Research Article

The Role of the Nuclear Envelope Protein MAN1 in Mesenchymal Stem Cell Differentiation¹

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ABSTRACT

Mutations in MAN1, a protein of the nuclear envelope, cause bone phenotypes characterized by hyperostosis. The mechanism of this pro-osteogenic phenotype remains unknown. We increased and decreased MAN1 expression in mesenchymal stem cells (MSC) upon which standard osteogenic and adipogenic differentiation were performed. MAN1 knockdown increased osteogenesis and mineralization. In contrast, osteogenesis remained stable upon MAN1 overexpression. Regarding a mechanism, we found that low levels of MAN1 facilitated the nuclear accumulation of regulatory smads and smads-related complexes, with a concurrently high expression of nuclear β -Catenin. In addition, we found adipogenesis to be decreased in both conditions, although predominantly affected by MAN1 overexpression. Finally, lamin A, a protein of the nuclear envelope that regulates MSC differentiation, was unaffected by changes in MAN1. In conclusion, our studies demonstrated that lower levels of MAN1 in differentiating MSC are associated with higher osteogenesis and lower adipogenesis. High levels of MAN1 only affected adipogenesis. These effects could have an important role in the understanding of the role of the proteins of the nuclear envelope in bone formation.

INTRODUCTION

The nuclear envelope (NE) serves as a bridge and scaffold that links the nucleoplasm and the cytoplasm. It facilitates the selective interchange of molecules between these two compartments, while also regulates cell survival and maintenance. The NE is formed by four main components: (1) the outer nuclear membrane, which is linked to the endoplasmic reticulum by phospholipids; (2) the inner nuclear membrane; (3) the nuclear pore complexes; and (4) the lamina (composed of lamin A, B and C) (Broers et al., 2006). NE proteins are key intermediates of the mechanotransduction process in which an extracellular stimulus is physically propagated through the cytoplasm, crosses the nuclear envelope, and generates a rapid genetic response by regulating gene transcription (Wang et al., 2009). In addition, proteins at the NE associate themselves in a tissue-specific manner. This binding pattern modulates and delivers molecular signals, which are faster than second messengers (Wang et al., 2009).

Amongst these proteins, lamin A is one of the main proteins at the NE that not only gives firmness to the nucleus, but also regulates a broad range of cellular processes that control signal transduction (Andres and Gonzalez, 2009). Lamin A binds NE transmembrane (NET) proteins forming networks that regulate replication, transcription, DNA repair, gene expression, and signaling/communication between the nucleus and the cytoplasm (Wilson and Foisner, 2010). In bone, lamin A is a key player for mesenchymal stem cell (MSC) survival and differentiation (Bermeo et al., 2015; Broers et al., 2006). Changes in lamin A expression affect osteoblast and adipocyte differentiation of MSC along with low bone formation and fat accumulation in the bone marrow (Akter et al., 2009; Li et al., 2011; Tong et al., 2011), which are the typical hallmarks of age-related bone loss and osteoporosis (Bermeo et al., 2014).

More than 80 NET proteins have been identified so far, most of them with unknown function. Amongst them, MAN1 is a NET protein containing the LEM (LAP, emerin, MAN1) domain, which is located in the amino-terminal position, and whose function is to bind several transcription factors (Caputo et al., 2006; Lin et al., 2005; Mansharamani and Wilson, 2005).

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MAN1 has two transmembrane domains and a carboxy-terminal RNA-recognition motif, which face the nucleoplasm playing important roles in cell function and differentiation (Pan et al., 2005). The principal reported function of MAN1 is the regulation of transforming growth factor- β (TGF- β) and bone morphogenetic protein (BMP) signaling pathways. This function is exerted through interactions with regulatory transcription factors known as Drosophila protein "suppressor of mothers against decapentaplegic" (smads) and the C. Elegans protein SMAS (Pan et al., 2005; Parnaik, 2008; Shaklai et al., 2007).

In the mesenchyme, the significance of this interaction starts when TGF- β ligands sense perturbations – mechanical stress, tissue injury, inflammation, etc. – in the extracellular matrix. As part of the response to these perturbations, TGF- β ligands become active chemo attractants that switch on the signaling pathways required for MSC mobilization with tissue repair purposes (Kamiya et al., 2008; Lowery et al., 2015; Wan et al., 2012). Amongst these pathways, receptors for TGF- β and BMP facilitate the phosphorylation and activation of the smads, which then form complexes (i.e. 1/5/8 and 2/3) that translocate into the nucleus and regulate gene transcription (Bengtsson, 2007; Chen et al., 2012; Heessen and Fornerod, 2007). MAN1 antagonizes TGF- β /BMP signaling by sequestering or binding both active phosphorylated (Psmads) and non-phosphorylated smads (Bourgeois et al., 2013; Gruenbaum et al., 2005; Konde et al., 2010), thus acting as a regulator of this tissue repairing process. MAN1 overexpression decreased smads and TGF- β /BMP-responsive promoters activity while also caused cell death in Hep3B and HEK293 cell lines (Pan et al., 2005). Similarly, deficiency of MAN1 caused that *Man1* Δ/Δ mice died during embryogenesis due to enhanced TGF- β signaling and high levels of smads 2/3-activated signal (Ishimura et al., 2006).

