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Effects of Prelamin A Processing Inhibitors on the Differentiation and Activity of Human Osteoclasts

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ABSTRACT

Osteoclast differentiation is a complex process involving cytoskeleton and nuclear reorganization. Osteoclasts regulate bone homeostasis and have a key role in bone degenerative processes. Osteolysis and osteoporosis characterize a subset of laminopathies, inherited disorders due to defects in lamin A/C. Laminopathies featuring bone resorption are characterized, at the molecular level, by anomalous accumulation of the unprocessed lamin A precursor, called prelamin A. To obtain a suitable cell model to study prelamin A effects on osteoclasts, prelamin A processing inhibitors FTI-277 or AFCMe were applied to peripheral blood monocytes induced to differentiate towards the osteoclastic lineage. Previous studies have shown that treatment with FTI-277 causes accumulation of non-farnesylated prelamin A, while AFCMe inhibition of prelamin A maturation causes accumulation of a farnesylated form. We demonstrate that monocytes subjected to FTI-277 treatment and mostly those subjected to AFCMe administration, differentiate towards the osteoclastic lineage more efficiently than untreated monocytes, in terms of number of multinucleated giant cells, mRNA expression of osteoclast-related genes and TRACP 5b activity. On the other hand, the bone resorption activity of osteoclasts obtained in the presence of high prelamin A levels is lower with respect to control osteoclasts. This finding may help the understanding of the osteolytic and osteoporotic processes that characterize progeroid laminopathies. J. Cell. Biochem. 105: 34–40, 2008.

KEY WORDS: PRELAMIN A; HUMAN OSTEOCLASTS; OSTEOCLAST DIFFERENTIATION; FTI-277; AFCMe

Laminopathies are an heterogeneous group of inherited disorders caused by mutations in the lamin A/C gene or in genes encoding proteins involved in lamin A processing or intermolecular interactions [Vlcek and Foisner, 2007]. Progeroid laminopathies, including Hutchinson-Gilford progeria, Mandibuloacral dysplasia, atypical Werner syndrome and Restrictive dermopathy, are characterized by premature aging, loss of subcutaneous fat and osteolysis of clavicles, mandible, and phalanges [Broers et al., 2006]. Osteoporosis is also observed in Hutchinson-Gilford progeria [Hennekam, 2006]. The common molecular feature of progeroid laminopathies has been determined in the altered post-translational processing of the lamin A precursor, which causes accumulation of prelamin A [Maraldi and Lattanzi, 2007]. In normal cells, the translated lamin A precursor undergoes a multi-step process of maturation including farnesylation of its C-terminus CaaX motif (CSIM), endoprotease cleavage of the last three aminoacids, methylation of the C-terminal cysteine by the enzyme ICMT and endoproteolytic removal of other 15 aminoacids at the protein carboxy terminus end [Rusinol and Sinensky, 2006]. Each of these post-translational modifications appears to be necessary for the subsequent reaction to occur. Hence, defects in protein farnesylation or cleavage impair maturation of lamin A and cause prelamin A accumulation. In Hutchinson-Gilford progeria cells a point mutation in the *LMNA* gene causes accumulation of an alternatively spliced truncated prelamin A form, which is carboxymethylated and farnesylated [Eriksson et al., 2003]. Restrictive dermopathy cells accumulate farnesylated prelamin A due to inactivating mutations of the specific prelamin A endoprotease ZMPSTE24 [Navarro et al., 2004]. In mandibuloacral dysplasia, a reduced rate of prelamin A maturation has been detected both in the case of *LMNA* mutations [Filesi et al., 2005] and

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in the case of *ZMPSTE24* gene mutations [Agarwal et al., 2003], leading to chromatin disorganization and anomalous transcription factor binding [Capanni et al., 2005; Filesi et al., 2005]. However, the pathogenic mechanism linking prelamin A accumulation to bone defects has not been unraveled. Involvement of osteoclast activity in bone resorption processes occurring in progeroid laminopathies appears likely, although defective osteoblast differentiation or proliferation can be also hypothesized. A hint for the involvement of osteoclasts in bone defects associated with lamin A/C mutations comes from the evidence that lamin A is able to sequester the transcription factor cFos [Ivorra et al., 2006], involved in osteoclast differentiation [Wan et al., 2007].

