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Journal of Cellular Biochemistry 116:2344–2353 (2015)

Journal of Cellular Biochemistry

Lamin A/C Acts as an Essential Factor in Mesenchymal Stem Cell Differentiation Through the Regulation of the Dynamics of the Wnt/β-Catenin Pathway

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ABSTRACT

Changes in the expression of lamin A/C, a fibrilar protein of the nuclear envelope, are associated with the cellular features of age-related bone loss. Reduced expression of lamin A/C inhibits osteoblastogenesis while facilitating adipogenic differentiation of mesenchymal stem cells (MSC) in vitro and in vivo. In this study we investigated the regulatory role that lamin A/C plays on the essential elements of the Wnt/β-catenin pathway, which are pivotal in MSC differentiation. Initially, we assessed the effect of lamin A/C gene (*LMNA*) overexpression on MSC differentiation while compared it to lamin A/C depleted MSC. Osteogenesis and gene expression of osteogenic factors were higher in *LMNA*-transfected MSC as compared to control. Conversely, adipogenesis and expression of adipogenic factors were significantly lower in *LMNA* transfected cells. Nuclear β-catenin was significantly higher (∼two fold) in MSC expressing higher levels of *LMNA* as compared to control with nuclear β-catenin levels being significantly lower (∼ -42%) in siRNA-treated MSC. Luciferase activity for β-catenin-mediated transcriptional activation was significantly higher in cells overexpressing *LMNA*. These data indicate that MSC overexpressing *LMNA* have higher osteogenic and lower adipogenic differentiation potential. In conclusion, our studies demonstrate that lamin A/C plays a significant role in the differentiation of both osteoblasts and adipocytes by regulating some of the elements of Wnt/β-catenin signaling during early MSC differentiation. J. Cell. Biochem. 116: 2344–2353, 2015. © 2015 Wiley Periodicals, Inc.

KEY WORDS: OSTEOBLASTOGENESIS; β-CATENIN; MESENCHYMAL STEM CELLS; LAMIN A; ADIPOGENESIS

Apart from being a structural barrier between the cytosol and the nuclear contents, the nuclear envelope also has several other important cellular functions. The nuclear membrane is a complex structure composed of multiple different proteins each having various functions including the regulation of gene expression, chromatin organization and communication with the cytoskeleton and transcriptional factor translocation across the nucleus [Heesen and Fornerod, 2007; Andres and Gonzalez, 2009].

Interest in the importance of the nuclear membrane proteins started from the discovery of a group of diseases known as laminopathies caused by mutations within several lamin-coding genes [Maraldi et al., 2011; Worman, 2012]. One of these laminopathies known as the Hutchinson-Gilford progeria syndrome (HGPS) is caused by the novo mutation in the lamin A/C (*LMNA*) gene, manifested by accelerated aging, lipodystrophy, alopecia, atherosclerosis, stiff joints and accelerated bone loss, which leads to repeated non-healing fractures [Paro et al., 2002]. At the cellular level, HGPS patients exhibit nuclear defects, abnormal chromatin structure and increased DNA damage, predominantly in tissues of mesenchymal origin [Scaffidi and Misteli, 2005,2008; Hennekam, 2006].

Lamin A/C is the end product of the post-translational processing of prelamin A, which involves farnesylation, endoproteolysis and methylation [Worman, 2012]. Although the HGPS phenotype is predominantly a result of the accumulation of an aberrant form of lamin A/C known as progerin, the fact that defects in lamin A/C processing are associated with accelerated aging and bone loss, has established a basis to propose that lamin

Sandra Bermeo and Christopher Vidal contributed equally to this manuscript.

Grant sponsor: Australian National Health and Medical Research Council NHMRC; Grant number: 632767.

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Manuscript Received: 18 August 2014; Manuscript Accepted: 1 April 2015

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 3 April 2015

DOI 10.1002/jcb.25185 • © 2015 Wiley Periodicals, Inc.

A/C plays a role not only in normal aging in general, but also in bone biology in particular [Vidal et al., 2012].

Several studies have suggested that pharmacological regulation of the steps involved in post-translational processing of prelamin A could play a role in the regulation of bone metabolism involving the differentiation of mesenchymal stem cells (MSC) into either osteoblasts or adipocytes [Duque et al., 2011; Rivas et al., 2007; Vidal et al., 2012]. In addition, lamin A/C knock-down is associated with low osteoblastogenesis and high levels of osteoclast differentiation in vitro [Akter et al., 2009; Rauner et al., 2009]. Furthermore, four-week-old lamin A/C knock-out mice show the typical features of age-related bone loss and a severe osteoporotic phenotype with fat accumulation in both bone and muscle [Li et al., 2011; Tong et al., 2011]. Overall, all the previous knock-down in vitro and in vivo experiments indicate an important role of lamin A/C in the pathogenesis of age-related bone loss and osteoporosis. However, the molecular mechanisms that explain the role of lamin A/C in MSC and bone metabolism remain poorly understood.

The post-translational processing of prelamin A that ends in lamin A/C release is followed by a set of protein-protein and protein-pori interactions in the nucleus that regulate multiple cell functions, including cell differentiation [Kubben et al., 2010]. Although the interactions between lamin A/C and other proteins/transcriptional factors in other cell models such as HeLa, COS, and C2C12 are well understood [Brachner et al., 2005; Markiewicz et al., 2006], the role of lamin A/C in the dynamics of MSC commitment into osteoblasts or adipocytes, and the changes in the osteogenic/adipogenic transcription factors that occur in the presence and absence of lamin A/C in MSC remain to be identified.

Therefore, the aim of the present study was to investigate the molecular mechanisms of MSC differentiation that are affected in either the absence or overexpression of lamin A/C. Using an in vitro model of overexpression/knock-down we have characterized the changes in MSC differentiation and the dynamics of the elements of Wnt/β-catenin signaling that would allow understanding of the role of lamin A/C in this process.

MATERIALS AND METHODS

PREPARATION OF THE LAMIN A/C CONSTRUCT (PCDNA_LMNA)

A first strand cDNA of the full sequence of the lamin A/C (*LMNA*) mRNA (ENST00000368300-1) was reverse transcribed using oligo-dT_{15} and 1 μg of DNAse-treated total RNA extracted from normal human osteoblasts (Lonza Bioresearch). The cDNA was used as template for PCR using a high fidelity Phusion® polymerase (Finnzymes) and oligonucleotides LMNA_F 5′-CTCCGAGCAGTCTCTGTCCT-3′ and LMNA_R 5′-AGGCAGATGTGGAGTTTCCTG-3′. The amplicon was cleaned and used as template for the second PCR using oligonucleotides harboring sequences for the restriction enzymes KpnI and XhoI and the same specific sequences as above. The PCR product was cleaned and digested respectively with these restriction enzymes, together with the pcDNA™3.1/HisA plasmid (Invitrogen). Following transformation, selected clones were checked by direct DNA sequencing followed by purification of plasmid DNA for transfections, which was prepared using the PureYield Plasmid Midiprep System (Promega). The protein product of this plasmid was checked by Western blotting following transfection in HeLa cells using anti-lamin A/C antibody (Santa Cruz Biotechnology; sc-20681) and anti-His tag (Cell Signaling).