Interestingly, heterozygous mutations of the MAN1 gene (*LEMD3*) in humans have been associated with rare genetic diseases characterized by increased bone mass (Burger et al., 2010; Hellemans et al., 2004; Mumm et al., 2007). Patients suffering from these mutations show either small, diffuse round areas of increased bone density (Buschke-Ollendorff syndrome), or severe

bone thickening that tends to be limited to one arm or leg (melorheostosis). Although the mechanisms explaining these abnormally high levels of ossification remain unknown, there is some evidence suggesting that it could be due to changes in TGF- β /BMP-responsive pathways. However, this hypothesis has not been tested in human models of MSC differentiation in the past.

In this study, we hypothesized that regulation of MAN1 expression in normal human MSC *in vitro* would facilitate osteoblastogenesis. In addition, considering that the role of MAN1 in adipogenesis is unknown, we also hypothesized that regulation of MAN1 in MSC has an inhibitory effect on adipogenesis, which would play an important role in the pathogenesis of age-related bone loss and osteoporosis. We expect that understanding the role of the proteins of the NE in bone biology would open a new potential set of future therapeutic targets for osteoporosis that deserve further exploration.

MATERIALS AND METHODS

Cell culture – All experiments were carried out using normal human MSC (Lonza Bioresearch, Basel, Switzerland) 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 five. 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.

Transfections – In preparation for transfection, MSC were seeded at a density of 5×10^4 cells/cm² in corresponding plates in MSCGM without antibiotics and incubated at 37°C in a humidified atmosphere of 5% CO₂. On the day of transfection, the MSCGM was changed to antibiotic-free MSCGM. All transfections were carried out using Lipofectamine 2000 (Invitrogen, Life Technologies) at 80% confluency following the manufacturer's protocol. MAN1 knockdown was obtained using 40 μ M siRNA (sc-43384, Santa Cruz Biotechnology, Dallas, TX, USA), 10 μ M siRNA control (sc-37007, Santa Cruz Biotechnology) with siRNA transfection medium (Santa Cruz

Biotechnology). For MAN1 overexpression, we used a cDNA_MAN1 plasmid generously provided by Dr. Roland Foisner (Max F. Perutz Laboratories, University of Vienna, Austria) (Brachner et al., 2005). 100 ng of the pcDNA_MAN1 construct was used. After an overnight incubation, MSCGM was aspirated and replaced with osteoblast or adipocyte induction medium.

Cell 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 μ M dexamethasone. Medium was changed every 3 days for two weeks. 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 μ M dexamethasone, 10 μ g/mL insulin, 0.2 mM indomethacin, 0.5 mM 3-isobutyl-1-methylxanthine, 10% FBS, 0.05 U/mL penicillin and 0.05 μ g/mL streptomycin, and Adipogenic Maintenance Medium (AMM [10 μ g/mL Insulin, 10% FBS, 0.05 U/mL penicillin and 0.05 μ g/mL streptomycin]) every three days for two weeks, until an adipogenic phenotype was obtained.

Cell viability – Cell viability upon transfection and under differentiation conditions was measured using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). Twenty-four hours after seeding the cells in 4x96 well plates, cells were transfected for 24h as previously described. After 24h in transfection conditions, media was replaced with differentiation media (osteogenic or adipogenic) (time 0) and survival was evaluated at timed intervals, (24, 48 and 72h) using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay following manufacturer's instructions. Survival was calculated as percentage of change compared with control at time 0.

Alkaline phosphatase (ALP) activity and staining – ALP catalyzes the hydrolysis of p-Nitrophenyl phosphate (pNPP) to p-Nitrophenol (Sabokbar et al., 1994). To measure ALP activity, after

removing the medium and washing with tris-buffered saline (TBS, 20 mM Trizma base, 150 mM NaCl at pH 7.5) the cells were fixed in 3.7% formaldehyde-90% ethanol for 30 seconds at room temperature, then the cells were incubated with 1 mg of pNPP/mL reaction buffer (NaHCO₃ 50 mM, at pH 9.6 with 1 mM MgCl₂) at 37°C for 20 min. The reaction was stopped by adding 50 µL of 3 N NaOH and mixing thoroughly. 100 µL of reaction were transferred to a micro plate and read in a FLUOstar OPTIMA luminometer (BMG Labtech). Values were corrected against cell viability as previously described (Qiu et al., 2010). For ALP staining, seven days after transfection and differentiation, the cells were washed with PBS, fixed and stained for semi-quantitative ALP activity using the 86C staining system (Cat. No. 86C, Sigma-Aldrich) according to manufacturer's instructions.

Alizarin Red (AR) staining and measurement – At week two of osteogenic differentiation, we assessed mineralization using AR standard staining and quantification (Bermeo et al., 2015). Briefly, 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 minutes at room temperature 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 14, 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 hour. The fixative was then aspirated, and cells were washed with 60% isopropanol before being allowed to dry completely. Cells were stained for 10 minutes 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 minutes and absorbance measured at 500 nm. For cell count, plates were counterstained with undiluted filtered hematoxylin (Cat. No. 0701, Amresco) for 10 minutes. Photomicrographs (magnification 200X) were taken using an IX50 Olympus inverted microscope (Olympus, Tokyo, Japan) and a Digital Sight DS-5M Nikon camera (Nikon Instruments, Inc. Melville, New York, NY). The number of blue stained nuclei was counted by a blinded observer in at least five randomly chosen fields per well.

