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Mechanical Forces-Induced Human Osteoblasts Differentiation Involves MMP-2/MMP-13/MT1-MMP Proteolytic Cascade

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ABSTRACT

Matrix metalloproteinase (MMP) family proteins play diverse roles in many aspects of cellular processes such as osteoblastic differentiation. Besides, mechanical forces that occur in 3D collagen gel promote the osteoblastic phenotype and accelerate matrix mineralization. Although MMPs have been involved in bone differentiation, the proteolytic cascades triggered by mechanical forces are still not well characterized. In this study, we have investigated the contribution of both proteolytic cascades, MMP-3/MMP-1 and MMP-2/MMP-13/MT1-MMP in the differentiation of human osteoblasts cultured in a floating type I collagen lattice (FL) versus an attached collagen lattice (AL). Compared to AL, contraction of human osteoblasts-populated FL led to a fast (1 day) induction of alkaline phosphatase (ALP), bone sialoprotein (BSP), osteoprotegerin (OPG), and Runx-2 expression. At day 4, osteocalcin (OC) overexpression preceded the formation of calcium-containing nodule formation as assessed by X-ray analyses. MMP-1 and MMP-3 were produced to similar extent by cells cultured in FL and AL, whereas contraction of collagen lattices triggered both mRNA overexpression of MMP-2, MMP-13, and MT1-MMP (i.e., MMP-14), and their activation as evidenced by Western blotting or zymographic analyses. Down-regulating MT1-MMP expression or activity either by siRNA transfection or supplementation of culture medium with TIMP-1 or TIMP-2 highlighted the contribution of that enzyme in OC, ALP, and OPG expression. MMP-2 and MMP-13 were more directly involved in BSP expression. So, these results suggest that the main proteolytic cascade, MMP-2/MMP-13/MT1-MMP, and more particularly, its initial regulator MT1-MMP is involved in osteoblast differentiation through mechanical forces. J. Cell. Biochem. 113: 760–772, 2012. © 2011 Wiley Periodicals, Inc.

KEY WORDS: OSTEOBLAST; COLLAGEN LATTICES; MATRIX METALLOPROTEINASES; DIFFERENTIATION MARKERS; BONE MINERALIZATION

The architecture of the microenvironment has been consistently found to modulate cell fate, which in a feedback process also affected the composition and the organization of the extracellular matrix. These modifications are a pivotal force-linked morphogenetic regulator of tissue differentiation [Boudreau and Weaver, 2006]. For example, 3D gels constituted of native type I collagen directed the differentiation of 3T3 adipocytes precursors [Hilliou et al., 1988] and of monocytes to macrophages [Jacob and Sudhakaran, 2001]. Several other investigations pinpointed the primary importance of $\alpha_2\beta_1$ integrin–collagen interaction in osteogenic differentiation using cell lines or bone marrow-derived cells [Riikonen et al., 1995; Mizuno et al., 2000]. In addition, the modification of the plasticity of the microenvironment, through mechanical forces influenced the phenotype of several cell types including osteoprogenitor cells [Akhouayri et al., 1999].

In vivo, the contraction of type I collagen, which provides sufficient strength to tissues, might contribute to fracture healing by drawing the bone ends together [Kinner et al., 2002] and is

Abbreviations used: MMP, matrix metalloproteinase; ALP, alkaline phosphatase; OC, osteocalcin; BSP, bone sialoprotein; OPG, osteoprotegerin; TIMP, tissue inhibitor of metalloproteinase.

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associated with collagenase(s)-mediated collagen remodeling [Berton et al., 2000]. Several endopeptidases belonging to the matrix metalloproteinase (MMP) family proteins, displayed collagenolytic activity [Nagase et al., 2006], and generation of mice deficient in those enzymes highlighted their involvement in bone development. Loss of MMP-2 by gene targeting in mice induced marked skeletal abnormalities with decreased bone mineralization [Inoue et al., 2006], and mice lacking both MMP-13 and MMP-9 presented impaired endochondral bone [Lieu et al., 2011]. MT1-MMP knock-out mice, in turn, evidenced several important defects such as dwarfism, osteopenia, and arthritis leading to their premature death [Zhou et al., 2000; Holmbeck et al., 2005]. Moreover, a series of investigations suggested the active participation of these enzymes during osteogenesis in vitro. For instance, in osteoblastic cell cultures, the mineralization generally observed at the latest stage of differentiation, was associated with increased level of osteocalcin (OC) and MMP-13 expression [D'alonzo et al., 2002]. Recently, a special attention has been paid to MT1-MMP since osteogenic cells from mice deficient in that enzyme failed to differentiate when transplanted into wild-type animals [Lu et al., 2010]. Further experiments on tibial-derived rat osteoblast showed that MT1-MMP could regulate alkaline phosphatase (ALP) expression, mainly localized in cell-forming nodules [Filanti et al., 2000; Manduca et al., 2009].

Collagenases are regulated at distinct level. Stepwise activation of those enzymes probably constitutes one critical event and involves a cysteine switch disrupting the coordination bound between a cysteine, within a consensus peptidic sequence in the enzyme prodomain and zinc in the active site [Nagase et al., 2006]. Pericellular collagenolysis involves two sets of proteolytic cascades that can be triggered differently, depending upon cell types and mechanical forces [Deryugina et al., 1998; Cozlin et al., 2006]. Besides, isotonic and isometric stresses have been shown to modulate differently ALP and OC levels in a time-dependent manner in osteosarcoma cells [Akhouayri et al., 1999; Parreno et al., 2008].

In this investigation, we provided evidence that contraction, as opposed to static stress, of human osteogenic cell-populated collagen lattices, enhanced the expression of several osteoblastic markers as ALP, Runx-2, BSP, and OPG. OC expression was maximally increased at day 4 of contraction and was followed by nodule formation. Such increase of osteoblastic differentiation markers paralleled the activation of the MT1-MMP/MMP-2/MMP-13 cascade. Use of MT1-MMP siRNA, together with culture medium supplementation with TIMPs allowed us to delineate the dual contribution of MT1-MMP and MMP-2/MMP-13 in directing osteoblast differentiation in this model.

MATERIALS AND METHODS

CELL CULTURES AND TREATMENTS

Trabecular bone specimens were obtained from human male and female during orthopedic surgery. The patients (50- to 70-year old) had no abnormality of bone metabolism and bone remodeling. All subjects denied having taken drugs and gave their informed consent according to Helsinki Declaration.

Thirty-five millimeter diameter plastic tissue cultures dishes were from Costar (represented in France by Dutscher, Blumath) and glasses dishes were obtained from Special Verre (Geispolheim, France).

Acid soluble collagen from rat-tail tendons was prepared according to a technique previously described [Gillery et al., 1989]. Tissue culture media, including fetal calf serum (FCS), Dulbecco's modified Eagle medium (DMEM), EDTA-Trypsin solution, collagenase (Clostridiopeptidase A), penicillin–streptomycin were from Gibco (Life Technologies, Eragny, France). All other reagents of the highest purity available were purchased from Prolabo (Paris, France).

Bone tissue samples were rinsed twice with Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (PBS), minced, and the tissue fragments were first freed from adherent connective and/or fatty tissue and further digested with 0.05% trypsin (w/v) for 10 min. Then, bone explants were subjected to three sequential collagenase digestions (1.4 mg/ml) for 30 min at 37°C in PBS. Released cells were grown at confluency in 25 cm^2 culture flasks in DMEM supplemented with 20% FCS. At first culture passage, cells were incubated in DMEM containing 10% FCS and always used at the third passage in the present investigation.