To evaluate the response of differentiating osteoclasts to accumulation of prelamin A, as it occurs in laminopathies featuring osteolysis [Maraldi and Lattanzi, 2007], here we use an experimental approach. Accumulation of two different forms of the lamin A precursor was induced in peripheral blood monocytes differentiating towards the osteoclastic lineage by drug treatment and the degree of osteoclast differentiation and the resorptive activity were evaluated. The first drug here employed, FTI-277, is a farnesyl transferase inhibitor known to induce accumulation of non-farnesylated prelamin A. The second compound here used is a non-peptidomimetic drug (N-Acetyl-S-farnesyl-L-cysteine-methyl ester, AFCMe) known to affect the activity of the prelamin A endoprotease ZMPSTE24 and to cause accumulation of farnesylated prelamin A. Based on these properties, both compounds can be used to test the effects of prelamin A accumulation in cells. In previous studies, FTI-277 was assayed in osteoclasts and it did not appear to impair osteoclast differentiation [Woo et al., 2005]. Our results here reported show that both FTI-277 and AFCMe, besides inducing prelamin A accumulation in differentiating peripheral blood monocytes, accelerate the formation of multinucleated cells and the expression of osteoclast specific differentiation markers, including cathepsin K and ATPase V0. However, osteoclasts obtained in the presence of FTI-277 or AFCMe have a reduced resorptive activity. These data suggest that accumulation of prelamin A may initially increase preosteoclast fusion, ultimately leading to formation of osteoclasts with reduced resorption activity. This finding may have relevant implications in the understanding of the osteolytic and osteoporotic processes that characterize progeroid laminopathies.

MATERIALS AND METHODS

OSTEOCLAST CULTURES AND DRUG TREATMENT

Osteoclasts were obtained from peripheral blood mononuclear cells (PBMC) as described previously [Avnet et al., 2007]. Fresh buffy coats (AVIS, Bologna, Italy) were diluted with PBS, layered over Hystopaque (Sigma, St. Louis, MO), and centrifuged at 900*g* for 30 min. The mononuclear cells were extracted from interphase of the PBS and Hystopaque and centrifuged at 400*g* for 5 min. Cells were rinsed in PBS and seeded on tissue-culture glass or plastic ware in DMEM (Euroclone, Milan, Italy) supplemented with 10% FCS (Hyclone, Logan, UT) and incubated at 37°C in a humidified 5% CO_2 atmosphere. Cells were seeded at the density of 3,000,000/cm^2. After 1 h, medium was discarded and replaced with differentiating medium containing RANKL 30 and 25 ng/ml M-CSF (Peprotech, Rocky Hill, NJ).

Non-farnesylated prelamin A accumulation was induced by farnesyl transferase inhibition using the peptidomimetic drug FTI-277 (Calbiochem). Samples were cultured in the presence of 10 μM FTI-277 in differentiation medium. FTI-277-containing medium was replaced every other day. Treatment with AFCMe was performed to accumulate farnesylated prelamin A. This drug was added to differentiation medium at the concentration of 1 μM and replaced every other day. 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 Hoecst 33258 (1.25 (g/ml). TRACP-positive cells containing 3 or more nuclei were considered to be differentiated osteoclasts. At 8 days multinucleated cells with 3 and more than 3 nuclei were counted. Experiments were done in triplicate, 25 microscopy fields at 40× magnification were evaluated for each sample. The results were expressed as the means ± SE of three different experiments.

IMMUNOFLUORESCENCE ANALYSIS

Immunofluorescence microscopy analysis was performed as follows. Cells cultured in differentiation medium for 2, 4, or 8 days were washed in PBS and fixed in 2% paraformaldehyde for 10 min at room temperature and permeabilized with 0.15% Triton X-100 for 5 min. Incubation with 4% BSA in PBS was performed to saturate non-specific binding. Lamin A/C was detected by a mouse anti-lamin A/C antibody (Novocastra, NLC-LAM-A/C), used 1:10 dilution for 1 h at 37°C and revealed by FITC-conjugated anti-mouse IgG for 1 h at room temperature. Prelamin A was detected by goat anti-prelamin A antibody (Santa Cruz, SC-6214, lot. J3105), used 1:100 dilution for 1 h at room temperature, and revealed by TRITC-conjugated anti-goat IgG applied for 1 h at room temperature. Some samples were further incubated with FITC-phalloidin for 30 min at room temperature to detect actin fibers. Nuclei were then counterstained with 4,6-diamino-2-phenylindole (DAPI). Slides were observed by a Nikon Eclipse E600 fluorescence microscope equipped with a digital camera or by a confocal laser scanning microscope equipped with argon and helium-neon lasers (Nikon, Tokyo, Japan).