Immunofluorescence microscopy – MSC were cultured in a four-chamber slides (BD Biosciences).

When reaching 80% confluency transfection was performed as described above. Upon transfection and 24 h of differentiation, cells were fixed, permeabilized and blocked according to primary antibody brand guidelines. Primary antibody solution contained 1% bovine serum albumin (BSA, Sigma-Aldrich), 0.3% Triton-X, 0.05% tween 20 and 1 X tris-buffered saline with either anti phospho-smad1/5 and phospho-smad2/3, or anti-total smad2/3 (Cat. No. 13820, 8828, 8685; Cell Signaling Technology), or smad1/5/8 (Cat. No. 6031-R; Santa Cruz Biotechnology). Anti-total smads antibodies recognize both active phosphorylated and inactive unphosphorylated smads. Cells were incubated with primary antibody overnight at 4°C with mild agitation and then with secondary antibody Alexa Fluor® 488 Donkey Anti-Rabbit (Invitrogen) for 1 h at RT. Following incubation slides were blocked again and incubated for 1.5 h with the second primary anti-LEMD3 (Cat. No. TA500794, Origene). Slides were washed and incubated with IgG secondary antibody conjugated with Alexa Fluor® 555 Donkey Anti-Mouse IgG (Invitrogen) for another hour followed by washing and counterstaining with diluted (1:5000) nuclear dye 4,6-diamidino-2-phenylindole (DAPI). Slides were mounted using a buffered glycerol with anti-fade (0.1 M phosphate buffer pH7.4, 90% glycerol, 5 mg/ml n-propyl gallate), visualized and photographed using a Leica TCS SP5 II confocal microscope and LAS AF Software (Leica Microsystems, Wetzlar, Germany). Single staining was done for or anti V5 (Cat. No. V8012; Sigma-Aldrich), lamin A (Cat No. sc-20680; Santa Cruz Biotechnology) and β -Catenin (Cat No. sc-59737; Santa Cruz Biotechnology).

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RT-PCR – mRNA from transfected and control cells was reverse transcribed into a first strand cDNA using a highly sensitive Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Bioline, London, UK), oligo-dt18 and 1 µg of total RNA extracted with RNeasy mini kit (QIAGEN) and treated with RNase-free DNase (QIAGEN) according to manufacturer instructions. The RNA was kept on ice and immediately quantified using Nanodrop 2000 spectrophotometer (Thermo Scientific, Life Technologies). qPCR was performed using the SensiMix SYBR No-ROX Kit (Bioline) following manufacturer's instructions and specific primers (Table 1) as previously described (Vidal et al., 2012b). Amplifications were performed in a rotor-based Corbett Rotor-Gene™ 3000 (QIAGEN) under the following cycling conditions: 95°C 10 min, 40 cycles of: 95°C 15 s, 60°C 15 s, 72°C 30 s.

Western blot – MSC were grown in 6-well culture plates and transfected with siRNA, cDNA-MAN1 or controls as stated above. After induction was complete, total cell fraction was extracted using PARIS™ Kit (Ambion, Life Technologies) following the manufacturers' protocol. Protein concentrations were measured using the bicinchoninic acid (BCA) protein assay reagent (Thermo Scientific) on Nanodrop 2000. 15 µg of protein sample were mixed with 50 mM Dithiothreitol (DTT) and 1 X Lithium dodecyl sulfate (LDS) sample buffer (Life Technologies), denatured at 95°C for 5 min. and loaded into a 4–12% polyacrylamide reducing gel (Life Technologies). The electrophoresis was done using 3-(N-morpholino)propanesulfonic acid (MOPS) buffer in a SureLock mini-cell tank (Invitrogen) for 70 min. at 150 V. Proteins were transferred to Polyvinylidene difluoride (PVDF) membranes (Thermo Scientific) in a mini trans-blot transfer cell (BIO-RAD, Hercules, CA, USA) at 350 miliampere (mA) for 50 min. Membranes were blocked with 5% BSA at RT for 1 h and blotted using 0.2 µg/ml of primary antibodies overnight at 4°C with mild agitation, followed by 1h incubation with secondary antibody conjugated with horseradish peroxidase (HRP) (final concentration 0.04 µg/ml). Following incubation, blots were washed

visualized by densitometry using SuperSignal West Femto chemiluminescent substrate for HRP (Thermo Scientific) and Quantity One 4.4.0 software (BIO-RAD). Quantification was performed using NIH ImageJ medical image software (<http://imagej.nih.gov/ij/download.html>).

Co-immunoprecipitation – Co-immunoprecipitation was performed to assess whether smads 2/3 complex physically interacts with MAN1 in MSC. Cells were transfected with cDNA-MAN1 plasmid and induced to differentiate into osteoblasts for 24 h followed by extraction of nuclear proteins with the NE-PER (Thermo Scientific) kit according to manufacturer's instructions. 100 µg of nuclear protein were pre-cleared with protein A/G plus agarose (Cat. No. sc-2003; Santa Cruz Biotechnology) for 2 h, followed by centrifugation for 3 min. at 2000 g. The supernatant was incubated with 1 µg of anti-LEMD3/smads 2/3 overnight at 4°C on a slow rotator. To determine possible false positives antibodies and beads controls were prepared. After incubation, 20 µL of protein A/G were added and incubated for 4h at 4°C on a slow rotator, followed by centrifugation as before. The pellet was washed and mixed with 30 µL of 2 X electrophoresis sample buffer (Santa Cruz Biotechnology) followed by incubation for 2 min. at 95°C. Samples were then subjected to electrophoresis and western blotting as described above using anti-smads 2/3, anti-LEMD3 and anti-MAN1 (Cat. No. sc-19785 and sc-50458; Santa Cruz Biotechnology).