Three-dimensional lattices cultures were performed with 1 mg soluble type I collagen from rat-tail in 2 ml of DMEM containing 2.5% FCS as previously described [Gillery et al., 1989]. Attached collagen lattices (AL) were obtained using, before cells seeding, nylon bars (Polylabo, Strasbourg, France) situated at the inner periphery of the dish, thus preventing the retraction of collagen by cells [Lambert et al., 1992]. Cells were counted with a Neubauer device and their DNA content, in collagen lattices, was quantified by fluorometric assay [Lorimier et al., 1996]. In standard conditions, 200,000 cells were seeded and lattice diameter were measured everyday after seeding by placing the dishes on a graduate rule fixed on a black surface. Collagen lattices were analyzed at days 1 and 4 after seeding. Experiments were reproduced with five different cell strains seeded into collagen lattice, except from transmission electron microscopy (TEM) or enzyme-linked immunosorbent assays (ELISA), which were done on two cell strains. For all cell strains, triplicate experiments were performed.

MORPHOLOGICAL STUDIES

Lattices were examined by scanning electron microscopy (SEM) and TEM.

For SEM, samples were rinsed with PBS and then, fixed in situ with 2.5% (w/v) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 for 2 h at 4°C. After two washings in PBS, the lattices were frozen in liquid nitrogen and cracked to reveal their inner structure. They were then dehydrated in grading ethanol, followed by an acetone/ethanol solution to a pure acetone. The samples were further dissociated with a critical point dryer and coated with Palladium-Gold (JEOL, ION Sputter JFC 1100). The observations were carried out with a JEOL JSM 5400 LV SEM.

For TEM, samples were fixed in 2.5% (w/v) glutaraldehyde in PBS at 4°C for 1 h without tension release for AG, then followed by successive washings in PBS and distilled water. The gels were

post-fixed in 2% osmium tetroxyde (Merck, Fontenay/Bois, France) for 1 h, dehydrated, and embedded in resin (Kit AGAR 100 Resin, Aga Scientific/Oxford Instruments, Orsay, France). Sections were stained with lead citrate and uranyl acetate before being examined with JEOL JEM 10-10 electron microscope.

ALKALINE PHOSPHATASE (ALP) ASSAY

Alkaline phosphatase activity was assayed using cellular extracts previously washed with PBS, then preincubated at 4°C in 0.1 M Tris–HCl, 0.25 M NaCl, 0.01% Triton X-100, 0.02% NaN_3 pH 10.2, and, finally, sonicated. The release of *p*-nitrophenol from *p*-nitrophenol phosphate at 37°C and pH 10.5 was measured spectrophotometrically at 405 nm according to Bowers et al. [1981] using an ALP 3.1.3.1 kit (Ref 1972596, Roche Diagnostics, Meylan France). Results are expressed in UI/L.

MEASUREMENTS OF LATTICE DEGRADATION

The degradation of collagen lattice was determined by quantifying the 4-hydroxyproline (4-OH-PRO) content in conditioned culture media at various periods of culture, as previously described [Bellon et al., 1985].

ASSAYS FOR MMP-1, MMP-2, MMP-3, AND MMP-13

MMPs were measured in conditioned media and cell extracts by double antibody sandwich ELISAs. ELISAs were carried out with Biotrack Kits (Amersham Pharmacia Biotech, Orsay, France; MMP-1: RPN 2610; MMP-2: RPN 2617; MMP-13: RPN 2621) and MMP-3 kit (Ref: QIA73-1) from VWR International (Strasbourg, France). These assays quantify the amount of MMPs present irrespective of whether they are free or complexed to their inhibitors. Also, antibodies detected latent and inhibited forms of MMPs.

GELATIN ZYMOGRAPHY

To determine whether human bone cells were capable to secrete pro as well as active gelatinases species (MMP-2 and MMP-9), conditioned culture media or cell lysates were electrophoresed on 10% SDS–polyacrylamide substrate gels containing 1 mg/ml of gelatin. Samples were diluted with equal volume of non-reduced sample buffer (Biorad, Marnes la Coquette, France) and electrophoresed at 4°C. After electrophoresis, the gels were washed twice for 30 min in 2.5% Triton X-100, then, once for 1 min in 0.1 M Tris–HCl, pH 7.4 containing 10 mM $CaCl_2$ and 0.02% NaN_3. Gels were then incubated in the same buffer for 24 h at 37°C to allow for digestion of the gelatin substrate. Proteolytic activity was visualized by staining the gels in 0.5% Coomassie Blue R250 containing 30% Propanol-2 and 10% acetic acid, followed by destaining in 7.5% acetic acid and methanol.

WESTERN BLOTS

Proteins contained in conditioned media or cell extracts were separated by 10% SDS–PAGE under reducing conditions (Dithiotreitol) and blotted onto nitrocellulose membrane (Immobilon P Millipore, Bedford, MA) with blotting apparatus (100 V, 110 min; Biorad, Ivry/Seine, France). Blots were blocked for 2 h with 50 mM Tris, 150 mM NaCl, pH 7.5 (TBS) containing 5% (w/v) non-fat dry milk (Biorad), and 0.1% Tween (w/v). The transferred proteins were probed with primary antibodies (1:1,000 monoclonal anti-human MMPs-1, -2, -3, -13; Calbiochem, France) diluted in 1% non-fat milk TBST. Blots were developed by chemiluminescence (Kit ECL/RPN 2069, Amersham Pharmacia Biotech).

REVERSE-TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR)

For RT-PCR analysis, total RNA was isolated from 2×10^5 human osteoblasts-like cells in lattices after treatment with Trizol reagent (Life Technologies, Paisley, UK) for 3 h. Total RNA was reverse-transcribed according to the manufacturer's instruction (Life Technologies, No. 8025SA). The primers for MMPs, urokinase, TIMP-1 and -2, bone proteins, and 18S RNA were synthesized by Invitrogen Life Technologies (Auckland, New Zealand). The sequences of primers are indicated in Table I. cDNA products were amplified for 28 cycles for 18S RNA and for 30 cycles for all other assays.

The PCR products were then electrophoresed on a 1% agarose gel containing ethidium bromide.

SiRNA TRANSFECTION

Two siRNA, according to optimization of selection, containing 25 nucleotides were used with the following sequences: MT1-MMP siRNA (1)—forward: 5′-AGAGAGCAGCAUCAAUCUUGUCGG3-′; reverse: 5′-CGACAAGAUUGAUGCUGCUCUCUU-3′ and MT1-MMP siRNA (2)—forward: 5′-AUGAUGAUCACCUCCGUCUCCUCC-3′; reverse: 5′-GAGGAGACCGAGGUGAUCAUCAUU3-′.

Osteoblast transfection was performed according to manufacturer instructions (Block-it transfection kit, Ref 13750-070, Invitrogen Life Technologies). To evaluate the specificity of the siRNA, a non-operative siRNA (Stealth RNAi Negative Control Duplexes) was used in parallel. A 50 nM concentration of siRNA was found to abolish MT1-MMP expression by >80% at day 4 of floating type I collagen lattice (FL) while having no effect on cell viability. Also such treatment did not modify the expression of other MMPs.

MINERALIZATION EVALUATION BY X-RAY MICROANALYSIS

For the evaluation of mineralization by X-ray microanalysis, samples were fixed in 2.5% (w/v) glutaraldehyde in PBS at 4°C for 1 h without tension release for AG, then followed by successive washings in PBS and distilled water. The gels were post-fixed in 2% osmium tetroxide (Merck) for 1 h, dehydrated, and embedded in resin (Kit AGAR 100 Resin, Aga Scientific/Oxford instruments, Orsay, France). Sections were then covered with a thin layer of carbon (10 nm) before being examinated with STEM-EDS (Energy Dispersive Spectrometry) Philips CM 30 electron microscope (250kV, 10 nA).

