



Osteoblasts from a mandibuloacral dysplasia patient induce human blood precursors to differentiate into active osteoclasts[☆]

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ABSTRACT

Mandibuloacral dysplasia type A (MADA) is a rare disease caused by mutations in the *LMNA* gene encoding A type lamins. Patients affected by mandibuloacral dysplasia type A suffer from partial lipodystrophy, skin abnormalities and accelerated aging. Typical of mandibuloacral dysplasia type A is also bone resorption at defined districts including terminal phalanges, mandible and clavicles. Little is known about the biological mechanism underlying osteolysis in mandibuloacral dysplasia type A. In the reported study, we analyzed an osteoblast primary culture derived from the cervical vertebrae of a mandibuloacral dysplasia type A patient bearing the homozygous R527H *LMNA* mutation. Mandibuloacral dysplasia type A osteoblasts showed nuclear abnormalities typical of laminopathic cells, but they proliferated in culture and underwent differentiation upon stimulation with dexamethasone and beta-glycerophosphate. Differentiated osteoblasts showed proper production of bone mineral matrix until passage 8 in culture, suggesting a good differentiation activity. In order to evaluate whether mandibuloacral dysplasia type A osteoblast-derived factors affected osteoclast differentiation or activity, we used a conditioned medium from mandibuloacral dysplasia type A or control cultures to treat normal human peripheral blood monocytes and investigated whether they were induced to differentiate into osteoclasts. A higher osteoclast differentiation and matrix digestion rate was obtained in the presence of mandibuloacral dysplasia type A osteoblast medium with respect to normal osteoblast medium. Further, TGFbeta 2 and osteoprotegerin expression were enhanced in mandibuloacral dysplasia type A osteoblasts while the RANKL/osteoprotegerin ratio was diminished. Importantly, inhibition of TGFbeta 2 by a neutralizing antibody abolished the effect of mandibuloacral dysplasia type A conditioned medium on osteoclast differentiation. These data argue in favor of an altered bone turnover in mandibuloacral dysplasia type A, caused by upregulation of bone-derived stimulatory cytokines, which activate non-canonical differentiation stimuli. In this context, TGFbeta 2 appears as a major player in the osteolytic process that affects mandibuloacral dysplasia type A patients.

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1. Introduction

The osteoblast, a bone-specific mesenchymal cell type, is responsible for the synthesis and secretion of most proteins of the organic bone extracellular matrix and also expresses genes that are necessary and sufficient to induce bone mineralization [1]. Osteoblasts have a

third function: they are required for osteoclast differentiation, and thereby for bone resorption. Two main genes required for osteoclast differentiation are expressed in osteoblasts, M-CSF and RANKL, which act as positive regulators of osteoclast differentiation. Moreover, osteoblasts express osteoprotegerin, a soluble TNFα receptor that acts as a decoy receptor for RANKL and negatively regulates osteoclast differentiation [2]. Thus, soluble factors produced by osteoblasts may be used to obtain differentiation of blood precursors into active osteoclasts [3].

Lamin A/C is a nuclear lamina constituent implicated in a number of inherited disorders affecting muscle, adipose tissue, and nerves or

[☆] In memory of Rossano.

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causing syndromes with bone defects and/or premature aging [4–6]. Among syndromic laminopathies, MADA shows a clinical phenotype characterized by partial lipodystrophy, slightly accelerated aging and osteolysis [7]. The biological mechanism implicated in the osteolytic process is not well understood. However, four recent papers shed light on that aspect of MADA. The paper by Rauner et al. [8], performed in human precursors where lamin A/C expression was knocked-down, showed that lamin A/C is required for proper osteoblast differentiation while absence of the nuclear lamina constituent activates RANKL-dependent osteoclastogenesis. The paper by Akter et al. [9], performed in human bone marrow precursors subjected to lamin A/C siRNA-mediated inhibition, presented evidence of increased differentiation of bone progenitor cells towards the adipogenic lineage. The latter result was also obtained in bone marrow progenitors from *Zmpste24* null mice, an animal model showing accumulation of prelamin A due to knockout of its specific endoprotease [10]. A paper published by our groups [11] showed that accumulation of prelamin A favors osteoclast differentiation from human peripheral blood monocytes. All these data argue in favor of an altered bone turnover associated with absence of mature lamin A and/or accumulation of the unprocessed lamin A precursor. The study we report in the present paper is the first one performed in human osteoblasts from a laminopathic patient affected by osteolysis. We show proper differentiation, but increased expression of TGFβ and osteoprotegerin in MADA osteoblasts. We further show that MADA osteoblast conditioned medium is able to activate osteoclast precursors at higher rate relative to medium derived from control osteoblast cultures. How osteoprotegerin and TGFβ could mediate the increased stimulation of osteoclast progenitors is discussed in the paper. The reported data suggest a key role for TGFβ in triggering the osteoclastogenic activity of MADA osteoblasts.

2. Materials and methods

2.1. Cell cultures and differentiation

Primary cultures of human osteoblasts (CTRL HOB) were obtained from normal bone removed during orthopedic surgery of three different patients, as previously described [12], and obtained according to all the local ethical committee issues. Informed consent had been obtained from patients. Isolated cells were maintained, in Dulbecco's Modified Eagle Medium supplemented with 10% FBS, 100 μmol/L ascorbic acid-2 phosphate and 10^{-8} M dexamethasone. [13].

Osteoclasts were obtained from peripheral blood mononuclear cells (PBMC) as described previously [11]. Fresh buffy coats (AVIS, Bologna, Italy) were diluted with PBS, layered over Hystopaque (Sigma, St. Louis, MO), and centrifuged at 900g for 30 min. The mononuclear cells were extracted from the interphase of the PBS and Hystopaque and centrifuged at 400g for 5 min. Cells were rinsed in PBS and seeded on tissue-culture glass or plastic ware in D-MEM supplemented with 10% FCS and incubated at 37 °C in a humidified 5% CO₂ atmosphere. Cells were seeded at the density of 3,000,000/cm². After 1 h, medium was discarded and replaced with conditioned medium from confluent cultures of CTRL HOB or MADA osteoblasts (MADA HOB) (25% conditioned medium and 75% normal medium). Conditioned medium was replaced every 4 days for 14 days. In order to verify the differentiation of mononuclear cells to osteoclasts, after 8 days of culture, cells were analyzed for tartrate resistant acid phosphatase (TRACP) activity by cytochemistry (Acid Phosphatase Leukocyte assay, Sigma), and stained with Hoechst 33258 (1.25 g/mL). TRACP-positive cells containing 3 or more nuclei were considered to be differentiated osteoclasts. If not differently specified, the average value for controls is the average of results of three different CTRL HOB cultures.