Runx2-related transcription factor 2 (RUNX2) and peroxisome proliferator-activated receptor gamma (PPAR γ) activation assays – Active RUNX2 binding to DNA was determined using the ELISA-based RUNX2 and PPAR γ activation TransAM™ kit (Active Motif, Rixensart, Belgium). The Trans-AM Runx2 Kit contains a 96-well plate on which an oligonucleotide containing either a consensus-binding site has been immobilized. The active form of RUNX2 or PPAR γ contained in nuclear extract specifically binds to their corresponding oligonucleotide. The primary antibody used in the Trans-AM Runx2 Kit recognizes an accessible epitope on RUNX2 or PPAR γ protein upon DNA binding. The addition of a secondary horseradish peroxidase-conjugated antibody provides a

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sensitive colorimetric readout easily quantified by spectrophotometry (450 nm). To quantify active binding, 10 µg of protein extract was obtained from osteoblasts or adipocytes under transfection conditions. The DNA binding was measured at 450nm by spectrophotometry. The calculations were done according to the manufacturer's instructions (Active Motif, Rixensart, Belgium).

Statistical analysis – Each experiment was repeated three times and results are shown as average with $\pm$ standard deviation. Statistical analysis of data comparing treated and untreated cells was performed with two-tailed, paired student's t-tests with the probability set at 0.05 as the level of significance. One-way ANOVA, using the IBM SPSS statistics version 20.0 software, was computed when more than two groups were compared. The genic expression indicators obtained by qPCR were analyzed using the REST software from QIAGEN (Pfaffl et al., 2002).

RESULTS

Transfection efficiency and cell survival – Considering that both decreased and increased expressions of MAN1 have been reported to cause cell death in other models (Pan et al., 2005), we tested several doses of siRNA and cDNA transfections aiming to identify the safest and more transfection-efficient doses for our differentiation models. For inhibition (siRNA) of MAN1, different concentrations of oligo (20-160 µM) were tested. MAN1 inhibition was further confirmed by RT-PCR, and MTS Formazan determined cell survival. A dose of 40 µM showed the most optimal and stable transfection efficacy for both osteogenic and adipogenic conditions (Fig. 1A, $p<0.001$), without affecting cell survival (Fig. 1B).

Similarly, we tested knock-in (overexpression) efficiency with three different concentrations of plasmid (100-500 ng) containing full-length human *LEMD3* (cDNA_MAN1) (Brachner et al., 2005). Although all the tested concentrations significantly increased MAN1 gene expression (an effect that was still present at day 14), the lowest dose (100 ng) was the only condition that showed high transfection efficacy at day 14 of osteogenic differentiation (Fig. 1C, $p<0.001$) without

concurrently affecting cell survival (Fig. 1B). In addition, presence of exogenous MAN1 in cDNA_MAN1-transfected cells was visualized 24 h post-transfection using immunostaining with anti-V5 tag, which confirmed the presence of transfected MAN1 in our osteogenic differentiation model (Fig. 1D).

Changes in MAN1 expression affect osteoblast differentiation of MSC – The mechanisms that explain the formation of heterotopic bone in patients suffering from MAN1 gene mutations remain unknown. To elucidate a potential molecular mechanism of this clinical observation, we used a model of knockdown (siRNA) and overexpression (cDNA_MAN1) of the MAN1 gene in osteogenic differentiating MSC *in vitro*. MSC were transfected (MAN1 siRNA or cDNA_MAN1) as previously described and then cultured in osteogenic media for up to 14 days. At day 7, ALP expression and activity were not affected by up or down regulation of the MAN1 gene (Fig. 2A, B). In contrast, at day 14, mineralization assessed by staining Ca^{2+} depots with AR showed that, whereas overexpression of MAN1 did not affect the mineralization, MAN1 knockdown significantly increased mineralization at a relatively early phase of osteoblast differentiation (Fig. 2C, $p<0.05$).

Subsequently, we tested changes in lamin A and osteogenic gene expression using RT-PCR of RNA obtained at day seven of differentiation. Lamin A gene expression was unchanged by either overexpression (Fig. 2D) or knockdown (Fig. 2E) conditions. Regarding the osteogenic genes, RUNX2 gene was increased by both high and low levels of MAN1 gene expression (Fig. 2D and E; $p<0.001$). Other osteoblast markers osteopontin (OPN), osteoprotegerin (OPG) and Wnt10b did not show significant changes in gene expression in either condition as compared with controls.

Subsequently, to confirm if the raise in RUNX2 gene was concomitantly associated with higher transcriptional activity, we measured RUNX2 activity using an ELISA-based method. We found that RUNX2 transcriptional activity was significantly increased under MAN1 siRNA conditions ($p<0.001$), with no changes being observed when MAN1 expression was increased (Fig. 2F).

