Journal of Cellular Biochemistry 104:1803–1817 (2008)

Inhibition of Osteoblastic Metalloproteinases in Mice Prevents Bone Loss Induced by Oestrogen Deficiency

Corinne Schiltz,[1,2] Caroline Marty,[1,2] Marie-Christine de Vernejoul,[1,2] and Valerie Geoffroy[1,2]*

[1]INSERM U606, Hôpital Lariboisière, Paris, France
[2]University René Diderot Paris 7, Paris, France

Abstract Matrix metalloproteinases (MMPs) are key mediators in extra-cellular matrix remodelling and implicated primarily in bone growth, and particularly in osteoclastic bone resorption. We hypothesise that MMPs have a role in the increased bone remodelling resulting from oestrogen deficiency. Transgenic (TG) mice overexpressing TIMP-1 in their osteoblastic cells and their wild-type (WT) littermates were ovariectomised. One month after surgery, bone mineral density (BMD) and bone microarchitecture were assessed. Primary cells from WT and TG mice were used to determine how TIMP-1 affects osteoclast and osteoblastic cells. The reduction of BMD induced by ovariectomy in WT mice was not observed in the transgenic mice. The transgene overexpression also dampened the post-ovariectomy increase in bone resorption in contrast to the WT mice. In vivo, osteoclastic surfaces and D-pyridinoline were not increased in TG mice, and ex vivo, the differentiation of osteoclasts from TG bone marrow precursor cells were unaffected by in vivo oestrogen deficiency or treatment. We showed also that TIMP-1 overexpression reduces and delays the osteoblastic proliferation and differentiation respectively, and reduced the generation of the active form of TGFβ1 in the supernatant of TG osteoblasts. Our findings support the hypothesis that in vivo inhibition of osteoblastic MMPs prevented the bone loss induced by oestrogen deficiency, with a significant decrease in bone resorption. This effect was presumably resulting from (1) a direct inhibition of osteoclastic resorption activity by the TIMP-1 and (2) the modification in the local activation of extra-cellular signalling factors such as TGFβ1 and the OPG/RANKL ratio. J. Cell. Biochem. 104: 1803–1817, 2008.

Key words: matrix metalloproteinases; osteoporosis; TIMP-1; transgenic mice; oestrogen; TGFβ1

Osteoporosis is a systemic disorder of bone tissues, defined as a generalised and significant reduction of bone mass, and deterioration of the microarchitecture of bone tissue, leading to greater bone brittleness, and susceptibility to fractures. Maintenance of skeletal mass depends on remodelling, a tightly coupled process involving bone formation and bone resorption. Estrogens deficiency plays a key role in the occurrence of osteoporosis in women. Menopause induces an increase in the remodelling rate of bone tissue, leading to an imbalance between the resorption and formation of bone, resulting in accelerated bone loss [Riggs et al., 2002]. In women, post-menopausal bone changes are only detected after several years [Kanis, 2002]. Therefore the ovariectomised mouse is considered as a classical model to use to study the acute effect of estrogens withdrawal [Raisz, 2005; Weitzmann and Pacifici, 2006]. It has led to considerable progress towards deciphering the molecular mechanism of the action of estradiol on bone. In this model,

Abbreviations used: ALP, alkaline phosphatase; BMD, bone mineral density; DPD, deoxypyridinoline; DXA, dual-energy X-ray absorptiometry; E2, 17beta-estradiol; OCL, osteoclast-like cells; OVX, ovariectomy; MMP, matrix metalloproteinase; PBS, phosphate buffer saline; qPCR, quantitative polymerase chain reaction; RT, reverse transcriptase; Sham, sham-operated; TG, transgenic; TIMP-1, tissue inhibitor of metalloproteinases type 1; WT, wild-type.

Grant sponsor: "Rhumatisme et Travail" Association; Grant sponsor: Institut National de la Santé et de la Recherche Médicale; Grant sponsor: Université René Diderot; Grant sponsor: EC 6th Framework Program (ANABONOS Network Program; Priority Thematic Area); Grant number: LSH-2002-2.1.4-3.

*Correspondence to: Valerie Geoffroy, INSERM U606, Hôpital Lariboisière, 2, rue Ambroise Paré, 75475 Paris Cedex 10, France. E-mail: valerie.geoffroy@larib.inserm.fr

Received 10 January 2008; Accepted 1 February 2008

DOI 10.1002/jcb.21747

several cytokines have been shown to be responsible for the increased bone resorption relative to bone loss following ovariectomy in mice [Weitzmann and Pacifici, 2006].

The matrix metalloproteinases (MMPs) constitute a family of secreted and cell-surface enzymes that process and degrade numerous pericellular substrates [for review see Sternlicht and Werb, 2001]. Their activities are regulated by specific inhibitors the tissue inhibitors of metalloproteinases (TIMPs) that contribute to controlling the local activities of MMPs in tissues. The ability to degrade or process extra-cellular proteins is essential for proper cell–cell and cell–matrix interaction. In bone tissue, the renewal of the extra-cellular matrix is a critical aspect of the process of bone matrix remodelling and maintenance. Both osteoblasts [Otsuka et al., 1984; Meikle et al., 1992; Koh et al., 2005; Varghese, 2006] and osteoclasts [Hill et al., 1994; Delaisse et al., 2000; Andersen et al., 2004; Hou et al., 2004] synthesise MMPs, and MMPs are known to play various significant roles in bone biology, notably during bone development [Delaisse et al., 2000; Stickens et al., 2004; Malemud, 2006]. Genetic human disease and knockout-mice have made clear the importance of MMP-2, -9, -13 and -14 in endochondral bone growth [Sternlicht and Werb, 2001; Stickens et al., 2004; Holmbeck et al., 2004; VanSaun and Matrisian, 2006]. The findings of other in vivo studies provide supporting evidence that osteoblastic MMPs play a role in adult bone remodelling: conditional inactivation of MMP-13, specifically in osteoblasts, causes increased trabecular bone that persists for several months [Stickens et al., 2004]. Furthermore, adult mice that expressed collagen that is resistant to MMP cleavage, exhibit a mild bone phenotype, with increased bone deposition [Zhao et al., 2000].

