HCl (pH 7.4), 1% sodium deoxycholate, 1% Triton
X-100, 0.1% SDS, 150 mM NaCl, 1 mM sodium orthovanadate
and 1 mM sodium fluoride. An amount of 25 or 50 μg of
cellular protein was loaded onto 10% or 4–15% gradient
acrylamide gels (Biorad Laboratories) and separated by
electrophoresis. Proteins were transferred onto a nitrocellulose
membrane (Biorad Laboratories) and membranes were incu-
bated with primary and horseradish-peroxidase-linked second-
ary antibodies. Chemiluminescence was detected using ECL
Kit (Amersham) according to the manufacturer’s instructions.
Scion Image software (http://www.scioncorp.com) was used
for quantitative analysis of phosphorylated AKT1 and total
AKT1 signals.

Materials

Unless otherwise specified, routine chemical reagents were
obtained from Sigma-Aldrich. Tissue culture reagents were
obtained from Invitrogen.
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Conflict of Interest statement. None declared.

REFERENCES