CELL CULTURE AND MSC TRANSFECTIONS

All experiments were carried out using normal human MSC (Lonza Bioresearch) obtained from bone marrow of 3–4 young male donors (approx. age 25 years old). Experiments were done using cells that were less than passage 5. MSC were expanded in MSC growth medium (MSCGM) (Lonza Bioresearch) with 10% fetal bovine serum (FBS) and were split by trypsinization when reaching 60–70% confluency. In preparation for transfection, MSC were seeded at a density of 5×10^4 cells/cm^2 in corresponding plates in MSCGM without antibiotics and incubated at 37°C in a humidified atmosphere of 5% CO_2. On the day of transfection, the MSCGM was changed to antibiotic free MSCGM. All transfections were carried out using Lipofectamine 2000 (Invitrogen) at 80% confluency following the manufacturer's protocol. Lamin knock-down (60%) was obtained using 50 pmol siRNA (Santa Cruz Biotech; sc-35776) as previously described [Akter et al., 2009]. For lamin A/C overexpression, 250 ng of the pcDNA_*LMNA* construct was used. After an overnight incubation, MSCGM was aspirated and replaced with osteoblast or adipocyte induction medium.

OSTEOBLAST AND ADIPOCYTE DIFFERENTIATION

MSC were induced to differentiate into osteoblasts using osteogenic induction medium (OIM) containing 10 mM glycerophosphate, 0.05 mM ascorbic acid and 0.1 μM dexamethasone. Medium was changed every 3 days. For adipocyte differentiation, MSC were cultured up to 90% confluency and then induced to differentiate into adipocytes by alternating between Adipogenic Induction Media (AIM), containing 0.1 μM dexamethasone, 10 μg/mL insulin, 0.2 mM indomethacin, 0.5 mM 3-isobutyl-1-methylxanthine, 10% FBS, 0.05 U/mL penicillin and 0.05 μg/mL streptomycin, and Adipogenic Maintenance Medium (AMM [10 μg/mL Insulin, 10% FBS, 0.05 U/mL penicillin and 0.05 μg/mL streptomycin]) every three days for three weeks, until an adipogenic phenotype was obtained.

TOTAL RNA ANALYSIS BY QPCR

Total RNA was extracted from differentiated cells using the PARIS™Kit (Ambion, Life Technologies) (Table I). Gene expression of osteogenic and adipogenic genes was measured by quantitative real-time PCR. Prior to cDNA synthesis, RNA was treated with RNAse free DNAse (Promega) for 30 min at 37°C followed by deactivation. First strand cDNA was synthesized using 200 ng of RNA as the template and random hexamers using the Tetro cDNA Synthesis Kit (Bioline, Australia, BIO-65043), incubated at 42°C for 1 h. Quantitative real-time PCR was performed using the SensiMix No-ROX Kit (Bioline) following the manufacturer's instructions and specific primers for Wnt7b and Wnt10b and the specific osteogenic genes Runt-related transcription factor 2 (RUNX2), osteopontin (OPN), osteocalcin (OCN), alkaline phosphatase (ALP), and osterix (OSX) with GAPDH as normalizer. As adipogenic genes we used peroxisome proliferator activator receptor γ2 (PPARγ2), Ap2, adiponectin, lipoprotein lipase (LPL), leptin and C/EBPα as

TABLE I. Primer Sequences for Osteogenic Genes by Real-time PCR

Gene	Forward 5′ -3′	Reverse 5′ -3′
RunX2	TTTGCACTGGGTCATGTGTT	TGGCTGCATTGAAAAGACTG
Osteopontin	ACTCTGGTCATCCAGCTGACTCGT	CTCCTAGGCATCACCTGTGCCATA
Osteocalcin	TGGCCGCACTTTGCATCGCTGG	CGATAGGCCTCCTGAAAGCCGATG
ALP	GACCCTTGACCCCCACAAT	GCTCGTACTGCATGTCCCCT
OSX	AGAGGTTCACTCGCTCTGACGA	TTGCTCAAGTGGTCGCTTCTG
LMNA	GGATGAGGATGGAGATGACC	CACGGGGGAGGCTGGGGAGAG
Wnt7b	AGCAAAGTGATGAGGAGACTGA	CTGGCTATGTGTTAGTGCCG
Wnt10b	TGACTTCTGTGAGCGAGACC	CACCCACTCTGTAACCTTGC

ALP: Alkaline Phosphatase; OSX: osterix; LMNA: Lamin A/C.
Annealing temperature for all PCR is 60°C

previously described [Vidal et al., 2012a]. All PCRs were performed in a Corbett Rotor-Gene™ 3000 (Qiagen Pty).

ALIZARIN RED (AR) STAINING AND MEASUREMENT

After osteogenic differentiation, we assessed mineralization using AR standard staining and quantification [Gregory et al., 2004]. Culture medium was aspirated and cells were washed with phosphate buffered saline (PBS), followed by fixation with 10% formalin. After fixation, cells were washed with PBS (pH4.2) and stained with AR for 10 min at room temperature (RT) followed by washing with tap water. After microscopic examination, AR was eluted with 10% cetylpyridinium chloride and measured by spectrophotometry at 560 nm.

OIL RED O (ORO) STAINING AND MEASUREMENT

ORO staining was used to assess adipocyte differentiation as an indicator of intracellular lipid accumulation. On day 21, culture medium was removed from tissue culture well and cells were rinsed with PBS once, followed by fixation using 10% formaldehyde in PBS for at least 1 h. The fixative was then aspirated and cells were washed with 60% isopropanol before being allowed to dry completely. Cells were stained for 10 min at RT with a diluted solution of ORO (66.6%) prepared from a 0.5% w/v ORO dissolved in isopropanol. Cells were then washed 4 times with running tap water to remove excess stain. ORO was eluted with 1 ml 100% isopropanol for 10 min and absorbance measured at 500 nm.