STATISTICAL ANALYSIS

Data were analyzed and statistically significant differences were determined using Student–Fischer's test. All values were expressed

TABLE I. Sequences of Primers Used for RT-PCR

Gene	Primer name	Sequence (5′ → 3′)	Size (bp)	T_m (°C)
Bone sialoprotein (BSP)	Forward	AATGAAAACGAAGAAAGCGAAG	450	60
	Reverse	ATCATAGCCATCGTAGCCTTGT		
Osteocalcin (OC)	Forward	GGCAGCGAGGTAGTGAAGAG	230	69
	Reverse	CTGGAGAGGAGCAGAACTGG		
Osteoprotegerin (OPG)	Forward	GAAGGGCGCTACCTTGAGAT	362	60
	Reverse	TGCCTCCTCACACAGGG		
Receptor activator of nuclear factor-kappa B ligand (RANKL)	Forward	GCTTGAAGCTCAGCCTTTTGCTCAT	412	65
	Reverse	GGGGTTGGAGACCTCGATGCTGATT		
Alkaline phosphatase (ALP)	Forward	TGAAATATGCCCTGGAGC	804	56
	Reverse	TCACGTTGTTCCTGTTCAG		
Runx-2	Forward	CTCACTACCACACCTACCTG	320	56
	Reverse	TCAATATGGTCGCCAAACAGATTC		
MMP-1	Forward	CGACTCTAGAAACACAAGAGCAAGA	786	58
	Reverse	AAGGTTAGCTTACTGTCACACGCTT		
MMP-2	Forward	GCCTGGTCAGTGGCTTGGGGTA	225	62
	Reverse	AGATCTTCTTCTTCAAGGACCGGTT		
MMP-3	Forward	GAACAATGGACAAAAGGATACAACA	729	55
	Reverse	GAACAATGGACAAAGGATACAACA		
MMP-13	Forward	GTGGTATGGGAAGTATCATCA	330	51
	Reverse	GCATCTGGAGTAACCGTATGG		
MT1-MMP	Forward	CGCTACGCCATCCAGGGTCTC	497	62
	Reverse	CGGTCATCATCGGGCAGCACA		
TIMP-1	Forward	TCA GGC TAT CTG GGA CCG CAG GGA	627	68
	Reverse	ACC ATG GCC CCC TTT GAG CCC CTG		
TIMP-2	Forward	CGA GAA ACT CCT GCT TGG GG	364	68
	Reverse	CTC GGC AGT GTG TGG GGT C		
Urokinase	Forward	CCTGCTTCTCTGCGTCCT	212	60
	Reverse	AAAAGTGACCATTCCCCTCA		
18S	Forward	GCGAATTCCTGCCAGTAGCATATGCTTG	126	60
	Reverse	GGAAGCTTAGGAGCGAGCGACCAAAGG		

as mean standard deviation (SD). Differences with $P < 0.05$ were considered as statistically significant.

RESULTS

TENSION FORCES INFLUENCE OSTEOBLAST MORPHOTYPE AND COLLAGEN REMODELING IN COLLAGEN LATTICES

Lattices were seeded with 200,000 human bone marrow osteoprogenitors cells in DMEM containing 2.5–10% serum and maintained as AL or allowed to contract (FL) for 7 days. Whatever the serum concentration used, no significant difference could be found in the extent of collagen gel retraction at day 1 of culture (Fig. 1A). However, the rate of gel retraction increased proportionally and significantly with serum concentration from days 2 to 7 of culture. Because no sign of cell death was observed in cells cultured with 2.5% serum, all others experiments were performed using this experimental condition, which let osteoblasts in a quiescent state from 1 to 4 days.

Human bone marrow cells in AL and FL were examined by TEM at day 4 of culture, where FL had retracted by 26%. Inside FL, osteoblasts exhibited a stellate or elongated appearance and their cytoplasm was characterized by the presence of numerous organelles without any particular orientation. Few cytoskeleton elements, as microfilaments, could be identified and no defined orientation of collagen fibers could be evidenced (Fig. 1A). On the contrary, within AL, osteoblasts exhibited a bipolar appearance and their cytoskeleton contained abundant bundles of microfilaments that paralleled the cell main body. Their cellular membrane was thicker and collagen fibers were aligned in the same direction as the long axis of the cell (Fig. 1B). Collagen remodeling was observed in FL by SEM with the presence of a few pericellular lysed matrix cavities (Fig. 1C). In turn, AL was characterized by osteoblasts lining up parallel to the surface of the lattice, and close contact between cells and collagen matrix was evidenced (Fig. 1D). In keeping with collagen remodeling, FL-conditioned medium contained an average amount of 82 ± 5 ng of collagen (as compared to 42 ± 10 ng for AL) as determined by OH-PRO content; it corresponded to 8.5% of total collagen present in FL.

LATTICE RETRACTION MODULATES OSTEOBLASTS DIFFERENTIATION

To assess the influence exerted by mechanical forces in collagen gels on the differentiation of human osteoblasts, ALP mRNA expression and activity, used widely as an osteoblast marker, were determined at days 1 and 4 in cells lysates of FL and AL cultures. At these stages of culture, whatever conditions, neither variation in cell growth nor apoptosis could be evidenced (data not shown). Although the expression of ALP (Fig. 2A1) and its activity (Fig. 2A2) decreased over time, ALP mRNA remained higher in FL compared with AL. A similar mRNA pattern was observed with Runx-2 (Fig. 2B), a master gene in osteoblast differentiation and function triggering the major home matrix genes in differentiating osteoblasts [Komori, 2006; Hayami et al., 2008].

Accordingly, compared with AL conditions, cell cultured within FL displayed higher mRNA level for bone sialoprotein (BSP) and osteoprotegerin (OPG), two other osteoblastic differentiation markers (Fig. 2B,C1). Most of the factors described to stimulate osteoclast formation and activity are known to favor RANKL