MAN1 regulates TGF- β and Wnt/ β -Catenin signaling pathways in MSC under osteogenic

conditions – The biological role of MAN1 is exerted via TGF- β signaling pathway mostly through the regulation of smads (Lin et al., 2005; Pan et al., 2005). As shown in Fig. 3A, MAN1 overexpression decreased nuclear concentration of Psmads 2 with smads complexes 1/5, 2/3 and 1/5/8 remaining mostly in the cytoplasm. In contrast, low levels of MAN1 allowed a higher nuclear expression of Psmad 2 and Psmad1/5, and markedly higher concentrations of the osteogenic complexes smads 2/3 and 1/5/8 within the nucleus of osteogenic differentiating MSC.

A further quantification of protein expression (Fig. 3B) revealed that MAN1 overexpression was associated with significantly lower levels of expression of Psmad 2 and smads 1/5/8 and 2/3 complexes ($p<0.01$). On the contrary, MAN1 knockdown was associated with high levels of protein expression of phosphorylated smad-2 and the smads complexes 1/5/8 and 2/3 ($p<0.01$). Taken together, these results indicate that changes in the expression in MAN1 during osteogenic differentiation of MSC influence the distribution of smads with predominantly nuclear concentrations observed in knockdown conditions, which also demonstrated to facilitate mineralization and bone formation (Fig. 2B).

Furthermore, considering that TGF- β and Wnts signaling pathways cooperate to regulate osteoblastogenesis in human MSC via β -Catenin (Zhou, 2011) – followed by strong activation of RUNX2 – and that the role of lamin A in osteoblastogenesis has been associated with changes in nuclear β -Catenin (Bermeo et al., 2015), we assessed those changes in nuclear β -Catenin induced by high and low levels of MAN1 expression in osteogenic differentiating MSC. As observed in Fig. 3C, low levels of MAN1 were associated with significantly higher levels total β -Catenin in the nucleus ($p<0.05$), a finding that could explain in part the observed increase in bone formation. On the other hand, no change in nuclear β -Catenin was observed during overexpression of MAN1 in osteogenic differentiating MSC.

Subsequently, we tested whether, as in other models (Bourgeois et al., 2013; Gruenbaum et al., 2005; Konde et al., 2010), high levels of MAN1 also sequester smads through specific MAN1/smad interaction. Using co-immunoprecipitation, we found that there is no interaction between MAN1 and smads in our model (Fig. 4).

Finally, considering that MAN1 as one of the main binding partners of lamin A in the nuclear membrane (Mansharamani and Wilson, 2005), we evaluated whether changes in MAN1 altered the expression of lamin A in our model. We found that, as per our RT-PCR findings (Fig. 2D and E), no changes in protein expression or distribution of lamin A were observed under any of our experimental conditions (Fig. 3D). These results indicate that increased osteoblastogenesis upon absence of MAN1 was not directly caused by changes in lamin A.

Changes in MAN1 expression prevent adipocyte differentiation in MSC – Adipogenesis takes place at the expense of osteoblastogenesis when factors needed for the latter are altered (Rosen and Buxsein, 2006). This phenomenon is explained by the fact that both lineages share the same cell origin, and that MSC turn into adipocytes when stemness is lost due to natural stimuli such as aging (Sethe et al., 2006) or inflammation (Lepperdinger, 2011). In the case of the NE, we have previously demonstrated that decreased and increased expression of lamin A improves and affects MSC fate toward the adipogenic lineage respectively (Akter et al., 2009; Bermeo et al., 2015).

To assess the adipogenic differentiation potential of human MSC upon overexpression and knockdown of MAN1, MSC were transfected as previously described and then treated with adipogenic medium for two weeks. As shown in Fig. 5A, both the number of adipocytes per well and the amount of eluted oil red O (ORO) were decreased by MAN1 overexpression ($p<0.001$). A similar although less dramatic effect was observed upon MAN1 knockdown ($p<0.01$). To assess the significance that changes in MAN1 expression have on adipogenic differentiation, we assessed gene expression at day two of differentiation under adipogenic conditions. PPAR γ was increased under both overexpression and knockdown conditions MAN1 ($p<0.001$). MAN1 overexpression

inhibited aP2 while knockdown was associated with increasing expression of CEBP α and low adiponectin ($p<0.001$) (Fig. 5B).

Finally, we assessed the activity of the main adipogenic transcription factor PPAR γ (Fig. 5C) at day two of differentiation by ELISA and absorbance values corrected against protein content. In spite of its increased gene expression, PPAR γ transcriptional activity was dramatically decreased upon overexpression of MAN1 as compared with control ($p<0.05$), an effect that corresponded with the significant inhibitor effect on adipogenesis by high levels of MAN1 (Fig. 5A), but which was not observed under knockdown conditions.

DISCUSSION

Considering that mutations in MAN1 are associated with hyperostosis in humans (Burger et al., 2010; Hellemans et al., 2004; Mumm et al., 2007), and that the mechanisms explaining this phenomenon remain unclear, we therefore hypothesized that changes in MAN1 expression could affect differentiation of human MSC into osteoblasts and adipocytes thus inducing alterations in mineralization and bone biology. We also investigated whether the role of MAN1 in MSC differentiation was independent of lamin A, and whether as in other models (Pan et al., 2005), MAN1 plays a role in the dynamics of some essential transcription elements of the BMP/TGF- β and Wnts/ β -Catenin pathways in differentiating MSC.