2.2. Gene expression analysis

RNA isolation and reverse transcription were performed as previously described [11]. Total RNA was isolated using Rneasy Mini Kit (Qiagen GmbH, Hilden, Germany) from semi-confluent osteoblast cultures following manufacturer's instructions. RNA was reverse transcribed into cDNA using the Advantage RT for PCR Kit (Clontech Laboratories, Palo Alto, CA). OPG, RANKL or TGFβ expression was evaluated by real time PCR, performed using a Light Cycler instrumentation (Roche Diagnostics), by amplifying 1 μg of cDNA and the Universal Probe Library system (Roche Applied Science). Probes and primers were selected using a web-based assay design software (ProbeFinder <https://www.roche-applied-science.com>). Forward and reverse primers are listed in Table 1. The amplification protocol was: 95 °C for 10 min, 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 1 s for 45 cycles, 40 °C for 30 s. The results are expressed as ratio between gene of interest and GAPDH reference gene. The experiments were performed in duplicate.

2.3. Resorption assay

To evaluate the resorption activity in osteoclast cultures we used the Osteolyse assay (Cambrex Bio Science Walkersville, Inc., Charles City, IA). Cells were cultured in conditioned medium from MADA osteoblast cultures or controls for 10 days. Supernatants were collected and the fraction of collagen Type I released in the medium as a consequence of the osteoclast-mediated resorption activity was quantified as follows. The Osteolyse assay provides a quantitative assessment of in vitro osteoclast-mediated degradation of human bone Type I collagen by directly measuring the release of Europium-labeled collagen fragments via time resolved fluorescence. At the end of incubation time (10 days), the collected cell culture supernatants were centrifuged, and 10 of the 200 μL of the supernatant were added to 200 μL of Fluorophore Releasing Reagent. The fluorescence of each well of the assay plate was determined in a time-resolved fluorescence fluorimeter (Wallac Victor, exc. 340 nm, ems. 615 nm). For the evaluation of the effect of the treatment with an anti-TGF-β-2 antibody, cells were incubated for ten days with the supernatants obtained from the CTRL HOB and MADA HOB cultures, as described above, added or not added with 0.03 μg/ml of a polyclonal IgG anti-TGF-β2 antibody (Abcam), or with 0.03 μg/ml of an IgG control antibody (polyclonal anti-emerin antibody, Santa Cruz Biotechnology).

2.4. ELISA

A multiple sandwich enzyme immunoassay was performed for the quantitative determination of OPG and sRANKL released from CTRL HOB or MADA osteoblasts, as previously described [14]. For OPG detection, the following antibodies were used: mouse anti-human OPG (2 mg/mL), goat biotinylated anti-human OPG (100 ng/mL) and recombinant human OPG as standard reference in a range of

Table 1
List of primers used in this study.

OPG (NM_002546.2)	Sense	GAAGGGCGCTACTCTTGAGAT
	Antisense	GCAAACTGTATTTTCGCTCTGG
RANKL (NM_003701.2, NM_033012.2)	Sense	TGATTCATGTAGGAGAATTAACAGG
	Antisense	GATGTGCTGTGATCCAACGA
TGFβ2 (NM_003238.2)	Sense	CCAAAGGGTACAATGCCAAC
	Antisense	CAGATGCTTCTGGATTATGTTAT
GAPDH (NM_002046.3)	Sense	AGCCACATCGCTCAGACAC
	Antisense	GCCCAATACGACCAAAATCC
COL1A1 (NM_000088.3)	Sense	CCCCTGAAAGAATGGAGAT
	Antisense	AATCCTCGAGCACCTCTGA
BGLAP (NM_199173.2)	Sense	GGCGCTACCTGTATCAATGG
	Antisense	TCAGCCAACCTCGTACAGTC
SPARC (NM_003118.2)	Sense	ACCCGCTTTTCGAGACC
	Antisense	CAAGATCCTTGTGATATCCTTCT

concentration between 0 and 2,000 pg/mL (R & D, Minneapolis, MN). For sRANKL detection, the following antibodies were used: goat anti-human RANKL (2 µg/ml) and biotinylated goat anti-human RANKL (0.2 µg/ml). The standard reference curve was set using recombinant human sRANKL (Preprotech) with a range of 0.01–50 ng/ml.

2.5. Alkaline phosphatase measurement

Alkaline phosphatase (ALP) was used as a marker of osteoblast differentiation. ALP content was evaluated as previously described [15]. Cytochemical alkaline phosphatase (ALP) analysis was performed directly on confluent cell culture plates using a commercial kit (ALP leucocyte, Sigma, kit no 86-R). Cells were observed with a light microscope and the presence of ALP was revealed by red precipitates.

ALP activity was measured in cell lysates, with a biochemical method based on the conversion of p-Nitrophenyl Phosphate Liquid Substrate (Sigma-Aldrich). Semi-confluent cells were lysed with 0.1% Triton-X 160 100 (Sigma) in PBS for 10 min at 37 °C. After 15 min at 37 °C, the product of p-nitrophenyl phosphate reaction (Sigma) was measured at 405 nm, and cell-associated ALP was expressed as M p-

nitrophenol formed/cells. The experiments were performed in duplicate.

2.6. Electron microscopy

Control and MADA HOB were fixed with 2.5% glutaraldehyde – 0.1 M phosphate buffer pH 7.6 for 1 h at room temperature, postfixed with 1% OsO₄ in veronal buffer for 1 h, dehydrated in an ethanol series, scraped, and embedded in epoxy resin. Ultrathin sections counterstained with uranyl acetate-lead citrate were observed with a Philips EM 400T electron microscope, operated at 100 KV. For each sample, 100 nuclei were evaluated.