IMMUNOFLUORESCENCE MICROSCOPY

To assess the role of lamin A/C in the translocation of β-catenin from the cytosol to the nucleus, MSC were cultured in a four-chamber slide (BD Biosciences). When reaching 80% confluency transfection was performed as described above. The 3 conditions tested were MSC with normal levels of lamin A/C (vector only), *LMNA* knock-down and overexpression. Cells were incubated at 37°C in 5% CO_2 in MSCGM overnight and the next morning this was changed to OIM supplemented with 20 mM lithium chloride (LiCl), which inhibits glycogen synthase kinase (GSK)-3 and hence protects β-catenin against phosphorylation and degradation thus assuring high levels of β-catenin in this model. After 24 h, cells were washed with ice-cold PBS (pH7.4) and fixed with 4% paraformaldehyde for 20 min followed by permeabilization (0.2% triton-X) for 15 min and blocked with 5% bovine serum albumin (BSA) for 1 h at RT. Primary antibody dilutions (1:100) were prepared in 1% BSA in TBS-T using anti-β-catenin (Santa Cruz Biotech; sc-59737) and incubated overnight at 4°C with mild agitation. Following incubation, cells were washed 3 times followed by 1 h incubation at RT with a fluorescently labeled (555 nm) anti-mouse secondary antibody (1:400). Following incubation and while protecting from light, cells were washed as before and incubated for 1.5 h with the second primary antibody for lamin A/C (Santa Cruz Biotech; sc-20680), prepared as before. Cells were washed and incubated with a fluorescently labeled (488 nm) anti-rabbit antibody for another hour followed by washing and counterstaining with the nuclear stain DAPI for 3 min. Slides were dried and mounted using a buffered glycerol with anti-fade (0.1 M Phosphate buffer pH7.4; 90% glycerol; 5 mg/ml n-propyl gallate). Slides were visualized and photographed using a Leica TCS SP5 II confocal microscope and LAS AF Software (Leica Microsystems CMS GmbH). Fluorescence intensity for nuclear β-catenin was measured using ImageJ software and analyzed statistically comparing vector, overexpression and knock-out *LMNA*. Using the area, integrated density and background readings we calculated corrected total cell fluorescence (CTCF) for each cell nucleus and cytoplasm. Then, nuclear/cytoplasmic ratio was calculated. Two independent investigators analysed a total of 25 cells in each condition.

WESTERN BLOTTING

To quantify the translocation of β-catenin to the nucleus and to determine how different levels of lamin A/C affect this translocation, we used western blotting from nuclear and cytoplasmic extracts. Human MSC were grown in 6-well plates and transfected with siRNA or pcDNA_*LMNA* using the same method as previously described. Twenty-four hours after induction, cells were washed with ice-cold PBS and nuclear and cytoplasmic fractions extracted using a PARIS™Kit (Ambion, LifeTechnologies) following the manufacturer's protocol. Protease inhibitors were added to the samples and concentrations were measured using the bicinchoninic acid (BCA) protein assay reagent (Thermo Fisher). 15 μg from each protein sample were loaded into a 4–12% polyacrylamide reducing gel in MOPS buffer (50 mM MOPS; 50 mM Tris–Base, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA; pH 7.7), followed by electrophoresis and Western transfer. Immunoblotting was done using primary antibodies, diluted in TBS-T (final concentration 0.2 μg/ml), for lamin A/C (Santa Cruz Biotech; sc-20680), total β-catenin (Santa Cruz Biotech; sc-59737) normalized with glyceraldehyde-3-phosphate

dehydrogenase (GAPDH) (Santa Cruz Biotech; sc-32233) and lamin B1 (Santa Cruz Biotech; sc-30264) for cytoplasmic and nuclear localization, respectively. Blots were incubated overnight at 4°C with mild agitation, followed by washing 3 times and 1 h incubation with secondary antibody conjugated with horseradish peroxidase (HRP) (final concentration 0.04 μg/ml). Following incubation, blots were washed 3 times in TBS-T and visualized using a chemiluminescent substrate for HRP. Quantification was performed using NIH ImageJ medical image software.

β-CATENIN / T-CELL FACTOR (TCF) TRANSACTIVATION ASSAY

To test whether lamin A/C affects the transcriptional activity of β-catenin we used a reporter assay system using TOPFLASH/FOPFLASH reporter plasmids (generously donated by Prof RT Moon, University of Washington School of Medicine, WA) [Veeman et al., 2003]. TOPFLASH harbor 6 consensus sequences for TCF/LCF while FOPFLASH has mutated sequences, thus acting as a negative control. MSC were cultured in 12-well plates and transfected with siRNA or pcDNA_*LMNA* as above. Twenty-four hours post-transfection, MSCGM was aspirated, cells washed with PBS, and OIM supplemented with 20 mM LiCl was added. A second transfection was carried out using lipofectamine 2000 as before and 800 ng of either TOPFLASH or FOPFLASH plasmid with renilla vector as a transfection efficiency control. The transfected cells were incubated overnight at 37°C in a humidified atmosphere of 5% CO_2. After incubation, the media was aspirated and cells washed with ice-cold PBS followed by adding 100 μl of passive lysis buffer and incubation at RT for 30 minutes with mild agitation. The lysates were stored at −80°C until analyzed. For the analysis, 20 μl from each sample were transferred to a 96-well plate. The assay was performed using the dual luciferase assay kit (Promega) following the manufacturer's instructions. The raw data was corrected for background and normalized using renilla reporter values that correct for the transfection efficiency within each well. Mean values for TOPFLASH for each condition were further corrected by the corresponding FOPFLASH mean value and expressed as the fold difference from the negative control. This experiment was replicated 4 times with a total of 8 replicates for each condition.

CO-IMMUNOPRECIPITATION (COIP)

CoIP was performed to assess whether β-catenin physically interacts with lamin A within the nuclear envelope. Cells were transfected with pcDNA_*LMNA* and induced to differentiate into osteoblasts for 24 h followed by extraction of nuclear and cytoplasmic proteins as described before. 100 μg of nuclear protein was pre-cleared with protein A/G agarose (Santa Cruz Biotech; sc-2003) for 2 h followed by centrifugation for 3 min at 2000 g. The supernatant was incubated with 1 μg of anti-lamin A/C (Santacruz Biotech; sc-7292) overnight at 4°C on a slow rotator. After incubation, 20 μl of protein A/G were added and incubated for 4 h at 4°C on a slow rotator, followed by centrifugation as before. The pellet was washed 3 times with 1 ml of ice-cold modified Dulbecco's PBS (Thermo Scientific) and after the final wash, 30 μl of 2 X electrophoresis buffer were added followed by boiling for 2 min at 95°C. Four controls were prepared: an agarose and lysate without antibody; an anti-lamin A antibody only control; an anti- β-catenin only control and a non-specific IgG (lysates and beads) control. Samples were then subjected to electrophoresis and western blotting as described above using anti-lamin A/C and anti-β-catenin. The results were confirmed by repeating the experiment using anti-β-catenin for immunoprecipitation.