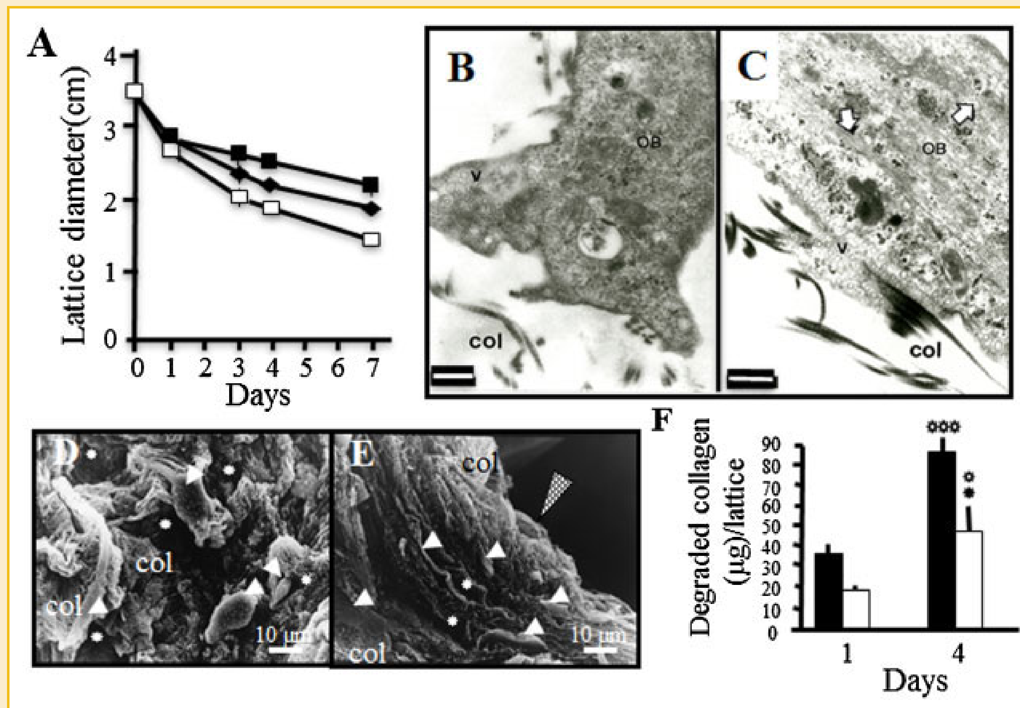

Fig. 1. Influence of mechanical forces on human osteoblast morphotype. A: Influence of FCS on lattice contraction. Lattices were seeded with 200,000 human osteoblasts containing □ 10% FCS, ◆ 5% FCS, ■ 2.5% FCS. B,C: Morphologic appearance of human osteoblasts cultured in free floating lattice (B), in attached lattice (C) as observed by transmission electron microscopy. OB, osteoblast; V, exocytotic vesicles; col, collagen. Bar: 500 µm; ⇨: cytoskeleton. D–F: Influence of lattice retraction on collagen remodeling. D,E: Scanning electron microscopy of floating (D) and attached (E) lattices. OB, ▷; ✹, lysed cavities; col, collagen; ▷, lattice surface. Bar: 10 µm. F: Level of collagen (OH-Pro determination: µg/lattice) present in the conditioned medium of free floating lattice (FL: ■) and attached lattice (AL: □). Data are means of triplicate determinations and bars represented the standard deviation: ★: $P < 0.05$ at day 4 between FL and AL; ☆ ☆ ☆: $P < 0.01$ between days 1 and 4 in FL; ☆: $P < 0.05$ between days 1 and 4 in AL.

expression by osteoblastic cells [Boyce and Xing, 2008]. Of note, expression of this molecule was much lower, that is, by 2-log order of magnitude as compared to OPG (Fig. 2C2). Furthermore, OPG/RANKL ratios increased with time of culture both in FL and AL (Fig. 2C2).

Osteocalcin, a late stage differentiation marker for osteoblasts, was expressed maximally, in contrast to other markers at day 4 in FL, whereas its mRNA level was nearly undetectable in all other experimental conditions (Fig. 2B). Since OC preceded immediately the onset of mineralization, any bone mineralization inside lattices was then investigated. Nodule formation determined by TEM could be identified at day 7 of culture in FL but not in AL (Fig. 3A). X-ray microanalysis indicated the presence of calcium in those nodules (Fig. 3B). Nodule calcification increased with time of culture as assessed by SEM (Fig. 3C). The presence of calcium or calcium phosphate within nodules could be identified by X-ray microanalysis (Fig. 3D), some of these nodules displaying earlier signs of crystallization (data not shown).

INFLUENCE OF MECHANICAL FORCES ON MMP AND TIMP EXPRESSION BY OSTEOBLAST-POPULATED COLLAGEN LATTICES

To establish the link between osteoblast differentiation, collagen remodeling, and collagenase induction, the levels of expression of MMP-1, MMP-13, MMP-2, MT1-MMP together with their activators, that is, uPA and MMP-3, and inhibitors, that is, TIMP-1 and TIMP-2, were analyzed by RT-PCR. Whatever conditions and time of culture, uPA mRNA remained undetected (Fig. 4A). At day 1 of culture, the level of MMP-3 and MMP-1 mRNA expression increased approximately twofold when cells are cultured within FL as compared to AL. Level of MMP-3 expression then dropped significantly, while MMP-1 level remained invariant. MMP-2 expression, in turn was not modified with time of culture and condition. On the contrary, at day 4 of culture, MT1-MMP and MMP-13 mRNA levels were mostly elevated in FL compared with AL conditions (Fig. 4A). Except MT1-MMP, those endopeptidases accumulated into culture media in which their protein levels were determined by ELISA (Fig. 4B). MMP-3 and MMP-2 secreted amounts were not significantly different between FL and AL. On the contrary, the quantity of MMP-1 and particularly MMP-13 produced by osteoblasts cultured in FL was significantly higher compared with AL. On a molar basis, the level of MMP-13 recovered in conditioned medium was 20- to 30-fold less than other MMPs (Fig. 4B). Besides, analysis of TIMP-1 and TIMP-2 expressions showed no significant difference in mRNA and protein levels at either day 1 or 4 of culture in FL or AL (Fig. 4C1,C2). Their concentration within culture media was also analyzed by ELISA (Fig. 4C3). On average, TIMP-1 and TIMP-2 levels were 3 and 0.6 ng/ml, respectively, at day 4 of culture, indicating that cells, under our experimental conditions, expressed large excess level of protease(s) over inhibitor(s).