2.7. Western blot analysis

Whole cellular lysates from CTRL HOB or MADA HOB were lysed in RIPA buffer [16] and subjected to Western blot analysis. Proteins were separated by a 5%–20% gradient SDS-PAGE. Protein bands were blotted onto a nitrocellulose membrane and subjected to immunolabeling using anti-TGF-beta rabbit polyclonal antibody (1:200 dilution, Abcam), to detect intracellular TGFbeta levels or anti-actin antibody (Santa Cruz), as a loading control. Anti-prelamin A (Sc-6214), anti-

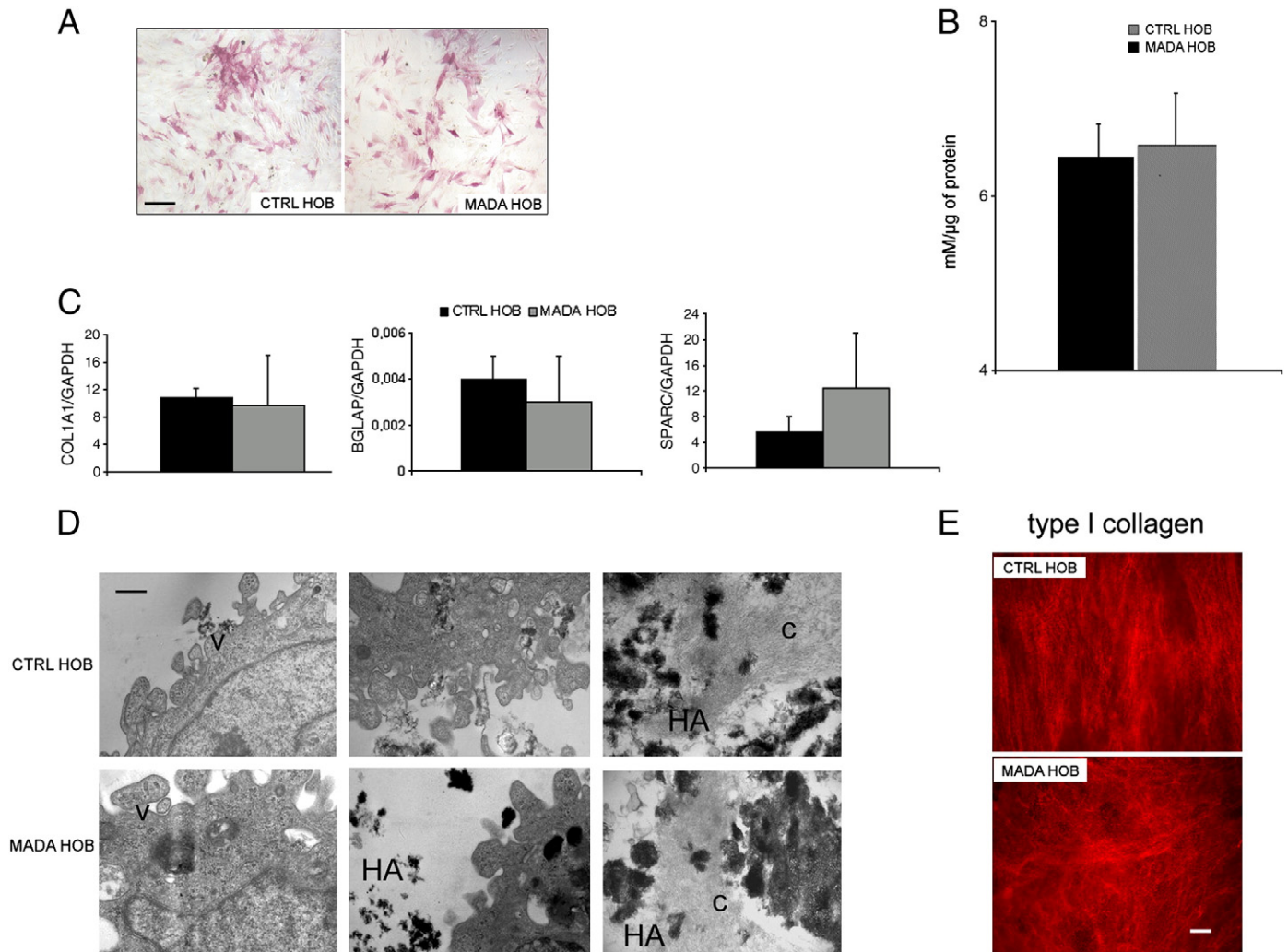


Fig. 1. Osteoblasts from MADA patient show normal differentiation. (A) Representative picture of the cytochemical ALP staining of control (CTRL HOB) and MADA (MADA HOB) osteoblast cultures. In (B) the histogram shows the mean \pm standard error of the ratios between millimoles of substrate converted by ALP in 1 min and the total amount of proteins. (C) COL1A1/GAPDH, BGLAP/GAPDH and SPARC/GAPDH mRNA levels, as determined by Real Time PCR in control and MADA osteoblasts (means \pm standard error). (D) The electron microscopy analysis of differentiated control and MADA osteoblasts shows secretion vesicles (v), hydroxyapatite crystals (HA) and collagen fibrils (c). (E) Collagen Type I immunofluorescence staining in differentiated control (CTRL HOB) and MADA osteoblasts (MADA HOB). Data from three different experiments were evaluated. Bars, 50 µm in (A), 1 µm in (D) and 10 µm in (E).

lamin A/C and anti-actin antibodies were purchased from Santa Cruz and used at 1:100 dilution. Primary antibodies were applied for 1 h at room temperature. Peroxidase-conjugated secondary antibodies (Sigma) were applied for 20 min at room temperature. Immunoblotted bands were revealed by the Amersham ECL® detection system. The intensity of bands was measured using a GS800 Densitometer (Biorad).

2.8. Immunofluorescence analysis

Cells were grown on glass coverslips and immunofluorescence staining was performed in proliferating CTRL HOB or MADA HOB fixed in 100% methanol. Anti-prelamin A Sc-6214 antibody was from Santa Cruz, anti-lamin A/C monoclonal antibody was from Novocastra, anti-collagen I antibody was from Sigma. Primary antibodies were applied overnight at 1:100 dilution, secondary fluorochrome-conjugated antibodies were applied for 1 h. DNA was counterstained using DAPI. Immunofluorescence microscopy was performed using a Nikon E600 epifluorescence microscope and a Nikon oil-immersion objective [100× magnification, 1.3 NA (numerical aperture)]. Photographs were taken using a Nikon digital camera (DXm) and NIS-Element BR2.20 software (Nikon). All images were taken at similar exposures within an experiment for each antibody. Image quantification was performed using NIS-Element BR2.20 software.

Images were processed using Adobe Photoshop 7.0 software (Adobe Systems).

2.9. Statistical analysis

Statistical analysis was performed with the StatView™ 5.0.1 software (SAS Institute, Inc., Cary, NC). Due to the low number of experiments, data were considered distributed not normally and the differences between groups were evaluated by a nonparametric test (Mann–Whitney U-test). The level for significance was taken as $P < 0.05$.