STATISTICAL ANALYSIS

Each experiment was repeated three times. Statistical analyses of data obtained from measurements of alizarin red, oil red O, and western blotting, comparing treated and untreated cells, was performed using ANOVA and/or independent sample t-tests with the probability at 0.05 as the level of significance. Real-time PCR statistics were worked out using REST software (Qiagen) [Pfaffl et al., 2002].

RESULTS

OSTEOBLAST DIFFERENTIATION IS INCREASED IN LAMIN A/C OVEREXPRESSING MSC

Two previous studies have reported that lamin A/C deficiency significantly compromises osteoblast differentiation from MSC in vitro [Akter et al., 2009; Rauner et al., 2009]. To investigate whether lamin A/C positively affects osteoblastogenesis, we transfected MSC with pcDNA_*LMNA* or empty vector and induced them to differentiate into osteoblasts. 45% transfection efficiency was obtained and maintained for at least two weeks. This transfection efficiency induced an increase of 250 fold in the *LMNA* gene expression, which decreased but was still significantly high (45 fold) on day 14 of differentiation as compared with vector-treated cells ($P < 0.001$, Fig. 1A). As shown in Figure 1B, overexpression of *LMNA* in MSC resulted in an increased deposition of calcium and mineralization when compared to empty vector control cells. This difference was statistically significant ($P < 0.001$) when measuring eluted alizarin red by spectrophotometry (Fig. 1B). In addition, the higher levels of *LMNA* expression in MSC also affected the expression of Wnts and major osteogenic genes. As shown in Figure 1C, in absence of LiCl, with the exception of OPN, gene expression was significantly increased for Wnt7b, Wnt10b and all the other osteogenic genes tested at the end of the 7th day of differentiation ($P < 0.01$). In contrast, this peak in gene expressions was delayed until week 2 in cells treated with LiCl, although reaching a similar high expression profile on week 2 of differentiation in non-treated MSC.

ADIPOCYTE DIFFERENTIATION IS DECREASED IN LMNA OVEREXPRESSING MSC

LMNA knock-down increased adipogenic differentiation in vitro [Akter et al., 2009]. Hence in this study, we investigated whether overexpressing *LMNA* would have the opposite effect on MSC, thus decreasing adipogenesis. The lipid droplets in adipocytes were stained with Oil-Red-O and quantified by spectrophotometric measurement. As shown in Figure 2A and B, overexpression of *LMNA* in MSC resulted in a reduced number of differentiated