Our findings are summarized in Figure 6. We found that low levels of MAN1 in human MSC were associated with higher osteoblastogenesis, increased nuclear accumulation of smads complexes, and higher gene expression and activity of the master osteogenic regulator RUNX2. Our molecular analysis of osteogenic pathways also demonstrated that low levels of MAN1 in osteogenic differentiating MSC are associated with high levels of nuclear β -Catenin, which strongly promotes RUNX2 activation and osteoblast differentiation. Overall, this effect could be explained by facilitation of translocation of smads and β -Catenin through the nuclear pori, a phenomenon that has been previously observed in other cell models of low MAN1 expression (Cohen et al., 2007).

In terms of the osteogenic factors activated by low MAN1 expression, only RUNX2 was strongly activated by the presence of high levels of smads within the nucleus. Activation of osteogenic pathways by osteogenic media would finally converge in the activation and nuclear translocation of the main osteogenic transcription factors (β -Catenin, smad2/3 or smad1/5/8), which activate RUNX2. After activation, specific domains in RUNX2 bind other effectors (smads), which in combination with nuclear mediator smad4 form the osteogenesis-activating complex (Javed et al., 2009; Lee et al., 2002). We consider that low levels of MAN1 facilitate this process by increasing the availability of the effector Psmads and β -Catenin within the nucleus, which could explain the solid effect on mineralization without a major effect on differentiation markers (i.e. ALP and OPN). In addition, this finding could also explain the clinical features in patients with mutations in MAN1 in which the predominant phenotype is associated with heterotopic ossification with abnormal and disorganized calcification.

On the other hand, we expected osteoblastogenesis to be diminished by increased levels of MAN1, an effect that would be expected if, as in other cell models (Bourgeois et al., 2013; Pan et al., 2005), high levels of MAN1 would physically interact with smads thus preventing their translocation to the nucleus while facilitating their degradation. Interestingly, we did not find any physical interaction (co-localization) between MAN1 and smads, thus suggesting that whereas low MAN1 conditions facilitate osteoblastogenesis through higher concentration of smads within the nucleus, high MAN1 is unable to prevent the translocation of smads in MSC due to lacking this physical interaction. Without this physical interaction, MAN1 would be unable to un-phosphorylate Psmads thus osteogenesis could still occur.

We also expected that a decrease in MAN1 expression would inhibit adipogenesis while mimicking the typical hallmarks of aging bone in which high osteoblastogenesis is balanced by low adipogenesis (Bermeo et al., 2014; Pei and Tontonoz, 2004). We found that both high and low levels of MAN1 affected adipocyte differentiation and function, being this effect more significant in MAN1 overexpressing MSC. In addition, MAN1 overexpression exerted a strong inhibitory

effect on PPAR γ mRNA and activity, closely corresponding to the levels in which adipogenesis was affected. Altogether, our data suggest that changes in MAN1 expression could affect adipogenesis. The mechanisms and implications of this finding in bone biology and the role of adipogenesis in MAN1 mutations in humans should be the subject of future studies.

Interestingly, the role of MAN1 in MSC differentiation appears to be independent of lamin A. Lamin A gene and protein expression were not affected by changes in MAN1. This is pivotal to understand the role and potential interactions of the proteins of the nuclear envelope in the pathogenesis of diseases associated with low (i.e. Hutchinson–Gilford Progeria Syndrome, HGPS) and excessive (Buschke-Ollendorff syndrome and melorheostosis) bone formation. Whereas lamin A exerts both genomic and translocation-facilitating effect on RUNX2 and β -Catenin (Bermeo et al., 2015), MAN1 seems to regulate this process by reducing the presence of osteogenic transcription factors within the nucleus. In terms of therapeutic implications of our findings, future anabolic treatments for osteoporosis could be targeted to stimulate lamin A processing (Vidal et al., 2012a), inhibit MAN1 expression, or combine these two effects in MSC. Any of these approaches would be expected to increase bone formation and mineralization.

In conclusion, we have identified a new potential mechanism that may partially explain the presence of hyperostosis in human subjects suffering from mutations in the MAN1 gene. Understanding the role of the proteins of the nuclear envelope in bone biology will not only have a significant impact in the developing of new therapies for these rare disorders but would also provide with new knowledge that could be used to understand and treat osteoporosis in the future.

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FIGURE LEGENDS

FIGURE 1. *Transfection efficiency and cell viability*

(A) Electrophoresis of RT-PCR products shows that consecutive transfections of siRNA MAN1 (siM) (40 μ M) in osteogenic and adipogenic differentiating MSC steadily decreased MAN1

expression in MSC up to day 14 of osteogenic differentiation. The figure illustrates MSC under osteogenic conditions which highly correlated with MSC under adipogenic conditions. MAN1 gene expression was significantly down regulated at all three time points (** $p<0.001$).

(B) Early cell survival was not affected by MAN1 knockdown (siRNA) in MSC under osteogenic conditions. Overexpression (cDNA_MAN1) affected cell survival at doses of 250 ng and above.

Similar results were obtained in transfected MSC under adipogenic conditions. (** $p<0.001$).