3. Results

The clinical phenotype of the affected patient, bearing an R527H homozygous *LMNA* mutation, was previously described [7,17]. The presence of osteoporotic vertebrae is not a common feature of all MADA patients, but it has been reported in a patient bearing a homozygous A529T *LMNA* mutation [18]. Osteoblasts from controls and MADA bone differentiated properly in osteoblast differentiation medium. Fig. 1A and B show comparable alkaline phosphatase staining in CTRL HOB and MADA HOB. Moreover, we did not detect a significant difference in the mRNA expression of the osteoblast markers related to bone matrix deposition, like Type I collagen (COL1A1), osteocalcin (BGLAP) and osteonectin (SPARC) that were

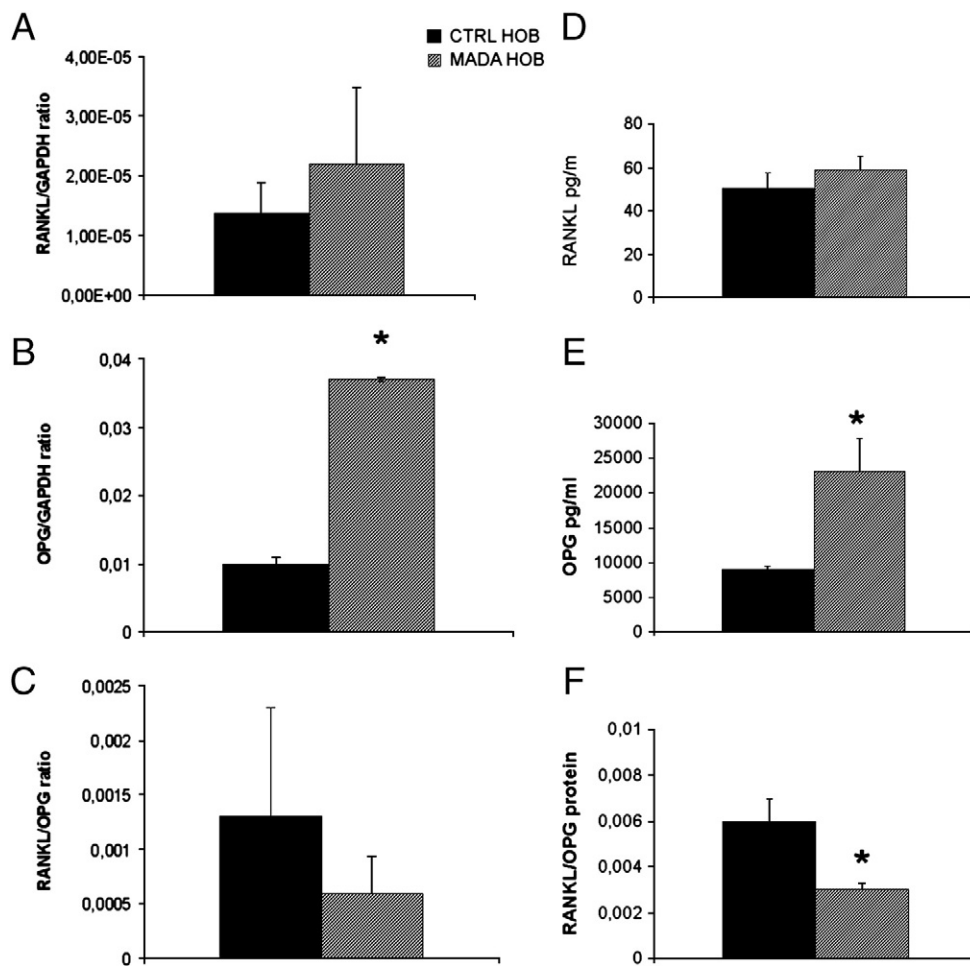


Fig. 2. Osteoprotegerin expression and release by MADA osteoblasts is significantly increased. (A) RANKL mRNA levels, as determined by real time PCR. (B) Osteoprotegerin mRNA levels, as determined by real time PCR. (C) RANKL/osteoprotegerin ratio (mRNA levels). (D) (RANKL protein levels, as determined by ELISA. Values are reported as means ± standard deviation of three different experiments. (E) Osteoprotegerin protein levels, as determined by ELISA. Values are reported as means ± standard error of three different experiments. * $p < 0.05$, statistically significant difference. (F) RANKL/osteoprotegerin ratio (secreted protein levels), statistically significant difference, $p = 0.02$. *, statistically significant difference $p < 0.05$; CTRL HOB, control osteoblasts; MADA HOB, MADA osteoblasts.

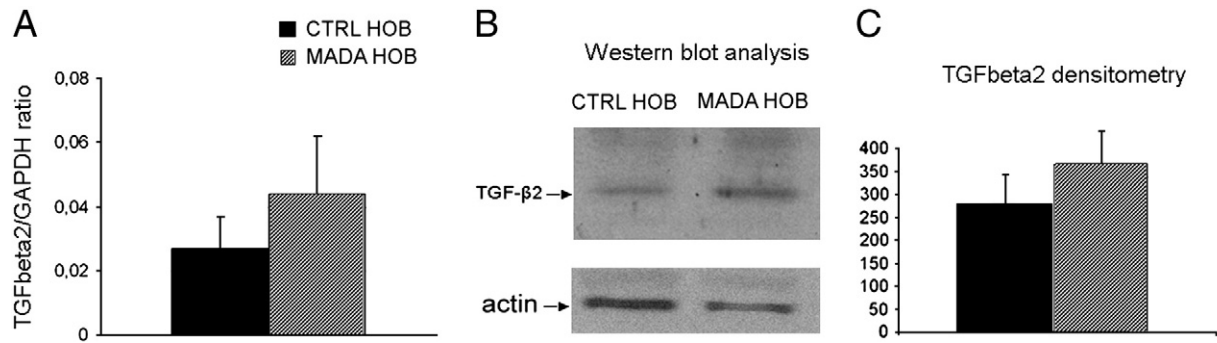


Fig. 3. TGFbeta 2 expression is significantly increased in MADA osteoblasts. (A) TGFbeta 2 mRNA levels in control and MADA osteoblasts. (B) Western blot analysis of TGFbeta protein in osteoblast cellular lysates. Beta actin staining shows equal loading of samples. (C) Densitometric analysis of TGFbeta band obtained in three different experiments. CTRL HOB, control osteoblast samples; MADA HOB, MADA osteoblast samples. Values are reported as means \pm standard error of three different experiments. * $p < 0.05$, statistically significant difference.

expressed at comparable levels in respect with control osteoblasts, as shown in Fig. 1C. Pictures reported in Fig. 1D show the ultrastructural morphology of differentiated control and MADA osteoblasts. MADA HOB showed altered nuclear morphology but proper chromatin organization. Moreover, hydroxyapatite crystals and collagen fibers were deposited as in control cell cultures (Fig. 1D). Normal collagen Type I deposition was supported by immunofluorescence analysis of the extracellular matrix protein (Fig. 1E, red staining). Collagen Type I

is a key constituent of bone extracellular matrix. Thus, we could not detect any defect in MADA HOB differentiation activity.