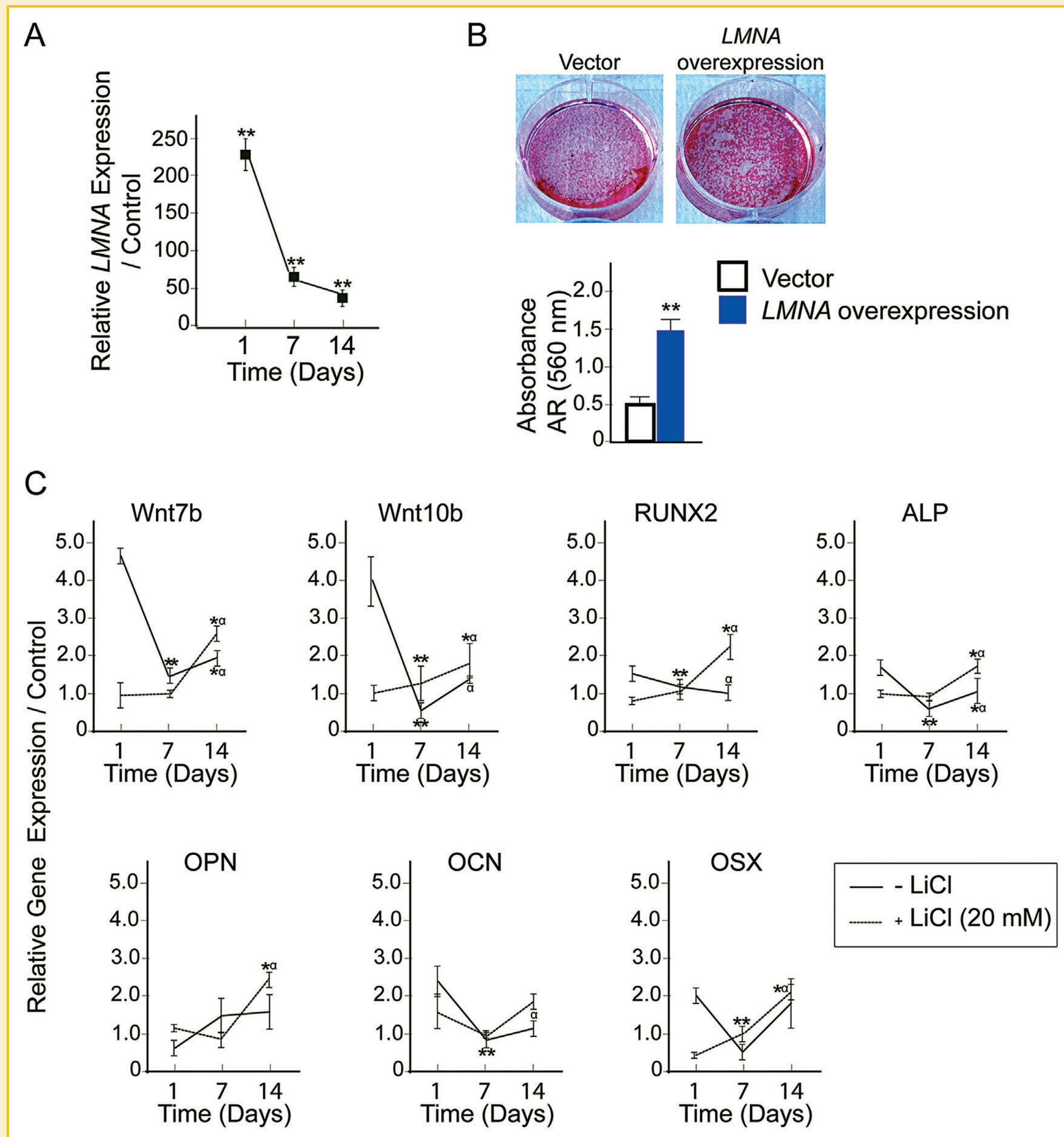

Fig. 1. Osteoblast differentiation is increased in lamin A/C overexpressing MSC. (A) 45% transfection efficiency was obtained and maintained for at least two weeks. This transfection efficiency induced an increase of 250 fold in the *LMNA* gene expression, which decreased but was still significantly high (45 fold) on day 14 of differentiation as compared with vector-treated cells (**$P < 0.001$). (B) Human MSC were transfected with pcDNA_*LMNA* and induced to differentiate into osteoblasts for up to 21days. Staining with alizarin red (AR) for mineral calcium deposition on day 21 (upper panel) showed that osteoblasts expressing higher levels of *LMNA* were more mineralized than those transfected with empty plasmid (vector). In addition, a statistically significant higher eluted AR was observed in the *LMNA* transfected MSC (lower panel) confirming higher mineralization when compared to control (**$P < 0.001$). (C) Osteogenic differentiating MSC were treated with either LiCl (20 mM) or vehicle for 14 days. Total RNA was extracted after 1, 7 and 14 days of differentiation and analyzed by quantitative real-time PCR. Levels of Wnt7b expression were significantly higher during the whole differentiation process in absence of LiCl. In contrast, high levels of Wnt7b were only observed after week 2 of differentiation in LiCl-treated MSC. In addition, LiCl induced a biphasic gene expression pattern in Wnt10b and most of the osteogenic factors. Untreated MSC showed increasing gene expression at day 1 followed by normalization. In contrast, LiCl-treated MSC showed a later gene induction (day 7), which usually persisted at day 14 of differentiation. **$P < 0.001$ day 1 vs. day 7 of differentiation; *$P < 0.01$ day 1 vs. day 14 of differentiation; $\alpha P < 0.01$ day 7 vs. day 14 of differentiation. RUNX2: Runt-related transcriptional factor 2; ALP: alkaline phosphatase; OPN: osteopontin; OCN: osteocalcin; OSX: osterix.

adipocytes, suggesting that lamin A/C negatively affected the adipogenic lineage commitment of MSC. Furthermore, following an increase in their expression on days 1 and 7, there was a significant decrease in the expression of Wnt7b, Wnt10b and all tested adipogenic markers ($P < 0.001$, Fig. 2C).

LAMIN A/C FACILITATES THE ENTRY OF β-CATENIN INTO THE NUCLEUS

Our experiments suggest that overexpression of lamin A/C shifts the MSC lineage commitment away from adipogenesis, thus stimulating osteoblastogenesis. We then hypothesized that lamin A/C may

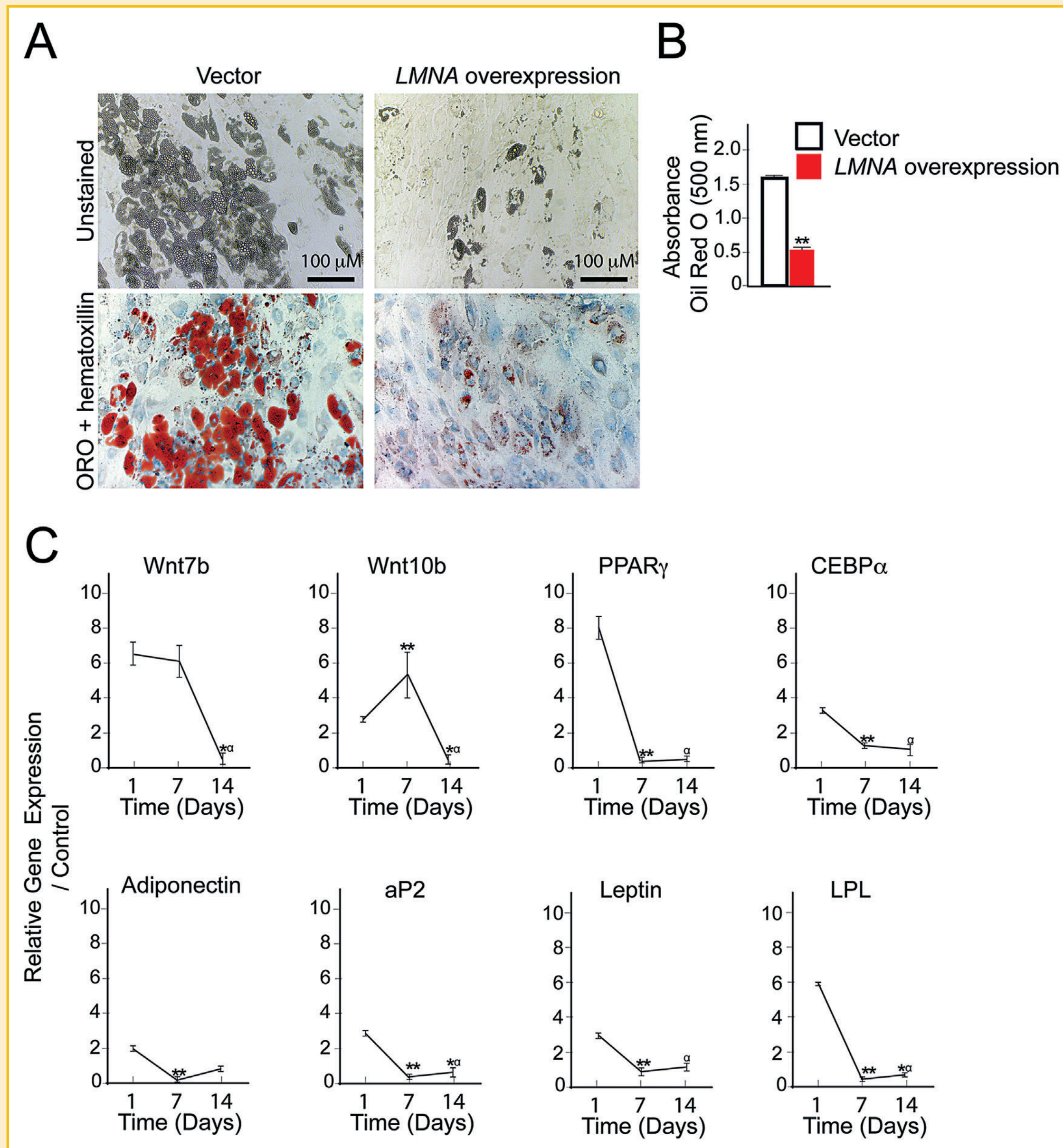

Fig. 2. Adipocyte differentiation is decreased in LMNA overexpressing MSC. MSC transfected with pcDNA_LMNA or empty vector were induced to differentiate into adipocytes. 21 days post-induction, cells were fixed and stained with the fatty acid stain Oil-Red-O (ORO) and visualized microscopically. (A) As can be seen, there was a reduced number of adipocytes in LMNA-transfected cells (red) than in the control. The upper panel shows unstained wells while the lower panel shows ORO staining + hematoxylin (40X magnification). (B) Following elution, there was a statistically significant reduction in ORO stain observed in LMNA transfected cells compared to control, thus confirming microscopic observations. (C) Total RNA was extracted after 1,7 and 14 days of differentiation and gene expression was analyzed by PCR. As shown, there was a statistically significant increase in both Wnt7b and Wnt10b both of which were significantly reduced after 14 days. Following a significant increase in gene expression at day 1, LMNA transfection of adipogenic differentiating MSC induced a statistically significant reduction in expression for all adipogenic genes tested after 7 and 14 days of differentiation. **$P<0.001$ day 1 vs. day 7 of differentiation; *$P<0.01$ day 1 vs. day 14 of differentiation; α$P<0.01$ day 7 vs. day 14 of differentiation. PPARγ2: Peroxisome proliferator activator receptor gamma 2; C/EBPα: CCAAT-enhancer-binding protein alpha; aP2: Adipocyte protein 2; LPL: lipoprotein lipase.