(C) For MAN1 overexpression, different concentrations of plasmid (100, 250 and 500 ng) tested for efficiency up to day 14 of osteogenic differentiation showed to significantly maintain high levels of gene expression at all time points. Similar results were obtained in transfected MSC under adipogenic conditions. (** $p<0.001$).

(D) Exogenous MAN1 from transfected cells was visualized 24 h post-transfection using immunostaining with anti-V5 tag confirming the presence of transfected MAN1 in our cell model.

FIGURE 2. *Effect of MAN1 knockdown and overexpression on osteoblast differentiation of MSC.*

Human MSC were transfected with either siRNA or plasmid cDNA_MAN1 and induced to differentiate into osteoblast. **(A)** After 7 days, staining for alkaline phosphatase (ALP) expression, a semi quantitative method for assessing early osteogenic differentiation, showed no changes upon either overexpression or down regulation of MAN1 as compared with cells transfected with empty plasmid or siRNA control, respectively. Percentage values of stained fractions were corrected against cell number obtained by counterstaining with DAPI. This result was in agreement with quantitation of ALP activity **(B)**, which was measured by spectrophotometry. Absorbance values were corrected against cell viability (Qiu et al., 2010).

(C) Mineralization at day 14, quantified using alizarin red, showed that osteoblasts expressing low levels of MAN1 were significantly more mineralized than siRNA controls (* $p<0.05$). In contrast, mineralization was not affected by MAN1 overexpression.

(D-E) Gene expression assessed by RT-PCR from total RNA obtained at day 7 of differentiation.

Lamin A expression was not affected by changes in MAN1 expression. Both, MAN1 overexpression (OE) and knockdown (KD) significantly up regulated the main osteogenic regulator RUNX2. Osteogenic markers, OPN and OPG along with Wnt ligand Wnt10b were not changed upon changes in MAN1. (** $p < 0.001$). RUNX2: Runt-related transcriptional factor 2; OPN: osteopontin; OPG: osteoprotegerin.

(F) Although RUNX2 gene expression was significantly increased in both conditions (D and E), its activity as a transcription factor was only increased upon MAN1 KD (** $p < 0.001$).

FIGURE 3. Changes in TGF- β and Wnt signaling upon MAN1 overexpression (OE) and knockdown (KD).

(A) Representative pictures of confocal microscopy upon double immunostaining (smads and MAN1) of MSC transfected with plasmid containing cDNA_MAN1 or siMAN1 after 24 h under osteogenic stimuli. Left panels show staining for active phospho-smad2 (Psmad, upper) and phospho-smad1/5 (lower); right panels show staining for total levels of osteogenic complexes smads 2/3 (smad2/3) and smads 1/5/8 (smad1/5/8). White arrows indicate nuclear localization of smads, which is more marked for all smads upon KD of MAN1 as compared with untransfected controls. Red arrows show cytoplasmic expression of smads, which is increased under MAN1 overexpressing conditions.

(B) Protein analysis by western blot and further quantification of total extracts obtained after transfections and 7 days of osteogenic differentiation. MAN1 overexpression was associated with significantly lower levels of expression of Psmad 2 and smads 1/5/8 and 2/3 complexes. MAN1 knockdown was associated with high levels of protein expression of Psmad-2 and the smads complexes 1/5/8 and 2/3. (* $p < 0.01$; ** $p < 0.001$).

(C) Nuclear accumulation of β -Catenin (white arrow) was demonstrated under MAN1 knockdown condition and confirmed by western blotting of total protein extracts (* $p < 0.05$).

(D) Lamin A protein expression was maintained under all experimental conditions.

U: untransfected, E: empty vector, OE: overexpression, SiC: siRNA control, KD: knockdown.

Scale bar: 10 μ m.

FIGURE 4. Immunoprecipitation of MAN1 and total smads.

Probe for MAN1 with anti-mouse (lanes 1-7), anti-rabbit (lane 8) and anti-goat (lane 9) antibodies.

1. Beads control (Nuclear proteins + anti-mouse antibody), 2. Input control: Pre-cleared nuclear input only (no antibody), 3. Beads control (Nuclear proteins + anti-rabbit antibody), 4. Beads control (Nuclear proteins + anti-goat antibody), 5. IP: Pre-cleared nuclear input and Tsmad1 antibody, 6. IP: Pre-cleared nuclear input and Tsmad2/3 antibody, 7. IP: Pre-cleared nuclear input and anti-MAN1 antibody (mouse), 8. IP: Pre-cleared nuclear input and anti-MAN1 antibody (rabbit), 9. IP: Pre-cleared nuclear input and anti-MAN1 antibody (goat). As indicated by arrows, we were able to precipitate MAN1 using 3 different antibodies (lanes 7, 8 and 9) but unable to detect it where precipitation was done with anti-smads (lanes 5 and 6). Boxes in lanes 8 and 9 indicate that they were probed separately and added to this figure.

FIGURE 5. Changes in MAN1 expression affects adipogenic differentiation of human MSC.

Human MSC were induced to differentiate in adipogenic medium under MAN1 knockdown (KD) or overexpression (OE). (A) Final adipogenic cell phenotype was evaluated by staining of fat with ORO at day 14 of differentiation. Fat production (ORO) and adipocyte number were reduced in both KD and OE conditions, with MAN1 OE showing a significantly higher inhibitory effect on adipogenesis than MAN1 KD. $**p < 0.001$.