Next, we examined by real-time PCR analysis the expression levels of genes, whose products are known regulators of osteoclast differentiation. Expression of the receptor activator of NF-κB ligand (RANKL) in MADA HOB was increased with respect to control cultures, although the difference was not statistically significant (Fig. 2A). On the contrary, a significant increase of osteoprotegerin

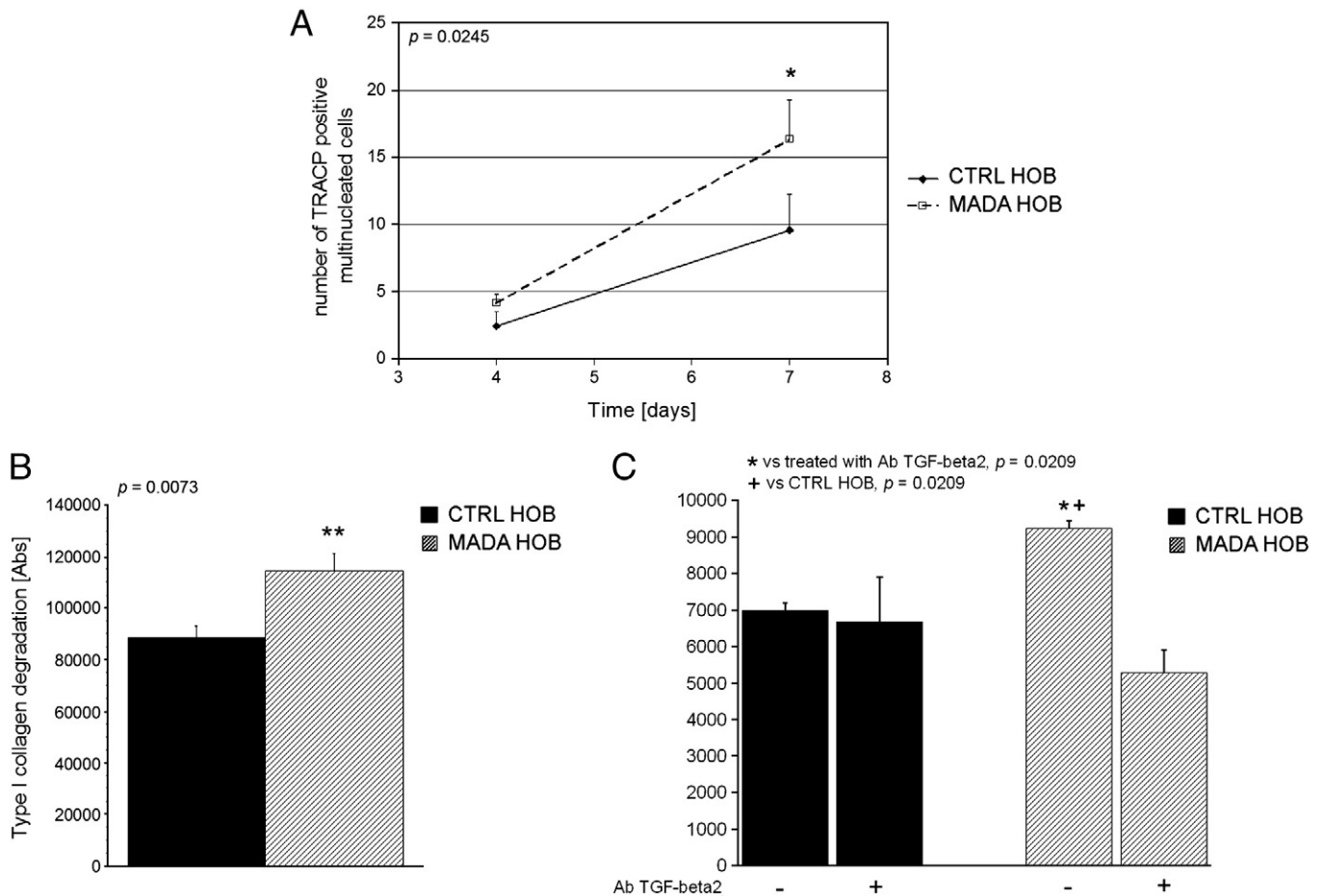


Fig. 4. Osteoclast differentiation and resorption activity is increased by MADA osteoblast conditioned medium. Control peripheral blood monocytes were induced to differentiate in the presence of conditioned medium from control (CTRL HOB) or MADA osteoblast (MADA HOB) cultures. (A) Number of TRACP positive multinucleated cells in cultures of PBMC treated with MADA conditioned medium. Mean \pm standard error, *, $P < 0.05$. (B) Collagen Type I degradation by osteoclasts obtained in the presence of MADA or control conditioned medium. Mean \pm standard error, **, $P < 0.01$. (C) Collagen Type I degradation by osteoclasts obtained in the presence of conditioned medium obtained from the MADA culture or from a CTRL HOB culture, added with an anti-TGF-beta 2 polyclonal antibody or not added. Mean \pm standard error, * or +, $p < 0.05$. The treatment with a control antibody (anti-emerin, rabbit polyclonal) did not produce any effect (7641,25 \pm 878,088 vs 6965,0 \pm 215,055 Abs, absorbance from the Type I collagen degradation assay of PBMC incubated with HOB CTRL supernatants added with or not added with a control antibody, respectively).