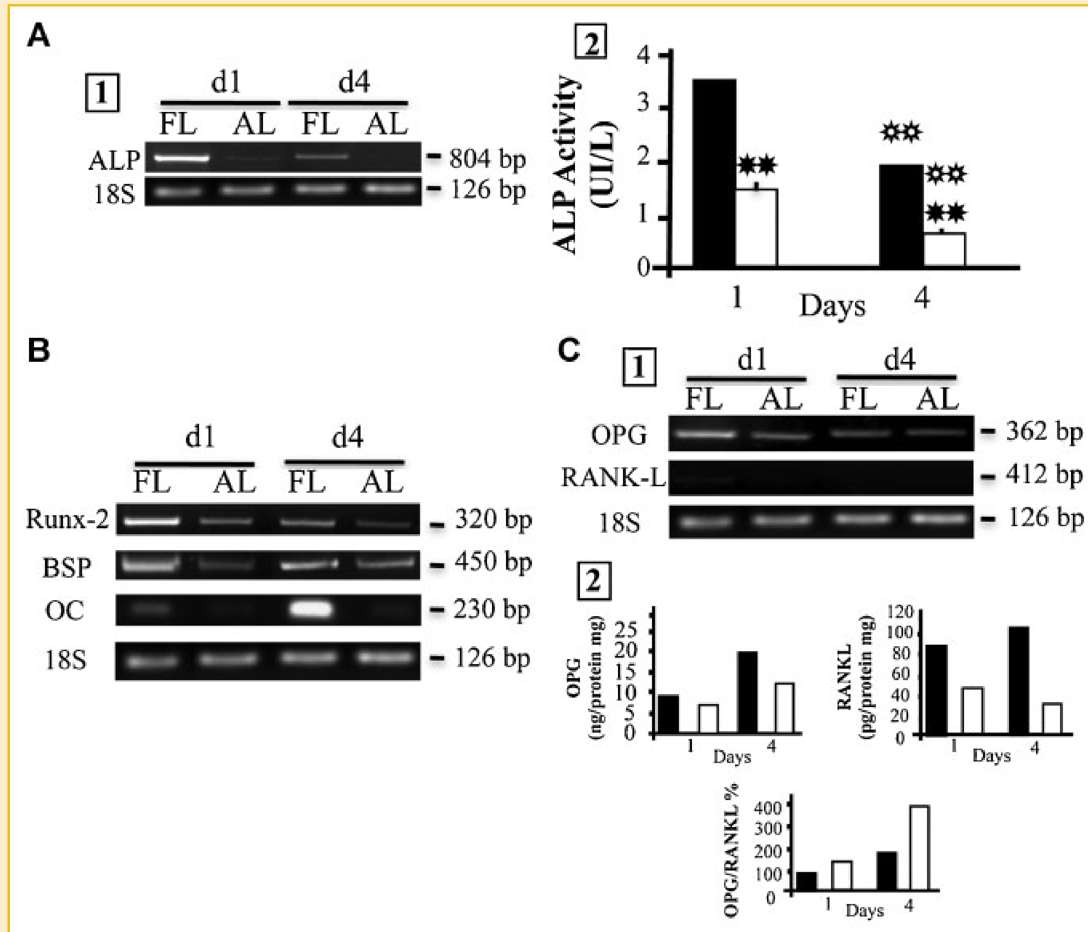

Fig. 2. Influence of mechanical forces on the expression of differentiation markers by human osteoblasts cultured in type I collagen lattices. FL, free floating lattice; AL, attached lattice; d1, day 1; d4, day 4; bp, base pairs. A: Alkaline phosphatase (ALP) activity. 1: RT-PCR analysis. 18S mRNA levels served as internal control. 2: ALP activity in cell lysates. ★★: $P < 0.01$ between FL (■) and AL (□) at the same day. ☆☆: $P < 0.01$ betweens days 1 and 4. B: RT-PCR analysis of Runx-2, bone sialoprotein (BSP), osteocalcin (OC). The figure is representative of data obtained from three different donors. C: Osteoprotegrin (OPG) and receptor activator of nuclear factor-kappa B ligand (RANKL) expression. 1: mRNA by RT-PCR analysis. 18S mRNA levels served as internal control. 2: Protein level as determined by ELISA. Expressed as ng (OPG) or pg (RANKL) per mg of protein. ■: Free floating lattice; □: attached lattice. Data shown are representative of data from two different donors.

RELATIONSHIPS BETWEEN EXPRESSION OF OSTEOBLAST DIFFERENTIATION MARKERS AND MMP LEVEL IN COLLAGEN FLOATING LATTICES

In order to seek a relationship in the osteoblast differentiation process between OC and MT1-MMP that were both increased at day 4 in FL (Fig. 4A,B), we then silenced the MT1-MMP expression by a siRNA approach. Transfection of osteoblasts with 2 siRNA targeting the MT1-MMP totally annihilated expression of this protease at day 4 of lattice contraction (Fig. 5). Scrambled siRNA, used as control, had no influence on MT1-MMP RNA level. SiRNA inhibition of MT1-MMP led to a nearly complete down-regulation of OC expression. Of note, ALP expression was similarly suppressed by cell transfection with MT1-MMP siRNA (not shown). On the contrary to other collagenases, MT1-MMP is activated intracellularly by furin-convertase(s) [Pei and Weiss, 1995] and vehiculated to osteoblast plasma membrane as an active enzyme [Knäuper et al., 2002]. At this site, it can directly modulate collagen remodeling or indirectly by triggering the MMP-2/MMP-13 collagenolytic cascade. Figure 6 indicated that contraction of collagen lattice led to MMP-2 and MMP-13 activation at day 4 of culture, determined both in conditioned medium and inside lattices by Western blotting and gelatin zymography. Of note, activation of the MMP-3/MMP-1 cascade was not evidenced, consistent with the low expression of uPA by osteoblasts cultured in collagen gels. Knockdown MT1-MMP expression by siRNA approach totally suppressed MMP-2 and MMP-13 activation. Thus, to differentiate any direct or indirect (through MMP-2 and MMP-13) action of MT1-MMP on the level of expression of osteoblast differentiation factors, FL-conditioned medium was supplemented by supra-physiological concentration of either TIMP-1 or TIMP-2. Indeed, TIMP-1 inhibits all MMPs except MT1-MMP while TIMP-2 suppresses indifferently the activity of MT1-MMP, MMP-2, and MMP-13 [Sabeh et al., 2009]. Accordingly, addition of 3 μg/ml TIMP-1 to FL did not modify MMP-2 activation at day 4 of culture contrary to TIMP-2 (3 μg/ml) supplementation (Fig. 7A). The expression of differentiation markers was analyzed in absence or presence of both TIMP-1 and TIMP-2 (Fig. 7B). Data

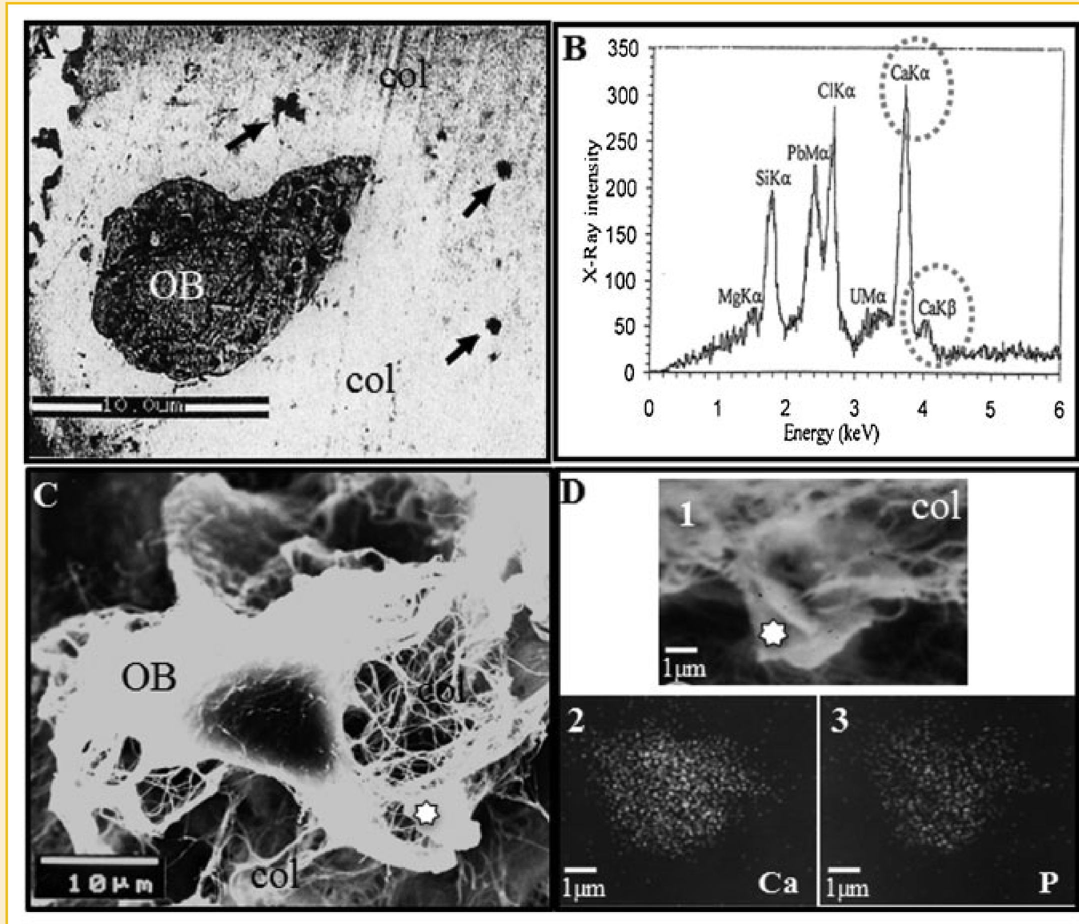

Fig. 3. Contraction of human osteoblast-populated collagen lattice induces mineralization. A,B: Transmission electron microscopy (day 7 of culture). Calcification nodules, ➡; OB, osteoblast; col, collagen. Elements within circles with dotted lines corresponded to calcium peaks in the nodule. C: Scanning electron microscopy (day 14 of culture). ✩, Bone mineralization; OB, osteoblast; col, collagen. D: Composition of the mineralization nodule (✩) by scanning electron microscopy associated to X-ray microanalysis. 1: Electronic image of the mineralization nodule found in free floating lattices at day 14. 2: Ca detection by X-ray microanalysis. 3: P detection by X-ray microanalysis.

obtained indicated that TIMP-2 but not TIMP-1 nearly abolished ALP and OPG expression, thus indicating the main contribution of MT1-MMP in the control of these two differentiation markers. MMP-2 and MMP-13 appeared to participate mainly in the control of BSP and OC expression that was similarly reduced in presence of both TIMP-1 and TIMP-2 (Fig. 7B). Higher level of inhibition of Runx-2 expression upon TIMP-2 treatment compared with TIMP-1 indicated that all members of the cascade were involved in the modulation of Runx-2 expression (Fig. 7B).