TRACP5b ACTIVITY ASSAY

Tartrate-resistant acid phosphatase isoform 5b (TRACP5b) in the supernatant of cell cultures treated or not treated with drugs at 4 days, was determined as a marker of osteoclast number using a commercial immunoassay (BoneTRAP®; SBA-Sciences, Oulu, Finland), according to the manufacturer's instructions. The active tartrate-resistant acid phosphatase isoform 5b (TRACP5b) in the supernatant of cell cultures treated or not treated with drugs at 4 days, was determined as a marker of osteoclast number using a commercial solid phase immunofixed enzyme activity assay (BoneTRAP®; SBA-Sciences), according to the manufacturer's instructions. The BoneTRAP® uses a highly characterized, specific anti-TRACP5b monoclonal antibody that binds only enzymatically active TRACP molecules, without binding any interfering inactive

fragments. Bound TRACP5b activity is then determined with a chromogenic substrate to develop color. The color intensity is directly proportional to the amount and activity of TRACP5b present in the sample.

SEMI-QUANTITATIVE RT-PCR

Total RNA was isolated using Rneasy Mini Kit (Qiagen GmbH, Hilden, Germany) from osteoclast cultures following manufacturer instructions. In particular, this technology combines the selective binding properties of a silica-based membrane with the speed of microspin technology. Before RNA extraction, cells were left untreated or treated with AFCMe or FTI for 8 days, as describe above. RNA was reverse transcribed into cDNA using the Advantage RT-for-PCR Kit (Clontech Laboratories, Palo Alto, CA). The RT-PCR for the specified human genes was determined using forward and reverse primers listed in Table I, and consisted in one denaturation at 94°C for 5 min, and then 30 cycles of amplification (denaturation at 94°C for 30 s, annealing at the specific temperature for 30 s, and extension at 72°C for 45 s), and then a final extension at 72°C for 7 min. Parallel reactions were performed for every assay using primers designed to amplify human β-actin. Specific cDNA for β-actin levels was assayed by denaturation at 94°C for 10 min, and then 30 cycles of amplification of denaturation at 94°C for 30 s, annealing at 65°C for 45 s and extension at 72°C for 30 s, and final extension at 72°C for 10 min. The products were separated by electrophoresis using 2% agarose gel stained with ethidium bromide (0.5 mg/ml). The pictures of the gel were transferred to the computer by camcorder and quantified by dedicated software for densitometric evaluation of the bands (Quantity One, Biorad Laboratories Headquarters, Hercules, CA). The specific bands were normalized to β-actin signals. Specific RT-PCR assay was repeated three times for each gene.

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 differentiating medium with or without drugs for 10 days. Supernatants were collected and the fraction of collagen 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 Type I bone 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 supernatant 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).

STATISTICAL METHOD

Statistical analysis was performed with the StatView™ 5.0.1 software (SAS Institute, Inc., Cary, NC). Due to the low number of the 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 $P < 0.05$.

RESULTS

ACCUMULATION OF PRELAMIN A BY FTI-277 AND AFCMe IN DIFFERENTIATING OSTEOCLASTS

Before evaluating the effect of FTI-277 and AFCMe treatment, we analyzed laminA/C levels in differentiating osteoclasts. Human monocytes from peripheral blood were completely negative for lamin A/C staining at seeding (data not shown), but after day 2 of culturing in M-CSF and RANKL-enriched medium (differentiation medium), cells showed lamin A/C localization at the nuclear envelope that persisted through an 8-day differentiation period (Fig. 1). On the contrary, prelamin A staining was completely absent in cells maintained in differentiation medium during the entire osteoclast differentiation process (Fig. 2A). Both FTI-277 and AFCMe treatment induced prelamin A accumulation at the nuclear rim of undifferentiated mononucleated monocytes at 2 days of culture. Prelamin A accumulation persisted also in preosteoclast cells at 4 days, and in multinucleated cells at 8 days of culture (Fig. 2 B,C). These multinucleated cells can be considered as fully differentiated active osteoclasts, as we previously showed [Avnet et al., 2006]. At 8 days, typical actin ring was observed in multinucleated osteoclasts in all conditions, while prelamin A was