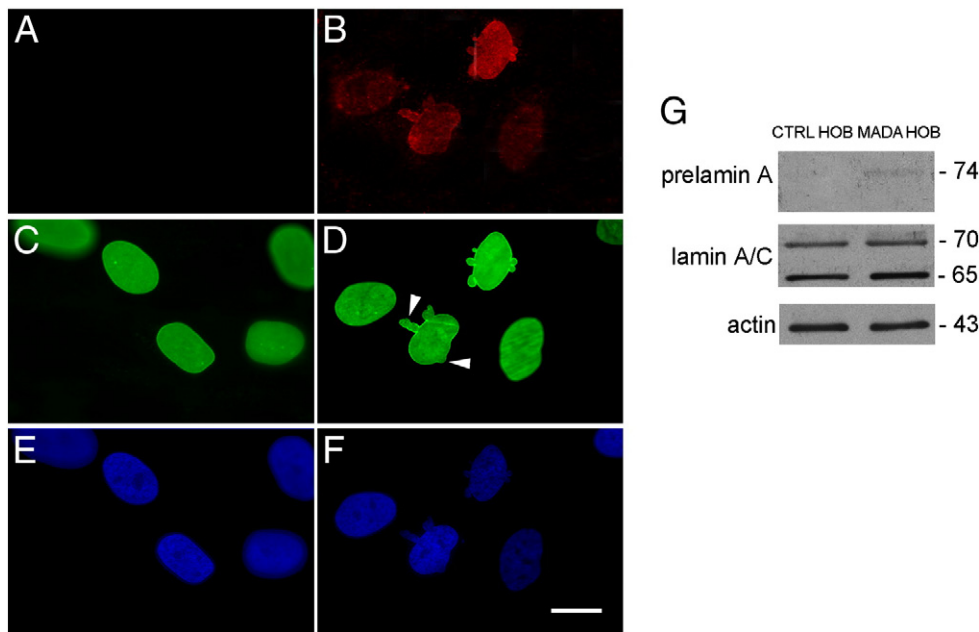


Fig. 5. Prelamin A and lamin A/C analysis in MADA osteoblasts. Proliferating CTRL HOB (A, C, E) or MADA HOB (B, D, F) were double-labeled using anti-prelamin A (A, B) and anti-lamin A/C (C, D) antibodies. TRITC-conjugated anti-goat IgG antibody (red) was used to reveal prelamin A, FITC-conjugated anti-mouse IgG was used to reveal lamin A/C. DAPI staining of nuclei (E, F) is shown. Bar, 10 μ m. (G) CTRL HOB and MADA HOB were subjected to Western blot analysis using anti-lamin A/C and anti-prelamin A Sc-6214 antibodies. Actin levels were evaluated to show equal loading of samples. Molecular weight markers are reported in kDa.

expression in MADA HOB was observed (Fig. 2B) and the RANKL/osteoprotegerin ratio of mRNA levels was diminished (Fig. 2C). Moreover, while RANKL secreted protein levels were comparable in controls and MADA HOB (Fig. 2D), protein levels of osteoprotegerin were consistently increased in MADA HOB, as determined by ELISA (Fig. 2E). Thus, the RANKL/osteoprotegerin ratio was diminished (Fig. 2F). Protein levels of osteoprotegerin were consistently increased in MADA HOB, as determined by ELISA (Fig. 2E). Moreover, the mRNA expression and the protein level of TGF β 2 were significantly increased (Fig. 3) in MADA HOB, further supporting an imbalance of bone metabolism-related signaling. The reduced RANKL/osteoprotegerin ratio found in MADA cells suggested reduced stimulation of osteoclast differentiation by the MADA osteoblast conditioned medium, since a high RANKL/osteoprotegerin ratio is required for osteoclast differentiation. Unexpectedly, the medium obtained from MADA HOB cultures triggered differentiation of normal peripheral blood-derived monocytes more efficiently than control osteoblast medium (Fig. 4A). Moreover, we found that the collagen matrix degradation activity of osteoclasts stimulated by MADA HOB conditioned medium was significantly increased with respect to CTRL HOB media-stimulated osteoclasts (Fig. 4B). Notably, when a neutralizing anti-TGF- β 2 antibody was added to the conditioned medium, the osteoclastogenic activity of MADA HOB supernatants was significantly impaired, whereas the activity of CTRL HOB supernatants was unaffected (Fig. 4C). Addition of a control antibody to conditioned media did not produce any effect.

In order to determine to which extent lamin A/C defects affected the nuclear phenotype of MADA osteoblasts, we labeled both prelamin A and lamin A/C by immunofluorescence staining. Fig. 5 shows the typical increase of prelamin A levels observed in a percentage of MADA cells. Here, 15% of MADA HOB showed prelamin A staining while the lamin A precursor could not be detected in CTRL HOB (Fig. 5). The honeycomb appearance of lamin A/C staining, which we recently linked to anomalous prelamin A intermolecular interactions [19], was observed in 60% of MADA HOB nuclei (Fig. 5B, arrowheads). Only 5% of CTRL HOB nuclei showed honeycomb structures (not shown). Prelamin A was detected by Western blot analysis in MADA HOB, but not in CTRL HOB,

while comparable levels of lamin A/C were observed in CTRL HOB and MADA HOB. Consistent with the low percentage of nuclei showing prelamin A staining, low prelamin A levels were indicated by the Western immunoblot.

4. Discussion

This study addresses the pathogenesis of bone defects found in laminopathies, using primary osteoblasts bearing the homozygous R527H *LMNA* mutation that causes MADA.

Our data show that MADA HOB derived from cervical vertebrae undergo normal differentiation and are able to produce calcified matrix in vitro. However, cytokine production in MADA HOB seems to be altered, as we report striking increase of osteoprotegerin and moderate increase of TGF β 2 expression. Importantly, despite the increase in osteoprotegerin levels, MADA HOB trigger osteoclast differentiation and resorption activity to a higher rate with respect to CTRL HOB, suggesting the activation of non-canonical differentiation mechanisms possibly mediated by TGF β -dependent signaling [20].

Previous reports showed that bone mineral density and osteoblast number are reduced in mouse models of laminopathies, including the *Zmpste24*^{−/−} mice, which show a progeroid phenotype associated with bone loss [10,21], and the progeria mouse model [22]. Based on these data, it appears likely that prelamin A accumulation, which occurs in the above mentioned experimental models and in MADA cells [23], causes an imbalance in osteoclast and/or osteoblasts leading to defective bone turnover and ultimately causing osteolysis. It is noteworthy that osteoclasts accumulating prelamin A, either as a result of protein mutation or under drug treatment, show normal or enhanced differentiation rate [11]. In fact, it has been shown that osteoclasts subjected to drugs inducing prelamin A accumulation proliferate at normal rate and differentiate more efficiently than the wild-type cells [11]. Here, we show normal differentiation rate of cultured MADA osteoblasts. These data have been obtained considering each cellular model independently of the other. However, the altered bone turnover observed in laminopathies featuring

prelamin A accumulation [24] is a result of a reciprocal interference between cells implicated in bone homeostasis [25]. In fact, we show that cytokine production is altered in MADA osteoblasts, whose secreted factors favor differentiation and matrix resorption activity of osteoclasts.