DISCUSSION

Cell-mediated contraction of collagenous matrix is a pivotal phase in the healing of tissues following injury. In skin, it accelerates wound closure by bringing wound edges while contributing to remodeling of the dermal matrix [Tomasek et al., 2002; Grinnell, 2003]. Similarly, the ability of osteoblasts to contract type I collagen matrices may help to bridge fractured bone cortices. A series of investigations using clonal cell lines, rat or human osteosarcoma cell lines, bone marrow cells from rat femur or isolated from the calvaria of neonatal mice, highlighted the contribution of mechanical forces in 3D collagen lattices in promoting the appearance of an osteoblastic phenotype and in accelerating the mineralization of the matrix [Akhouayri et al., 1999; Buxton et al., 2008; Parreno et al., 2008]. During contraction of collagen lattices, $\alpha_2\beta_1$ integrin acts as a mechano-receptor and initiates signaling cascades that leads to the up-regulation of MMP-1 as observed in fibroblasts and several osteosarcoma cell lines [Langholz et al., 1995; Riikonen et al., 1995]. It also initiates a signaling cascade leading to dephosphorylation of Akt and subsequently to cell apoptosis [Fujisaki and Hattori, 2002]. Indeed, primary osteoblastic cells cultured in 3D collagen lattices exhibit a low rate of proliferation, and cell death occurs at the end of contraction phase [Buxton et al., 2008]. The balance between bone formation and resorption is partly regulated by MMPs. In this setting, gene expression of osteogenic markers such as ALP and BSP can be substantially decreased in dense collagen gels treated with a broad spectrum MMP inhibitor [Buxton et al., 2008]. In turn, over-expression of Runx-2, a transcription factor involved in osteoblast

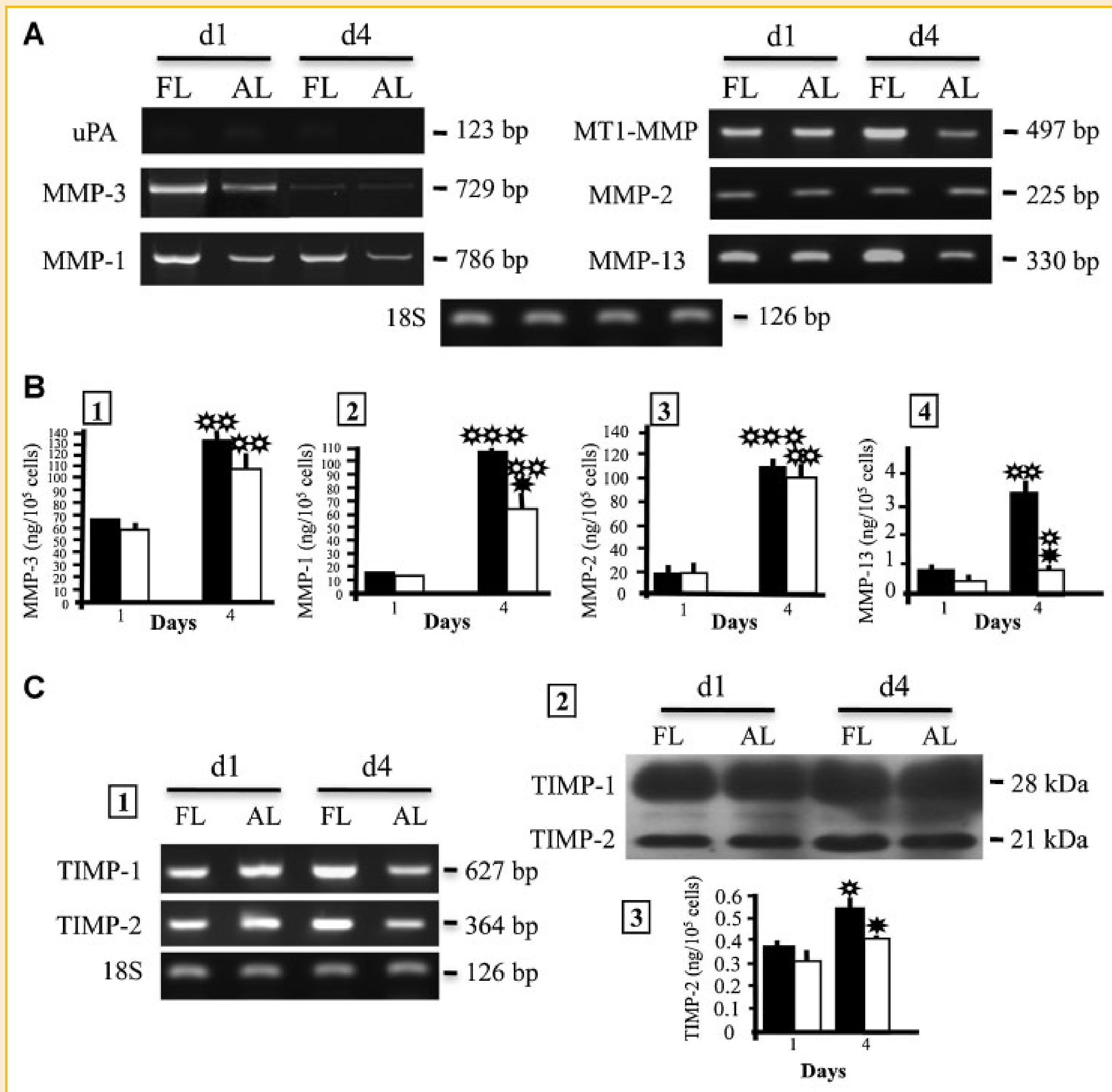

Fig. 4. Influence of mechanical forces on urokinase (u-PA), matrix metalloproteinases (MMP), and tissue inhibitors of metalloproteinases (TIMP) expression by human osteoblasts cultured in type I collagen gels. FL, free floating lattice; AL, attached lattice; d1, day 1; d4, day 4; bp, base pairs. A: RT-PCR analyses of proteinases. 18S mRNA levels served as internal control. B: Secreted MMP levels (ng/10^5 cell) as determined by ELISA. ■, free floating lattice (FL); □, attached lattice (AL). ☆: Refers to statistical difference with time of culture. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. ★: Refers to statistical difference between FL (■) and AL (□). $^{*}P < 0.05$. C: TIMP expression. 1: RT-PCR analysis. 18S mRNA levels served as internal control. 2: Reverse gelatin zymography. The position of individual TIMP and their corresponding molecular mass are indicated. 3: Determination of TIMP-2 secretion by ELISA. ■: Free floating lattice (FL); □: attached lattice (AL). ☆: Statistically different ($P < 0.05$) with time of culture. ★: Statistically different ($P < 0.05$) between FL and AL.

differentiation [Marie, 2008], and MMP-13 expression [Jimenez et al., 1999], in mice leads to a dramatic bone loss [Schiltz et al., 2010]. Emerging data indicate that osteoblast differentiation is accompanied by MMP expression, which in turn, as a feedback mechanism, can regulate cell fate and differentiation [Manduca et al., 2009; Lu et al., 2010].