(B) Gene expression of adipogenic markers showed significant increase in peroxisome proliferator-activated receptor gamma (PPAR γ) and aP2 induced by MAN1 OE. On the other hand, when MAN1 was down regulated, PPAR γ , CEBP α and adiponectin were up regulated. $**p < 0.001$.

(C) PPAR γ activity assessed by a DNA-binding assay was significantly reduced by OE conditions. $*p < 0.05$.

FIGURE 6. Role of MAN1 in mesenchymal stem cell (MSC) differentiation. Osteoblastogenesis (**A and B**) is initiated by either bone morphogenic protein (BMP)/transforming growth factor beta (TGF- β)- or Wnt/ β Catenin/Lamin A-activated pathways. BMP/TGF- β interaction activates the formation of active smads complexes. Low levels of MAN1 (**A**) allow the entry of smads complexes to the nucleus thus facilitating osteoblast differentiation. In presence of high levels of MAN1 expression (**B**), smads are not phosphorylated thus they remain at the cytoplasm. In addition, other osteogenic pathways such as Wnt/ β Catenin/Lamin A are unaffected thus assume the regulation of osteoblast differentiation via nuclear translocation of β Catenin. On the other hand, both low and high levels of MAN1 expression affect adipogenesis (**C and D**). In either case, Peroxisome-proliferator-activated receptor gamma (PPAR γ) expression is increased, however its capacity to induce adipogenic genes is significantly affected in the presence of high levels of MAN1.

ECM: extracellular matrix, ONM: outer nuclear membrane, INM: inner nuclear membrane, LRP: Lipoprotein receptor-related protein, FZD: Frizzled, GSK3: glycogen synthase kinase 3, APC: adenomatous polyposis coli, TCF/LEF: T-cell factor/lymphoid enhancer-binding factor, GC: Glucocorticoids, IGF1: Insulin-like growth factor 1, PI3K: Phosphatidylinositol 3 phosphate, PKB: Protein kinase B, CREB: cAMP-response-element-binding protein, SREBP1: sterol-regulatory-element-binding proteins, RXR: retinoid X receptor.

Table 1. Primer Sequences

	Primer sequences 5' - 3' (forward/reverse)	Accession Number
Adiponectin	GGCCGTGATGGCAGAGAT TTTCACCGATGTCTCCCTTAGG	NM_004797
aP2	AAAGAAGTAGGAGTGGGCTTTGC CCCCATTACACTGATGATCAT	NM_001442
C/EBP α	AAGAAGTCGGTGGACAAGAACAG	NM_004364

	TGCGCACCGCGATGT	
GAPDH	GAAATCCCATCACCATCTTCC AAATGAGCCCCAGCCTTCTC	NM_002046
LMNA	LMNAGGATGAGGATGGAGATGACC CACGGGGGAGGCTGGGGAGAG	NM_170707
LPL	TCCGCGTGATTGCAGAGA CGCTCGTGGGAGCACTTC	NM_000237
OPG	AGAGAAAGCGATGGTGGATG CGGTGGCATTAAATAGTGAGATG	NM_094332
OPN	ACTCTGGTCATCCAGCTGACTCGT CTCCTAGGCATCACCTGTGCCATA	NM_001040058
PPAR γ 2	TCCATGCTGTTATGGGTGAA TCAAAGGAGTGGGAGTGGTC	NM_015869
RUNX2	TTTGCACTGGGTCATGTGTT TGGCTGCATTGAAAAGACTG	NM_001024630
WNT10B	TGACTTCTGTGAGCGAGACC CACCCACTCTGTAACCTTGGC	NM_003394

Figure 1

Figure 2

Figure 3

Figure 4

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Figure 5

Figure 6

Table 1. Primer Sequences

	Primer sequences 5' - 3' (forward/reverse)	Accession Number
Adiponectin	GGCCGTGATGGCAGAGAT TTTCACCGATGTCTCCCTTAGG	NM_004797
aP2	AAAGAAGTAGGAGTGGGCTTTGC CCCCATTCACACTGATGATCAT	NM_001442
C/EBP α	AAGAAGTCGGTGGACAAGAACAG TGCGCACCGCGATGT	NM_004364
GAPDH	GAAATCCCATCACCATCTTCC AAATGAGCCCCAGCCTTCTC	NM_002046
LMNA	LMNAGGATGAGGATGGAGATGACC CACGGGGGAGGCTGGGGAGAG	NM_170707
LPL	TCCGCGTGATTGCAGAGA CGCTCGTGGGAGCACTTC	NM_000237
OPG	AGAGAAAGCGATGGTGGATG CGGTGGCATTAAATAGTGAGATG	NM_094332
OPN	ACTCTGGTCATCCAGCTGACTCGT CTCCTAGGCATCACCTGTGCCATA	NM_001040058
PPAR γ 2	TCCATGCTGTTATGGGTGAA TCAAAGGAGTGGGAGTGGTC	NM_015869
RUNX2	TTTGCACTGGGTCATGTGTT TGGCTGCATTGAAAAGACTG	NM_001024630
WNT10B	TGACTTCTGTGAGCGAGACC CACCCACTCTGTAACCTTGGC	NM_003394

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