On the other hand, the low level of prelamin A detected in MADA osteoblasts suggests that not only accumulation of the toxic lamin A precursor, but also the homozygous *LMNA* mutation itself may interfere with fundamental interactions of lamin A, thus causing altered expression of osteoblast-derived factors. The finding that osteoprotegerin was strikingly increased in MADA osteoblast cultures would have suggested reduced osteoclast activation by osteoblast conditioned medium [2]. Contrary to what was expected, osteoclasts differentiating in that medium showed a significantly increased bone resorption activity. These data indicate that a molecular pathway not involving the canonical RANKL/osteoprotegerin balance [26] might be implicated in osteoclast activation in MADA. Along this line, the finding that TGFβ2 levels are increased in MADA osteoblasts, while inhibition of TGFβ2 by a neutralizing antibody reduces the osteoclastogenic potential of MADA-HOB conditioned medium, might implicate this cytokine in osteoclast activation. Consistent with this hypothesis, it has been demonstrated that, in the absence of RANKL, TGFβ2 is able to induce osteoclast differentiation and activity in a RANKL-independent way, which is not regulated by osteoprotegerin levels [20]. We hypothesize that increased differentiation and resorption activity triggered by MADA conditioned medium might reflect a TGFβ2-mediated activation, which should overcome osteoprotegerin inhibition. Interestingly, a similar increase in TGFβ2 and osteoprotegerin levels has been found in serum from aged women and it has been shown to increase with age [27].

One could speculate that the cytokine changes observed in MADA osteoblasts could be associated with the premature aging phenotype [28] or could independently cause clinical defects, such as osteoporosis, typical of both progeroid laminopathies and aging [6,25,29]. Moreover, a clear link between another nuclear envelope protein, MAN1, and TGFβ2 expression and activity has been established and implicated in bone disease [30], consistent with the increasing evidence that proteins at the nuclear periphery regulate TGFβ2 and Smad dependent pathways [5,6,31].

It is noteworthy that TGFβ2 has been implicated in the activation of transcription factors that regulate nuclear positioning in osteoclasts [32], a role also emerging for lamin-dependent pathways. The following implications of the observed phenomena on MADA pathogenesis and treatment can be envisaged. First, the relationship between lamin A/mutated lamin A or prelamin A and TGFβ2 production deserves investigation, as well as the mechanism underlying overexpression of osteoprotegerin. Intriguingly, osteoprotegerin increase has been reported in several disorders, also found in MADA, such as altered adipose tissue metabolism and type 2 diabetes [33,34]. Secondly, the possible presence of anomalous levels of TGFβ2 in patients' serum should be evaluated. Finally, provided that elevated levels of TGFβ2 are detected in serum, drugs capable of reducing TGFβ2 production/activity could prove useful to rescue the bone phenotype in MADA patients.

Acknowledgments

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References

- [1] M.Q. Hassan, J.A. Gordon, M.M. Beloti, C.M. Croce, A.J. Wijnen, J.L. Stein, G.S. Stein, J.B. Lian, A network connecting Runx2, SATB2, and the miR-23a-27a-24-2 cluster regulates the osteoblast differentiation program, *Proc. Natl. Acad. Sci. U.S.A.* 107 (2010) 19879–19884.
- [2] H.L. Wright, H.S. McCarthy, J. Middleton, M.J. Marshall, RANK, RANKL and osteoprotegerin in bone biology and disease, *Curr. Rev. Musculoskelet. Med.* 2 (2009) 56–64.
- [3] J. Costa-Rodrigues, C.A. Teixeira, P. Sampaio, M.H. Fernandes, Characterisation of the osteoclastogenic potential of human osteoblastic and fibroblastic conditioned media, *J. Cell. Biochem.* 109 (2010) 205–216.
- [4] R. Akter, D. Rivas, G. Geneau, H. Drissi, G. Duque, Effect of Lamin A/C Knockdown on Osteoblast Differentiation and Function, *J. Bone Miner. Res.* (2008).
- [5] N.M. Maraldi, G. Lattanzi, V. Cenni, A. Bavelloni, S. Marmioli, F.A. Manzoli, Laminopathies and A-type lamin-associated signalling pathways, *Adv. Enzyme Regul.* 50 (2010) 248–261.
- [6] S. Marmioli, J. Bertacchini, F. Beretti, V. Cenni, M. Guida, A. De Pol, N.M. Maraldi, G. Lattanzi, A-type lamins and signaling: the PI 3-kinase/Akt pathway moves forward, *J. Cell. Physiol.* 220 (2009) 553–561.
- [7] G. Novelli, A. Muchir, F. Sangiuolo, A. Helbling-Leclerc, M.R. D'Apice, C. Massart, F. Capon, P. Sbraccia, M. Federici, R. Lauro, C. Tudisco, R. Pallotta, G. Scarano, B. Dallapiccola, L. Merlini, G. Bonne, Mandibuloacral dysplasia is caused by a mutation in LMNA-encoding lamin A/C, *Am. J. Hum. Genet.* 71 (2002) 426–431.
- [8] M. Rauner, W. Sipos, C. Goettsch, A. Wutzl, R. Foisner, P. Pietschmann, L.C. Hofbauer, Inhibition of lamin A/C attenuates osteoblast differentiation and enhances RANKL-dependent osteoclastogenesis, *J. Bone Miner. Res.* 24 (2009) 78–86.
- [9] R. Akter, D. Rivas, G. Geneau, H. Drissi, G. Duque, Effect of lamin A/C knockdown on osteoblast differentiation and function, *J. Bone Miner. Res.* 24 (2009) 283–293.
- [10] D. Rivas, W. Li, R. Akter, J.E. Henderson, G. Duque, Accelerated features of age-related bone loss in *zmpste24* metalloproteinase-deficient mice, *J. Gerontol. A, Biol. Sci. Med. Sci.* 64 (2009) 1015–1024.
- [11] N. Zini, S. Avnet, S. Ghisu, N.M. Maraldi, S. Squarzone, N. Baldini, G. Lattanzi, Effects of prelamin A processing inhibitors on the differentiation and activity of human osteoclasts, *J. Cell. Biochem.* 105 (2008) 34–40.
- [12] I. Amato, G. Ciapetti, S. Pagani, G. Marletta, C. Satriano, N. Baldini, D. Granchi, Expression of cell adhesion receptors in human osteoblasts cultured on biofunctionalized poly-(epsilon-caprolactone) surfaces, *Biomaterials* 28 (2007) 3668–3678.
- [13] D. Granchi, S.R. Baglio, I. Amato, A. Giunti, N. Baldini, Paracrine inhibition of osteoblast differentiation induced by neuroblastoma cells, *Int. J. Cancer* 123 (2008) 1526–1535.
- [14] D. Granchi, I. Amato, L. Battistelli, S. Avnet, S. Capaccioli, L. Papucci, M. Donnini, A. Pellacani, M.L. Brandi, A. Giunti, N. Baldini, In vitro blockade of receptor activator of nuclear factor-kappaB ligand prevents osteoclastogenesis induced by neuroblastoma cells, *Int. J. Cancer* 111 (2004) 829–838.
- [15] E. Cenni, G. Ciapetti, D. Granchi, C. Fotia, F. Perut, A. Giunti, N. Baldini, Endothelial cells incubated with platelet-rich plasma express PDGF-B and ICAM-1 and induce bone marrow stromal cell migration, *J. Orthop. Res.* 27 (2009) 1493–1498.
- [16] E. Mattioli, M. Columbaro, C. Capanni, S. Santi, N.M. Maraldi, M.R. D'Apice, G. Novelli, M. Riccio, S. Squarzone, R. Foisner, G. Lattanzi, Drugs affecting prelamin A processing: Effects on heterochromatin organization, *Exp. Cell Res.* 314 (2008) 453–462.
- [17] R. Pallotta, G. Morgese, Mandibuloacral dysplasia: a rare progeroid syndrome. Two brothers confirm autosomal recessive inheritance, *Clin. Genet.* 26 (1984) 133–138.
- [18] T. Koshi, J. Takahashi, T. Momose, A. Nakamura, A. Sakurai, T. Wada, K. Yoshida, K. Wakui, T. Suzuki, K. Kasuga, G. Nishimura, H. Kato, Y. Fukushima, Mandibuloacral dysplasia and a novel LMNA mutation in a woman with severe progressive skeletal changes, *Am. J. Med. Genet. A* 143A (2007) 2598–2603.
- [19] C. Capanni, R. Del Coco, E. Mattioli, D. Camozzi, M. Columbaro, E. Schena, L. Merlini, S. Squarzone, N.M. Maraldi, G. Lattanzi, Emerin-prelamin A interplay in human fibroblasts, *Biol. Cell* 101 (2009) 541–554.
- [20] I. Itonaga, A. Sabokbar, S.G. Sun, O. Kudo, L. Danks, D. Ferguson, Y. Fujikawa, N.A. Athanasou, Transforming growth factor-beta induces osteoclast formation in the absence of RANKL, *Bone* 34 (2004) 57–64.
- [21] I. Varela, S. Pereira, A.P. Ugalde, C.L. Navarro, M.F. Suarez, P. Cau, J. Cadinanos, F.G. Osorio, N. Foray, J. Cobo, F. de Carlos, N. Levy, J.M. Freije, C. Lopez-Otin, Combined treatment with statins and aminobisphosphonates extends longevity in a mouse model of human premature aging, *Nat. Med.* 14 (2008) 767–772.
- [22] S.H. Yang, D.A. Andres, H.P. Spielmann, S.G. Young, L.G. Fong, Progerin elicits disease phenotypes of progeria in mice whether or not it is farnesylated, *J. Clin. Invest.* 118 (2008) 3291–3300.
- [23] C. Capanni, E. Mattioli, M. Columbaro, E. Lucarelli, V.K. Parnaik, G. Novelli, M. Wehnert, V. Cenni, N.M. Maraldi, S. Squarzone, G. Lattanzi, Altered pre-lamin A processing is a common mechanism leading to lipodystrophy, *Hum. Mol. Genet.* 14 (2005) 1489–1502.
- [24] V.J. Cunningham, M.R. D'Apice, N. Licata, G. Novelli, T. Cundy, Skeletal phenotype of mandibuloacral dysplasia associated with mutations in ZMPSTE24, *Bone* 47 (2010) 591–597.
- [25] G. Duque, D. Rivas, Age-related changes in lamin A/C expression in the osteoarticular system: laminopathies as a potential new aging mechanism, *Mech. Ageing Dev.* 127 (2006) 378–383.
- [26] F. Hemingway, R. Taylor, H.J. Knowles, N.A. Athanasou, RANKL-independent human osteoclast formation with APRIL, BAFF, NGF, IGF I and IGF II, *Bone* (2011).