interact with β-catenin in the nucleus in a way that would facilitate osteoblastogenesis. Initially, we assessed whether lamin A/C has a regulatory role in the dynamics of β-catenin during MSC differentiation by overexpressing (pcDNA_*LMNA* construct) and knocking down (siRNA) the *LMNA* gene. As shown in Figure 3A, levels of β-catenin within the nucleus corresponded with higher levels of lamin A/C when compared to those expressing physiological levels. Conversely, MSC expressing less lamin A/C showed significantly lower levels of β-catenin within their nuclei. In addition, quantification of the relative nuclear/cytoplasmic β-catenin in osteogenic differentiating MSC, showed significantly lower levels in siRNA-treated cells and significantly higher levels in *LMNA* overexpressing cells as compared to empty vector-treated MSC ($P<0.01$) (Fig. 3B).

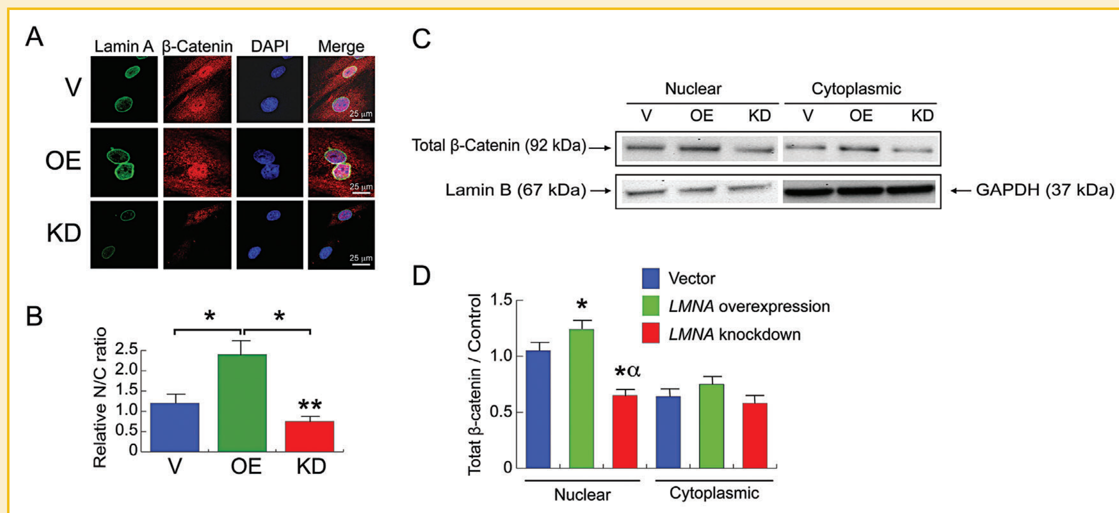

Fig. 3. Lamin A/C increases nuclear β-catenin and facilitates its transcriptional activity. MSC were transfected for 24 h with LMNA siRNA (KD), pcDNA_LMNA (OE) or empty vector (V) while induced to differentiate into osteoblasts in the presence of LiCl. Differentiation was followed by immunostaining and imaging using a Leica TCS SP5 II confocal microscope. All images were taken with a 63x oil immersion objective using the same fluorescence intensity. The figure shows representative cells for each condition. (A) Accumulation of β-catenin (red) was higher in MSC overexpressing LMNA (OE) when compared to MSC expressing normal (V) or lower (KD) levels of lamin A/C. As shown, increased lamin A/C expression (dense, strong green ring) can be clearly seen within the nuclear membranes of LMNA OE cells. (B) Fluorescence intensity for nuclear β-catenin was measured using ImageJ software and analyzed statistically comparing V, OE and KD. Using the area, integrated density and background readings we calculated corrected cell fluorescence (CTCF) for each cell nucleus and cytoplasm. A total of 25 cells in each condition were analyzed. High levels of β-catenin nuclear/cytoplasm (N/C) ratio were observed in MSC expressing high levels of LMNA, while the lowest accumulation of nuclear β-catenin was observed in cells expressing low levels of LMNA, suggesting that lamin A/C facilitates the accumulation of β-catenin in the nucleus. *$P < 0.01$; **$P < 0.001$ for V vs. KD. (C) Using the same model of MSC transfected with pcDNA_LMNA or LMNA siRNA, nuclear and cytoplasmic proteins were extracted and analyzed by western blotting for β-catenin. Image is representative of three separate experiments. (D) Luminescence intensity was measured and quantified using ImageJ and was corrected for lamin B1 or GAPDH for nuclear and cytoplasmic fractions respectively. Nuclear β-catenin was higher in MSC overexpressing LMNA when compared to both normal (V) and LMNA KD MSC, with the lowest level observed in the latter. $\alpha P < 0.01$ for KD vs. OE; * $P < 0.01$ vs. vector.

To confirm our immunofluorescence findings, we performed western blotting using nuclear and cytoplasmic proteins extracted from the same early differentiating osteoblasts. As shown in Figure 3C and D, nuclear β-catenin was significantly higher in the MSC overexpressing lamin A and significantly lower in lamin A/C siRNA-treated MSC ($P < 0.05$). In addition, nuclear β-catenin expression was significantly higher in lamin A/C overexpressing MSC and significantly lower in lamin A/C knock-down MSC ($P < 0.05$). The differences between the 3 conditions were non-significant for the cytoplasmic fractions.

LAMIN A/C INCREASES NUCLEAR β-CATENIN AND FACILITATES ITS TRANSCRIPTIONAL ACTIVITY

To assess the significance of high levels of nuclear β-catenin induced by high levels of lamin A/C expression, we tested the ability of β-catenin to form a complex with the lymphoid enhancer-binding factor (TCF), a well-known transcription factor activated by the presence of β-catenin in the nucleus. We co-transfected MSC with either the pcDNA_*LMNA* construct or *LMNA* siRNA, and TOPFLASH or FOPFLASH luciferase reporters. In the presence of overexpressed lamin A/C, luciferase activity was increased >100 fold while it was significantly decreased when lamin A/C was knocked down (Fig. 4A). This data suggest that lamin A/C plays an important role not only in the translocation and accumulation of β-catenin in the nucleus, but also in its activity during osteoblastogenesis.