TABLE I. List of Primers

Primer sequence (5′-3′)	NCBI sequence viewer (accession No.)	Product size (bp)	Annealing temperature (°C)
β-actin	NM_001101	838	65
5′-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3′			
5′-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3′			
Cathepsin K	BC016058	615	63
5′-TTCCCGCAGTAATGACACC-3′			
5′-TTTCCCCAGTTTTCTCCCC-3′			
TRACP	BC025414	171	58
5′-CTTTCTACCGCCTGCACTTC-3′			
5′-GCTGTTTCTTGAGCCAGGAC-3′			
Vitronectin receptor	NM_002210	547	59
5′-GGCTTCGCCGTGGATTTCTT-3′			
5′-TGCTGTAAACATTGGGGTCGT-3′			
ATPase V0 subunit A3 (TCIRG1)	NM_006019	500	61
5′-CCCCGCTCCCTACACCATCA-3′			
5′-CTCCGAGGACCACCCCAAAG-3′			

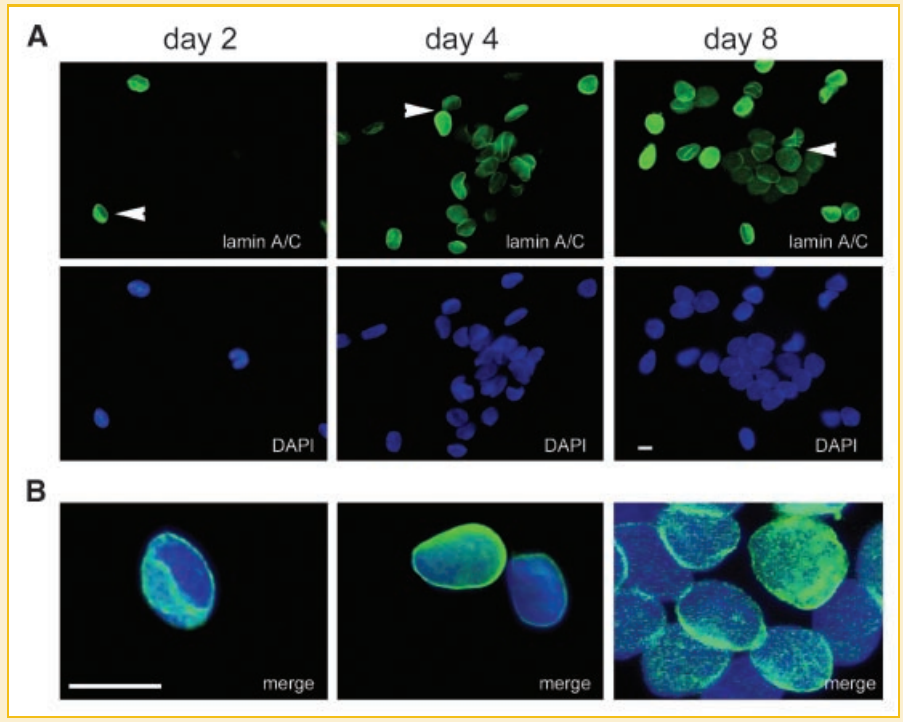

Fig. 1. Lamin A/C detection in PBMC induced to differentiate into osteoclasts by RANKL and M-CSF. A: Lamin A/C was stained by a specific mouse monoclonal antibody and revealed by FITC-conjugated anti-mouse IgG (green). Nuclei were counterstained with DAPI. Cells were labeled at days 2, 4, or 8 in differentiation medium. B: Higher magnification of nuclei indicated by arrowheads is shown in the merged images (merge) to better allow visualization of lamin A/C staining at the nuclear rim. Bar, 10 μm.

localized at the nuclear rim only for multinucleated cells treated with FTI-277 or AFCMe, as evaluated also by confocal microscopy analysis (Fig. 2). However, nuclear morphology, including nuclear envelope shape and mean nuclear diameter were not affected by drug treatments (Fig. 2).