In this line, use of transgenic mice bearing a collagenase-resistant COL-1 indicated that collagen hydrolysis and remodeling play pivotal function in bone homeostasis [Zhao et al., 2000; Inoue et al., 2006]. The majority of investigations with MMP-deficient mice focused on the role of MT1-MMP, MMP-2, and MMP-13 invalidation in ossification [Zhou et al., 2000; Inoue et al., 2006]. In this setting, amplified effects could be obtained by combined deletion of 2 MMPs such as MMP-13 and MMP-9 [Stickens et al., 2004]. MT1-MMP$^{-/-}$ mice exhibited several defects in skeletal development and die at a 3-week old age, thus impeding any experiment aiming at studying the contribution of this MMP in model of bone fracture repair [Zhou et al., 2000]. Nevertheless, it has been noticed that stages leading to bone regeneration display analogies with embryonic skeletal development. For instances, at early stage of development, MT1-MMP was mainly involved in the vascularization of chondroepiphyse, in keeping with major

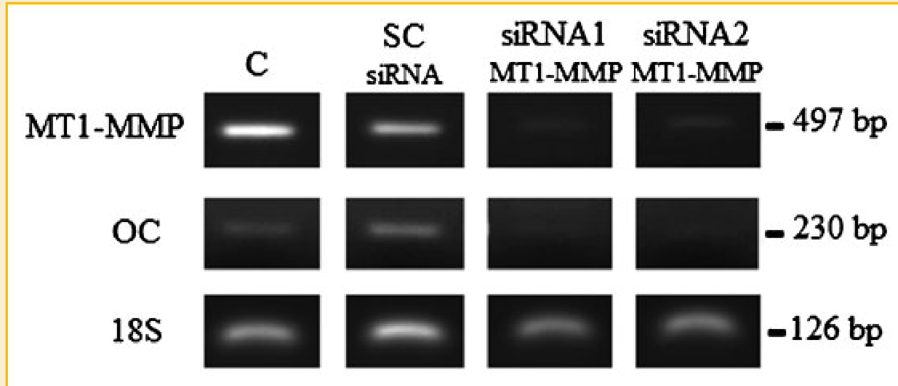

Fig. 5. Involvement of MT1-MMP in osteocalcin (OC) expression by human osteoblasts in floating type I collagen gels. Osteoblasts within lattice were transfected either by siRNAs (siRNA-1 and siRNA-2) or scrambled (SC) siRNA targeting the MT1-MMP. MT1-MMP and OC were analyzed by RT-PCR at day 4 of retraction. C, Control without cell transfection; bp, base pairs. 18S mRNA levels served as internal control.

contribution of this enzyme in angiogenesis [Robinet et al., 2005], whereas at later stage, it controlled osteoblasts fate through the organization of the osteocyte process network [Zhou et al., 2000; Karsdal et al., 2004; Holmbeck et al., 2005]. MMP-2 mice mutations led to less severe bone remodeling defects [Mosig et al., 2007] but similarly as proposed for MT1-MMP [Karsdal et al., 2004], it was mainly involved in the maintenance of osteocytes network [Inoue et al., 2006]. Recently, Lieu et al. [2011], using a tibia fracture in mice mid-diaphysis evidenced a significant delay in bone remodeling showing the critical role of this enzyme in bone repair. Conversely, MMP-13, as a potent type II collagen-degrading enzyme and articular cartilage destruction, was proposed to play a major function in preparing both cartilage and bone matrices for remodeling, therefore favoring MT1-MMP-mediated vascularization [Stickens et al., 2004; Kosaki et al., 2007]. As a whole, and in keeping with several in vitro data including ours, in vivo models using transgenic mice highlight the combined action of MMP-2 and MT1-MMP at late stage of osteoblast differentiation.

To deeper understand the role of MMP in the differentiation of osteoblasts and the contribution of mechanical forces, human bone marrow-derived osteoprogenitors cells were cultured in 3D type I collagen microenvironment under either attached or retracted (floating) conditions. Similarly as observed in woven bone within areas of fracture healing, collagen fibrils within retracted lattice are arranged in many directions, and collagen remodeling can be evidenced in the pericellular space. Several factors influenced the retraction of osteoblast-populated FL such as serum [Buttle and Ehrlich, 1983]. The relationships between differentiation markers and MMP expression and activation, was studied at 1–4 days of culture, a period where cell proliferation and/or apoptosis was not

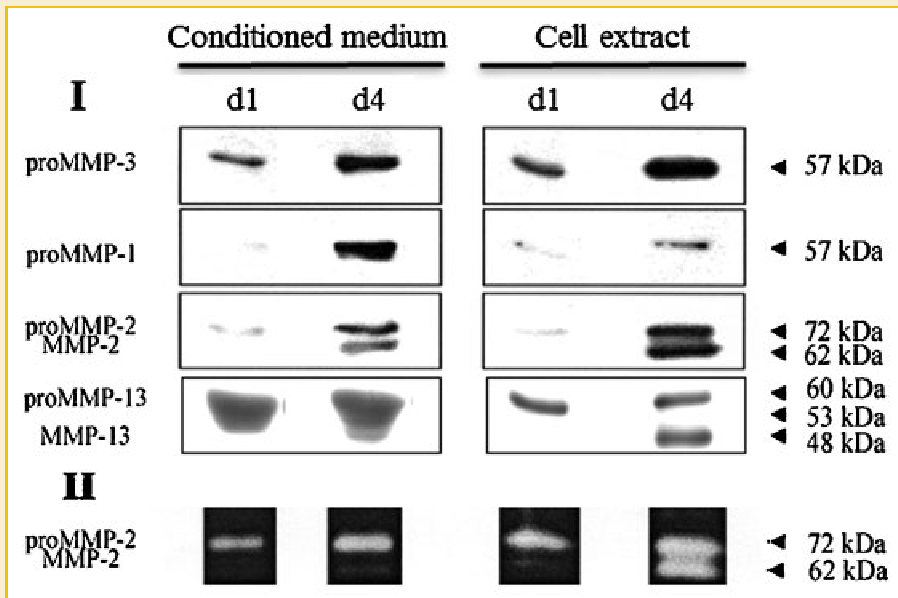

Fig. 6. Triggering of MMP activation cascades in human osteoblast-populated retracted type I collagen gels. d1, day 1; d4, day 4. I: Western blot analyses: the positions of latent and active forms of individual enzymes are indicated by arrows. II: Gelatin zymography: latent (72 kDa) and fully active (62 kDa) MMP-2 are identified.

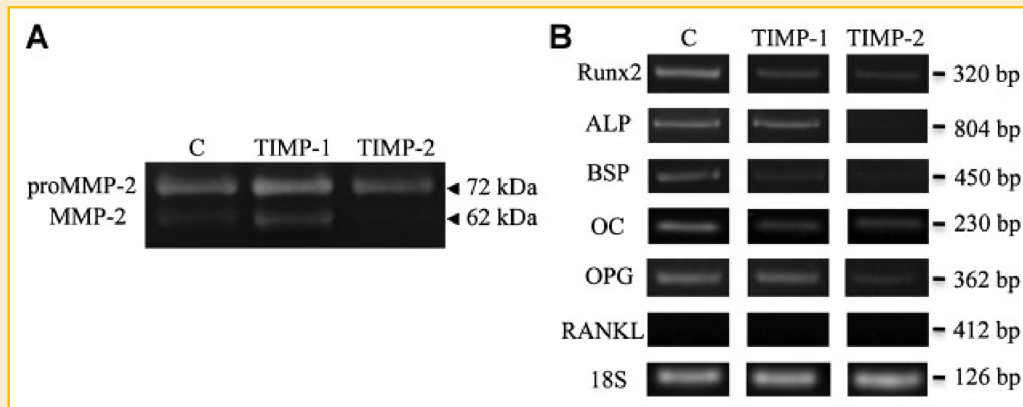

Fig. 7. Influence of the over-supplementation of TIMP-1 (3 μg/ml) and TIMP-2 (3 μg/ml) on the expression of differentiation markers by human osteoblasts in floating 3D collagen gels. C, Cell control without TIMP supplementation. A: Effect of TIMP supplementation on proMMP-2 activation by gelatin zymography. B: Effect of TIMP supplementation on the expression of several markers of osteoblast differentiation. 18S mRNA levels served as internal control. bp, base pairs. Experiment has been repeated with two different donors.

observed. Also experiments were performed at the lowest concentration of serum, that is, 2.5% to exclude any major interference of serum proteinases or inhibitors in obtained data.