LAMIN A PHYSICALLY INTERACTS WITH β-CATENIN WITHIN THE NUCLEUS OF OSTEOGENIC DIFFERENTIATING MSC

The high levels of nuclear β-catenin accumulation that were observed in lamin A/C-overexpressing MSC indicate that the translocation of β-catenin is facilitated by lamin A/C, and possibly by a direct physical interaction. To assess whether lamin A/C physically interacts with β-catenin we extracted nuclear proteins from cells transfected with pcDNA_*LMNA* and immunoprecipitated them with anti-lamin A/C and β-catenin (Fig. 4B columns 5, 6a, and 6b) followed by western blotting for lamin A/C. As shown in Figure 4B, β-catenin was co-immunoprecipitated with lamin A/C suggesting that both molecules physically interact within the nucleus.

DISCUSSION

In this study, we used an established model of differentiating MSC to characterize the role of lamin A/C during their differentiation into osteoblasts and adipocytes. We found that lamin A/C positively affects osteoblast differentiation by facilitating the presence of β-catenin into the nucleus and by inducing high levels of expression of Wnt ligands Wnt7b and Wnt10b while facilitating their osteogenic function. Conversely, high levels of lamin A/C inhibited adipogenesis.

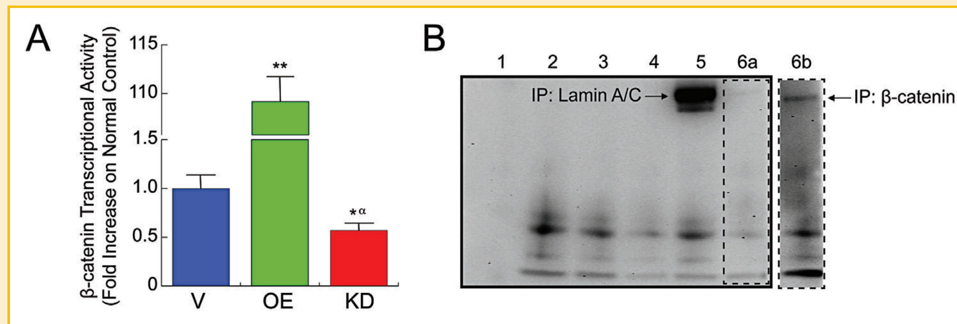

Fig. 4. Lamin A/C facilitates the osteogenic action of β-catenin and physically interacts with β-catenin in osteogenic differentiating MSC. (A) To measure the transcriptional activity of β-catenin and hence its ability to bind to TCF/LCF we used a luciferase reporter system using TOPFLASH/FOPFLASH. The highest transcriptional activity was observed in MSC overexpressing LMNA (**$P< 0.001$), while the lowest activity was in the LMNA KD MSC. *$P<0.05$ compared to vector; $\alpha P< 0.001$ when compared to MSC overexpressing LMNA. (B) To establish whether lamin A/C physically interacts with β-catenin within the inner-nuclear membrane, MSC were transfected with pcDNA_LMNA and nuclear proteins were extracted 24 h post-differentiation followed by co-immunoprecipitation (coIP) using both anti-lamin A/C and ant-β-catenin antibodies. Four controls were prepared: an agarose and lysate without antibody (column 1); an anti-lamin A antibody only control (column 2); an anti- β-catenin only control (column 3), and a non-specific IgG (lysates and beads) control (control 4). Western blot analysis of protein following IP with anti-lamin A/C antibody was positive for β-catenin (Column 5) (30 sec exposure). In addition, a reverse co-IP was performed using anti-β-catenin antibody for precipitation, while anti-lamin A/C was used for western blotting. The area surrounded by the broken line indicates an image of longer exposure time (60 sec) for chemiluminescence reaction. As shown in column 6b, lamin A/C was detected in the lysate confirming that lamin A/C physically interacts with β-catenin.

Lamin A and C are the major proteins of the lamina, which make part of the nuclear envelope, and are known to be involved in the regulation of gene expression through interplay with transcriptional factors, chromatin-associated proteins and other signal transduction molecules [Andres and Gonzalez, 2009]. The interest in studying lamin increased after the discovery that mutations within lamin A/C, or within genes that code for proteins known to interact with lamin A/C, cause diseases characterized by accelerated aging [Maraldi et al., 2011]. Interestingly these diseases affect organs and cells of mesenchymal origin, which are characterized by abnormal fat deposition in various organs similar to what is observed in bone with normal aging.

In a previous study we reported that lamin A/C knocked down by siRNA decreased the osteogenic potential of MSC while at the same time facilitating adipogenesis [Akter et al., 2009]. Inhibition of lamin A/C resulted in a significant reduction in mineralization and ALP expression, accompanied by a significant reduction of several osteogenic genes including OCN. Besides that, although the expression of the transcriptional factor RUNX2 was not affected, lamin A/C knock-down resulted in a decreased DNA binding of RUNX2, suggesting that lamin A/C plays a very important role in osteogenic complex formation and the activation of transcription in differentiating MSC.

This in vitro experiment was followed by the description of a comprehensive bone phenotype in lamin A/C knock-out (lmna$^{-/-}$) mice that showed low levels of osteoblastogenesis and high levels of marrow fat infiltration. Using the same animal model, Tong et al. [2011] reported that *Lmna*$^{-/-}$ mice had significantly higher levels of fat infiltration in both bone and muscle, a finding that correlates with observations in frail older individuals. Additional to this finding, the authors reported that lower Wnt10b and β-catenin was complemented with higher expression of major adipogenic factors in both bone and muscle of *Lmna*$^{-/-}$ mice, suggesting that lamin A/C modulates Wnt/ β-catenin signaling. Nevertheless, and despite all this previous evidence demonstrating a pivotal role of lamin A/C in fat and bone formation, the differentiation pathways that were regulated by lamin A/C during MSC differentiation remained unknown.

In the present study we assessed whether *LMNA* overexpression has the opposite effect of knock-down and thus improves osteoblast differentiation. This would have an important therapeutic relevance since processing of prelamin A into lamin A/C could become a target to regulate the typical changes of aging, frailty and osteoporosis in older people [Vidal et al., 2012]. Using an expression vector for lamin A/C we overexpressed lamin A/C in differentiating MSC when compared to cells transfected with an empty vector. We found that mineralization was significantly increased after 14 days of differentiation in lamin A/C transfected MSC. These findings correlated with higher levels of the major osteogenic genes. Interestingly, we found biphasic osteogenic characteristics in *LMNA*-transfected MSC treated with LiCl. Although the levels of osteoblastogenesis and mineralization on day 21 of differentiation was similar for LiCl-treated and untreated conditions, the high levels of β-catenin induced by LiCl in osteogenic differentiating MSC appeared to overpower the capacity of lamin A/C to regulate osteoblastogenesis, as indicated by a reduction in the expression of Wnts and most of the osteogenic genes, thus suggesting that the osteogenic effect of lamin A/C occurs downstream its interaction with β-catenin within the nucleus.