EFFECT OF FTI-277 AND AFCMe ON OSTEOCLAST NUMBER AND DIFFERENTIATION

At 8 days of culture, TRACP-positive multinucleated cells were present in all the conditions (Fig. 3A–F), however the number of multinucleated cells with more than 3 nuclei was slightly increased after prelamin A processing inhibitors treatments (Fig. 3G). In particular, there was a significant increase in the number of cells with more than 100 nuclei, relative to control samples, suggesting a higher rate of cell fusion (Fig. 3H). This result was supported by the evaluation of TRACP 5b activity at 4 days of culture. TRACP 5b is the TRACP isoform specifically expressed by active osteoclasts, and its level is directly related to osteoclast number [Alatalo et al., 2000]. Therefore TRACP 5b activity was slightly induced by FTI-277, and significantly upregulated by AFCMe treatment ($P < 0.05$, Fig. 3I).

To analyze the effect of drug treatment on osteoclast differentiation we also evaluated the level of the mRNA expression of the osteoclast markers ATPase V0, cathepsin K, vitronectin receptor and TRACP by semi-quantitative RT-PCR. After 8 days of culture, all examined mRNAs were increased in drug-treated cells (Fig. 4). However, significant upregulation were observed only for ATPase V0 and cathepsin K mRNAs in AFCMe treated cells, accumulating farnesylated prelamin A, and ATPase V0 and TRACP mRNAs in FTI-277 treated cells, accumulating non-farnesylated prelamin A ($P < 0.05$), whereas vitronectin receptor expression was not significantly enhanced (Fig. 4). Thus, both increase in osteoclast number and mRNA expression values of osteoclast markers demonstrated that prelamin A accumulation affects in a positive way the osteoclast formation.

EFFECT OF FTI-277 AND AFCMe ON COLLAGEN DEGRADATION

To analyze if prelamin A accumulation influences osteolytic activity we used a collagen resorption assay. At 10 days of culture, FTI-277- or AFCMe-treated osteoclasts showed a decreased collagen degradation activity (Fig. 5). In fact, resorption activity was significantly inhibited by FTI-277 treatment ($P < 0.005$), and only slightly inhibited by AFCMe treatment. This result demonstrates that prelamin A accumulation leads to formation of less functional osteoclasts.

DISCUSSION

A number of experimental results reported in the recent years has provided evidence of a major role of lamin A in nuclear functions. Lamin A has been implicated in chromatin anchorage at the nuclear periphery, nuclear envelope organization through interaction with emerin, nesprin and lamin B, regulation of cell cycle exit and cellular differentiation through interplay with the nuclear lamina protein LAP2α and modulation of the transcriptional activity through transcription factor binding [Broers et al., 2006; Vlcek and