- [27] X.Y. Wu, X.P. Wu, X.H. Luo, H. Xie, H. Zhang, Y.Q. Peng, L.Q. Yuan, Y.B. Jiang, E.Y. Liao, The relationship between the levels of gonadotropic hormones and OPG, Leptin, TGF-beta1 and TGF-beta2 in Chinese adult women, *Clin. Chim. Acta* (2010).
- [28] A. Garg, L. Subramanyam, A.K. Agarwal, V. Simha, B. Levine, M.R. D'Apice, G. Novelli, Y. Crow, Atypical progeroid syndrome due to heterozygous missense LMNA mutations, *J. Clin. Endocrinol. Metab.* 94 (2009) 4971–4983.
- [29] M. Prokocimer, M. Davidovich, M. Nissim-Rafinia, N. Wiesel-Motiuk, D. Bar, R. Barkan, E. Meshorer, Y. Gruenbaum, Nuclear lamins: key regulators of nuclear structure and activities, *J. Cell. Mol. Med.* (2009).
- [30] E. Konde, B. Bourgeois, C. Tellier-Lebegue, W. Wu, J. Perez, S. Caputo, W. Attanda, S. Gasparini, J.B. Charbonnier, B. Gilquin, H.J. Worman, S. Zinn-Justin, Structural Analysis of the Smad2-MAN1 Interaction That Regulates Transforming Growth Factor-beta Signaling at the Inner Nuclear Membrane, *Biochemistry* 49 (2010) 8020–8032.
- [31] A. Muchir, J. Shan, G. Bonne, S.E. Lehnart, H.J. Worman, Inhibition of extracellular signal-regulated kinase signaling to prevent cardiomyopathy caused by mutation in the gene encoding A-type lamins, *Hum. Mol. Genet.* 18 (2009) 241–247.
- [32] S.W. Fox, K.E. Evans, A.C. Lovibond, Transforming growth factor-beta enables NFATc1 expression during osteoclastogenesis, *Biochem. Biophys. Res. Commun.* 366 (2008) 123–128.
- [33] D.T. Ashley, E.P. O'Sullivan, C. Davenport, N. Devlin, R.K. Crowley, N. McCaffrey, N.M. Moyna, D. Smith, D.J. O'Gorman, Similar to adiponectin, serum levels of osteoprotegerin are associated with obesity in healthy subjects, *Metabolism* (2010).
- [34] E.P. O'Sullivan, D.T. Ashley, C. Davenport, N. Devlin, R. Crowley, A. Agha, C.J. Thompson, D. O'Gorman, D. Smith, Osteoprotegerin and biomarkers of vascular inflammation in type 2 diabetes, *Diabetes Metab. Res. Rev.* 26 (2011) 496–502.