We then evaluated the nuclear translocation of β-catenin in the presence of high levels or in the absence of lamin A/C in osteogenic differentiating MSC. Luciferase assays further confirmed β-catenin activities in binding to TCF/LCF, a critical step in osteoblastogenesis [Zhou et al., 2008]. Our results indicate that nuclear translocation of β-catenin is not only facilitated by the presence of lamin A/C but also that a physical interaction occurs between lamin A/C and

β-catenin, thus acting as a facilitator of DNA transduction, which in differentiating MSC would mean an increase in transcriptional activation of important osteogenic genes such as RUNX2 as previously reported [Akter et al., 2009].

Another important finding in this study is the fact that lamin A/C plays a role in the activation of Wnts ligands (Wnt7 and Wnt10) that are critical regulators of osteoblast differentiation and the commitment of MSC to differentiate into osteoblasts rather than into adipocytes [Zhou et al., 2008]. This increase in Wnt expression is a result of the accumulation of β-catenin within the nucleus and of its increased transcriptional activity as a result of *LMNA* overexpression. Evidence on the importance of Wnt signaling in bone physiology and osteoblast function are the various loss- and gain-of-function mutations described within the Wnt co-receptor low density lipoprotein-related protein (LRP)-5 that is associated with osteoporosis pseudoglioma syndrome and high-bone mass phenotype [Balemans et al., 2007]. Knock-down of Wnt7b resulted in complete failure of mineralization while that of Wnt10b was less dramatic due to a compensatory increase inWnt7b. The high levels of Wnt7b and Wnt10b induced by lamin A/C overexpression in our cell model seem to be enhanced by increased β-catenin levels, which also suggests a role for lamin A/C downstream its interaction with β-catenin, thus playing an important role in promoting osteoblastogenesis and preventing adipogenesis, as was previously reported [Zhou et al., 2008; Mak et al., 2009].

In this study, we observed that similar to other cell models [Bogulavsky et al., 2006; Tilgner et al., 2009], overexpression of lamin A/C suppressed adipocyte differentiation from MSC. Although the exact mechanism of how lamin A/C affects adipogenesis remains unclear, it has been suggested that it is due to changes in expression of the binding partner emerin, which would affect the dynamics of and the expression and activation of PPARγ [Tilgner et al., 2009]. Interestingly, we observed that MSC induced to differentiate into adipocytes and expressing high levels of lamin A/C showed a ~6 fold higher expression in both Wnt7b and 10b when compared to normal MSC at the first week of differentiation under adipogenic conditions. Although this initial increase in the expression of both Wnt proteins could be due to the effect of dexamethasone present in the adipogenic media, it has been reported that early activation of Wnt10b inhibits adipogenic differentiation of MSC by suppressing PPARγ [Bennett et al., 2005], which was in clear agreement with our gene expression profiling thus suggesting that this suppression acts as a molecular switch that favors MSC differentiation into osteoblasts while decreasing adipogenesis.

In conclusion, our findings are relevant to the features of age-related bone loss and osteoporosis, which are characterized by an increased infiltration of fat within the bone marrow, accompanied by lower osteoblast numbers (Fig. 5). From the molecular point of view, age-related bone loss has been associated with changes in Wnt10b expression, which leads to an age-progressive osteopenia primarily due to decreased bone formation rather than to increased bone resorption [Stevens et al., 2010]. The results that we report here complement our previous reports on the role of lamin A/C in MSC differentiation and provide a molecular mechanism involving Wnt/β-catenin, which is a pivotal osteogenic pathway. Taken together, our evidence indicates that the presence of normal levels of lamin A/C is required to maintain the integrity of the bone structure. From a therapeutic perspective, inducing the post-translational processing of prelamin A into lamin A/C could become a new therapeutic approach to age-related muscle and bone loss in the near future.

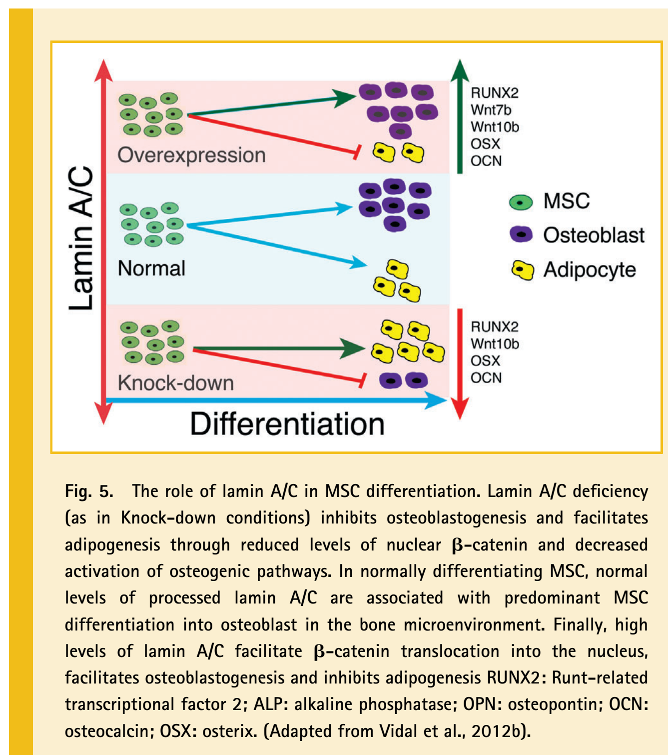

Fig. 5. The role of lamin A/C in MSC differentiation. Lamin A/C deficiency (as in Knock-down conditions) inhibits osteoblastogenesis and facilitates adipogenesis through reduced levels of nuclear β-catenin and decreased activation of osteogenic pathways. In normally differentiating MSC, normal levels of processed lamin A/C are associated with predominant MSC differentiation into osteoblast in the bone microenvironment. Finally, high levels of lamin A/C facilitate β-catenin translocation into the nucleus, facilitates osteoblastogenesis and inhibits adipogenesis RUNX2: Runt-related transcriptional factor 2; ALP: alkaline phosphatase; OPN: osteopontin; OCN: osteocalcin; OSX: osterix. (Adapted from Vidal et al., 2012b).

ACKNOWLEDGEMENTS

This study was supported by the Australian National Health and Medical Research Council (NHMRC 632767) and the Nepean Medical Research Foundation.The authors have no conflicts of interest to declare.

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