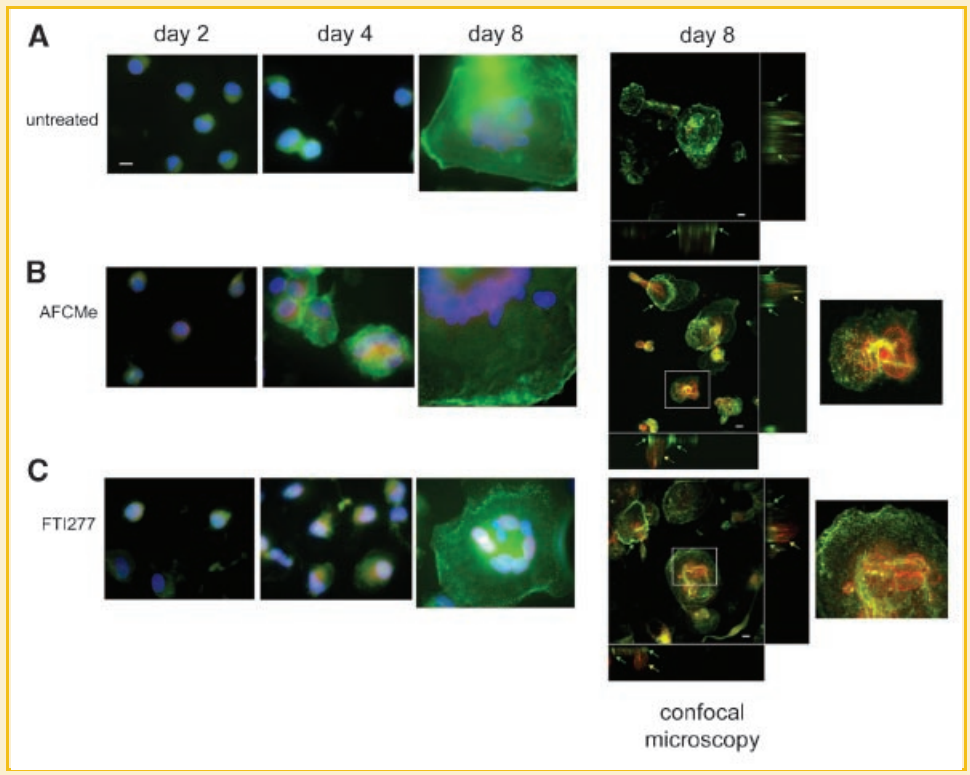

Fig. 2. Prelamin A detection in AFCMe- or FTI-277-treated PBMC induced to differentiate into osteoclasts by RANKL and M-CSF. Prelamin A was stained by specific antibody (Santa Cruz, SC-6214) and revealed by TRITC-conjugated anti-goat IgG (red). FITC-conjugated phalloidin (green) was used to stain actin fibers. Nuclei were counterstained with DAPI. Cells left untreated (A) or treated with 1 μM AFCMe (B) or 10 μM FTI-277 (C) were labeled at days 2, 4, or 8 in differentiation medium. Confocal images of osteoclasts at day 8 in differentiation medium are shown in the right column. Pictures represent overlaid images of 36 optical scanned XY-sections (0.4 μm thickness). Z-sections of one chosen scanned XY-section are also shown below (XZ-section), or on the right (YZ-section). In all images F-actin forms the characteristic actin-ring (green arrows). Bars, 10 μm.

Foisner, 2007]. Prelamin A, the precursor protein transiently formed during lamin A maturation, has been recently shown to play a role both in chromatin organization [Lattanzi et al., 2007; Mattioli et al., 2008] and transcriptional regulation [Capanni et al., 2005]. Moreover, it has been implicated in the pathogenesis of the most severe laminopathies, including those featuring bone disease [Maraldi and Lattanzi, 2007]. Therefore, it appears noteworthy to find out the role of lamin A and prelamin A in cells regulating bone omeostasis, such as osteoclasts.

In the reported study we show that accumulation of prelamin A in peripheral blood monocytes induced to differentiate into osteoclasts by the receptor activator of NFkB ligand (RANKL), increases the rate of cell differentiation, but leads to the formation of osteoclasts with reduced resorption activity.

Previous studies performed with FTI-277 and AFCMe showed that both drugs efficiently trigger prelamin A accumulation in human cells. FTI-277 accumulates a prelamin A form which is not processed to mature lamin A due to absence of the farnesylated residue at the C-terminus CSIM sequence. On the other hand, hindrance of the substrate binding site in the endoprotease ZMPSTE24 by AFCMe impairs cleavage of farnesylated prelamin A and leads to accumulation a farnesylated precursor. Regarding the effects of farnesylated or non-farnesylated prelamin A on cell function, we recently showed that prelamin A forms accumulated by FTI-277 or AFCMe alter chromatin arrangement in human fibroblasts. Similar effects on chromatin organization and downstream effects on differentiation and activity of osteoclasts appear likely. Here we failed to show major changes in nuclear morphology or chromatin organization in drug-treated pre-osteoclasts, although further studies are needed to evaluate these aspects. On the other hand, the effects of prelamin A on the differentiation rate of pre-osteoclasts appear to involve early steps of cell differentiation. In fact, PBMC cultures subjected to prelamin A processing inhibitors show a significant increase in the number of multinucleated cells at early differentiation stages with respect to controls. Signaling steps including possible NFATc interaction with lamin A or activation of NF-kB-regulated events warrant further investigation. Here we show that TRACP and cathepsin K expression are upregulated in prelamin A-accumulating cells. Although these factors are known to mediate bone resorption [Avnet et al., 2006; Habermann et al., 2007], osteoclasts obtained in our experimental model failed to show efficient resorptive activity. This finding implies that prelamin A triggers other pathways or alters the timing of gene expression thus leading to formation of osteoclasts with reduced resorption activity. Interestingly, it has been demonstrated that osteoclasts lacking resorptive activity play a role in the regulation of osteoblast number [Karsdal et al., 2008], suggesting a function independent on bone resorption.