In our experimental conditions, osteoblasts in 3D FL expressed higher level of Runx-2 transcriptional factor as well as ALP, BSP, OC, and OPG compared to cells in attached lattices. Intense ALP mRNA expression was observed as early as following 1 day of culture in retracted lattice. Although post-transcriptional regulation was described to be involved in the control of ALP activity in osteoblastic cells, variations in enzyme activity matched those in mRNA levels whatever cell culture conditions. Runx-2 mRNA expression was similarly up-regulated in retracted lattices and in keeping with the presence of binding sites for this transcription factor in several genes implicated in osteoblast differentiation, BSP and OC were overexpressed in parallel. Strikingly, BSP can reciprocally display a direct influence on osteoblast differentiation by increasing Runx-2 activity [Gordon et al., 2007]. OC was expressed maximally at day 4 of contraction in agreement with a later stage of differentiation before the onset of mineralization [Akhouayri et al., 1999; D'alonzo et al., 2002].

OPG and RANKL expressions have been linked to osteoclastogenesis. Indeed, the number of osteoclast and their activation were directly related to the level of OPG/RANKL ratio [Boyce and Xing, 2008]. Of note, RANKL expression was 2-log of magnitude lower than OPG in attached or retracted lattices, and in both systems, OPG/RANKL increased with days of culture, thus increasing osteoblast differentiation. X-ray microanalysis allowed us to evidence the formation of nodules in retracted but not in attached lattices at day 7 of culture corresponding to an average 30% of the gel. As also observed with osteosarcoma cell lines in a similar model [Akhouayri et al., 1999], deposition of minerals composed of calcium and phosphorus necessitates longer, at least 14 days of culture; also extent of mineralization can be enhanced by supplementing culture medium with betaglycerophosphate not used in our investigation.

The expression of neutral proteinases involved in two proteolytic cascades, that is: uPA/MMP-3/MMP-1; MT1-MMP/MMP-2/MMP-13 involved in collagen remodeling was analyzed in parallel. Expression of urokinase, an enzyme whose up-regulation is often associated with high healing capacity, was not or only weakly expressed by human bone marrow osteoblast-like cells whatever our experimental conditions, and contraction of the lattices had no influence, as compared to gel maintained under tension, on MMP-3 and MMP-2 production. Levels of MMP-1 mRNA and protein, whose expression is regulated by $\alpha_2\beta_1$-collagen interaction [Riikonen et al., 1995] in retracted lattices were only increased 1.8-fold at day 4 of culture. In turn, a significant enhancement of MMP-13 expression both at the mRNA and protein level was evidenced at later stage of culture. However, level of secreted enzyme never exceeded 4 ng/10^5 osteoblasts, an amount 50-fold lower, on average, than that of other MMPs produced by lattices. Expression of this enzyme is widely considered as a marker of the osteoblast phenotype. Indeed, Tuckerman et al. [2000] have shown that ALP, OC, and MMP-13 were expressed exclusively by cells involved in endochondrial or membranous ossification. However, such low amount of recovered extracellular MMP-13 might be linked to the high rate of endocytosis of this enzyme. Indeed, binding of MMP-13 to a high affinity 170 kDa receptor on osteosarcoma cells led together with the participation of low-density lipoprotein-related receptor to its rapid elimination from culture medium [Barmina et al., 1999].

MT1-MMP, whose expression was mainly up-regulated in retracted lattice, that is, 4.5-fold at day 4, has been considered as a master gene in driving osteoblast differentiation. Level of its expression was found to change with cell maturation from no signal in bone marrow cells to a lower one in periosteal cells and strong expression in osteoblasts. To that line, bone marrow osteogenic cells derived from MT1-MMP-deficient mice present severe deficient osteogenic activity and inhibiting its activation in rat preosteoblasts using a furin convertase inhibitor, led to partial suppression of ALP expression [Manduca et al., 2009]. Thus, we hypothesized that MT1-MMP was mainly driving the osteogenic differentiation during the contraction of osteoblast-populated FL, and expression of this enzyme was annihilated by a siRNA approach. MT1-MMP silencing, which does not elicit compensatory changes in MMP-2 and MMP-13 expression, was found to almost totally suppress OC expression.

Although other investigations have shown that MT1-MMP can directly trigger cell signaling and influence cell apoptosis, either directly or indirectly through TGFβ activation [Karsdal et al., 2002], neither proliferation nor apoptosis were affected by MT1-MMP silencing, at least within 4 days of lattice cultures.

Although, such data indicated that MT1-MMP was an active partner in the modulation of osteoblast differentiation, they do not ascertain whether this enzyme was acting directly or indirectly through activation of proMMP-2 and proMMP-13. Activity of those enzymes as well as other MMPs are controlled by TIMPs; expression of either TIMP-1 or TIMP-2 was unaffected by lattice retraction, and level of secreted MMPs, except MMP-13, largely exceeded those of their natural occurring inhibitors. Low level of uPA expression and generated plasmin impeded the formation of the MMP-3/MMP-1 activation cascade as revealed by Western blotting. On the contrary, high level of MT1-MMP expression together with low TIMP-2 production triggered MMP-2 as well as MMP-13 activation. The level of active MMP-2, an enzyme broadly expressed in the fracture callus, might be further exacerbated by two different mechanisms involving osteoblastic differentiation markers: either by ALP, able to dephosphorylate MMP-2 [Sariahmetoglu et al., 2007] and to increase its activity, or by BSP which was described to induce limited gelatinase activity in latent MMP-2 [Karadag and Fisher, 2006], and also to restore the activity of TIMP-2–MMP-2 complexes [Teti et al., 1998]. Thus, either MMP-13 or most probably MMP-2 could participate in the modulation of osteoblast differentiation. In this investigation, MMP-2 silencing by siRNA led to unspecific responses and to delineate the direct participation of either MT1-MMP or MMP-2 (and MMP-13) in the modulation of expression of osteoblastic markers, we supplied cultures with excess TIMP-1 or TIMP-2. Indeed, TIMP-1 can inhibit MMP-2 and MMP-13 while having no inhibitory capacity against MT1-MMP, whereas TIMP-2 was able to suppress indifferently the activity of the three collagenases. Analysis of data revealed that MT1-MMP was mainly involved in ALP up-regulation, thus confirming data obtained recently using rat preosteoblasts [Manduca et al., 2009], and human mesenchymal stem cells from bone marrow [Lu et al., 2010]. In turn, MMP-2 whose activity can be increased by BSP, is mainly implicated in the overexpression of this marker, thus creating an amplification loop of osteoblast differentiation [Jain et al., 2008]. Runx-2 expression seems to involve the participation of all collagenases in collagen retracted lattices.

Although it is well known that compared to in vivo models, in vitro studies present severe limitations such as absence of inflammation and vascularization. Altogether, data presented suggested that MT1-MMP might be an important element in facilitating fracture healing of human bone. This endopeptidase might display pleiotropic effects such as: (i) revealing hidden cues in collagen microenvironment; (ii) activating growth factor as proTGFβ; (iii) cleaving transglutaminase-2 [Nakano et al., 2010] and enhancing its ATPase activity.

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