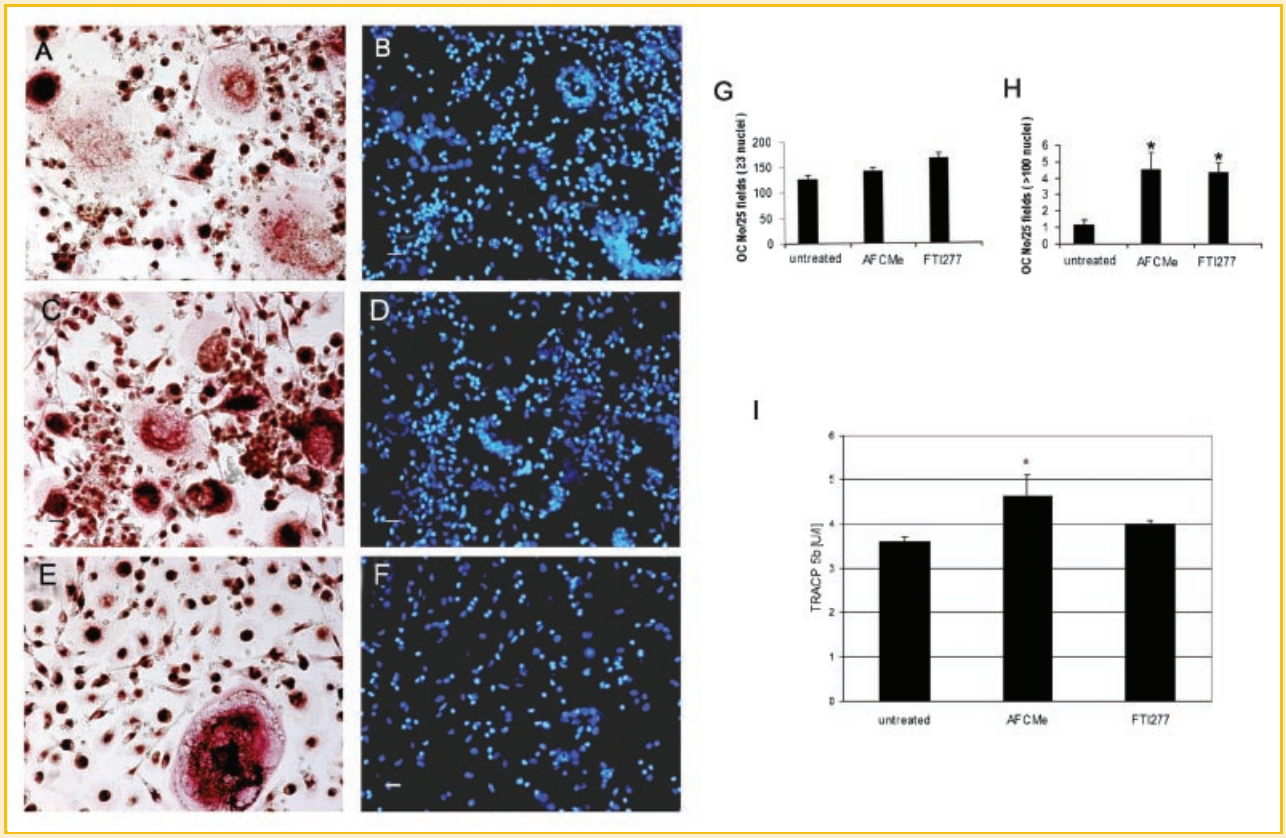

Fig. 3. TRACP-positive multinuclear cells in AFCMe- or FTI-277-treated PBMC cultures. Human mononuclear cells, induced to differentiate by RANKL and M-CSF from PBMC, were left untreated (A,B) or treated with AFCMe (C,D) or FTI-277 (E,F) for 8 days. Cells were then stained for TRACP expression (TRACP is revealed by red precipitates in representative pictures) (in A, C, and E), and with Hoechst 33258 (in B, D, and F) (Bars, 20 μm). In all conditions multinuclear TRACP positive cells were evident. G,H: Quantitative analysis of osteoclast formation. Analysis was performed on six countings of the TRAP positive cells that showed three or more than three nuclei (G), or more than 100 nuclei (H). Data were expressed as mean ± SE. I: TRACP5b activity was measured in the supernatant of PBMC cultures induced to differentiate by M-CSF and RANK-L, and treated with AFCMe or FTI-277, or not treated. Results represent means ± SE. Asterisks indicate statistically significant differences relative to untreated samples, $P < 0.05$.

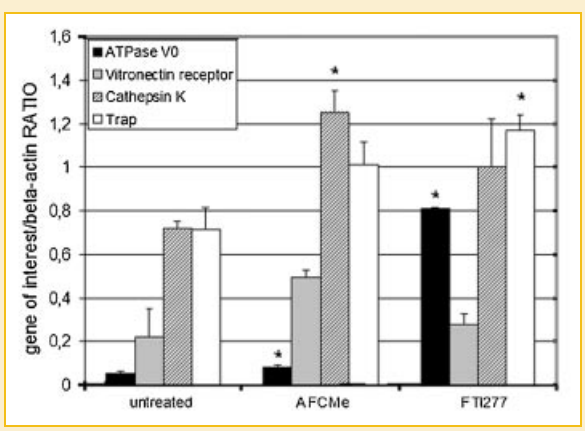

Fig. 4. mRNA expression of osteoclast marker genes following AFCMe or FTI-277 treatment. Cells were cultured for 8 days in the absence (untreated) or presence of AFCMe or FTI-277. The products were separated by electrophoresis and specific bands were quantified by dedicated software for densitometric evaluation. Each amplified product of the respectively gene was normalized to β-actin signals determined in parallel for each sample. RT-PCR was replicated three times. Asterisks indicate statistically significant differences relative to untreated samples, $P < 0.05$.

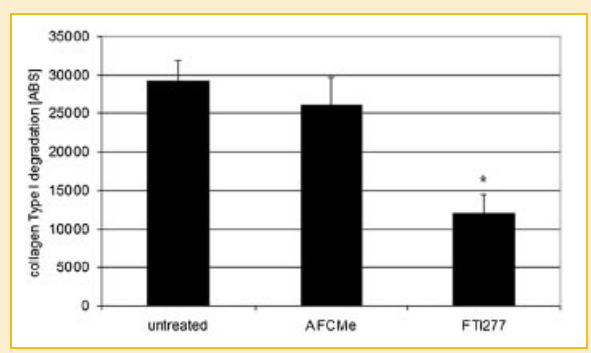

Fig. 5. AFCMe and FTI-277 effects on osteoclast-mediated collagen degradation. Collagen resorption activity of PBMC were evaluated by the Osteolyse assay after 10 days in differentiating condition, with or without drugs. Resorption activity was significantly inhibited by FTI-277 treatment, and only slightly inhibited by AFCMe treatment. Asterisk indicates statistically significant differences relative to untreated samples, $P < 0.005$.

Regarding the pathogenesis of laminopathies featuring bone defects, the present study suggests a role for misregulated osteoclast production. Other factors warrant investigation including the functionality of osteoblasts accumulating prelamin A and the production of osteoclast stimulating factors such as the RANK ligand (RANKL) by osteoblast populations bearing pathogenetic *LMNA* mutations. It is noteworthy that mature lamin A amount is reduced in aged osteoblasts, suggesting a relationship between protein processing and cellular aging [Duque and Rivas, 2006]. Finally, it is worth mentioning that the use of farnesylation inhibitors such as FTIs in the therapy of progeroid laminopathies must consider an accurate monitoring of the bone effects, based on the susceptibility of osteoclasts to FTIs treatments [Young et al., 2006]. However, since AFCMe effects on osteoclast resorptive activity are mild, while FTI-277 clearly reduces matrix resorption, we suggest that FTIs treatment could help the recovery of bone defects in progeroid laminopathies.

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