A potential role of MMPs in the pathological destruction of cartilage and bone matrix in inflammatory diseases, such as rheumatoid arthritis and osteoarthritis, has been the focus of much investigation for several decades [Burrage et al., 2006]. In contrast, the specific role of MMPs in the bone loss associated with estrogens deprivation has not been fully investigated [Williams et al., 1999].

We therefore decided to test the ability of the metalloproteinase inhibitor TIMP-1 to reduce the bone loss induced by ovariectomy in mice. To do this, we used an in vivo model in which the expression of TIMP-1 is driven by the collagen alpha-1 type-I mouse promoter in osteoblasts. We used histomorphometric and molecular analysis on long bones and vertebrae to provide evidence of the role of TIMP-1 in trabecular bone remodelling. We reported that TIMP-1 overexpressing females until 2.5 months of age displayed low bone turnover combined with a positive bone balance, that lead to an increase in the volume and density of trabecular bone [Geoffroy et al., 2004]. That study demonstrated that osteoblastic MMPs are implicated in the bone resorption in vivo, and suggested sex hormone regulation of resorption mediated by osteoblastic MMPs. Our data further suggested that TIMP-1 overexpression in osteoblasts affects bone volume and bone density only in situation of high bone turnover and rapid activation of bone resorption, that is, young growing females or adult females treated with parathyroid hormone [Geoffroy et al., 2004; Merciris et al., 2007]. In support of our hypothesis that osteoblastic MMPs could be crucial in situations of high bone remodelling, we show that (1) overexpression of TIMP-1 in osteoblasts prevents post-ovariectomy trabecular bone loss, (2) in the tissues, the mechanism consisted mainly of decreased osteoclastic resorption and (3) the regulation of the bioavailability of molecules such as TGFβ1 by MMPs that affects osteoblastic and osteoclastic functions may be a key mechanism in these effects.

MATERIALS AND METHODS

Animals

Four-month-old transgenic female mice with established overexpression of TIMP-1 and their wild-type littermates (10th and 11th generations) were used for the experiments [Geoffroy et al., 2004]. Wild-type and transgenic mice from the same litters were housed 8 per cage, under a 12/12 h light dark cycle at 21°C and allowed free access to water and chow from the beginning of the experiment in full compliance with the French government animal welfare policy. Animals were then subdivided randomly into six groups, and either sham-operated (ovaries were exteriorised, but not removed; SHAM), ovariectomised (OVX), or ovariectomised (bilateral ovariectomy), and given subcutaneous estrogen replacement therapy (15 μg 17beta-estradiol/kg body weight/day, 5 days

a week), (OVX + E2; Sigma, St. Louis, MO). After surgery, the mice were housed in individual cages, and provided with 3 g of chow per day to prevent weight gain. Dosing was started 1 day after surgery, and continued until sacrifice. Body weights were measured weekly, and the E2 injections were adjusted accordingly. Mice were killed by cervical dislocation under anaesthesia 2 months (first set of experiment) or 1 month (second set of experiment) after surgery. In the second set of experiment, the mice were subcutaneously injected with two fluorochrome labels in order to monitor dynamic bone parameters. Oxytetracycline (20 mg/kg; Pfizer, Amboise, France) and 10 mg/kg of calcein (Sigma) were administrated by *ip* injection 7 and 2 days before necropsy, respectively. Immediately after sacrifice, the uteri were removed and their weights were measured. Mice form the second set of experiment, were evaluated for densitometry at the femur and the caudal vertebrae and histomorphometry at the lumbar vertebrae. Long bones were harvested for both primary culture of the precursor cells present in the bone marrow and evaluation of genes expression in the whole bone.

Dual-Energy X-Ray Absorptiometry (DXA)

DXA measurement was performed using a PIXImus instrument (version 1.44; Lunar) in ultrahigh resolution mode (resolution, 0.18 mm × 8.18 mm). The precision and reproducibility of the instruments had previously been checked. The coefficient of variation was <2% for all the parameters evaluated. The machine was calibrated daily using a phantom provided by the manufacturer.

In the first set of experiment, a longitudinal evaluation of the bone mineral density (BMD; mg/cm^2) was realised at the femora and the caudal vertebral at the beginning of the experiment, and 30 and 60 days after surgery. In the second set of experiment, BMD was evaluated before and 1 month after surgery before sacrifice.

DPD Measurement

Accurate deoxypyridinoline measurement requires overnight fasting before collecting the urine. DPD was measured with the IMMULITE Pyrilinks-D in vitro diagnostic reagent. To correct for flow variations, DPD results were normalised to the urinary creatinine concentration (ADVIA System).

Histomorphometric Analysis of Bone

The L4-L6 lumbar vertebrae were excised at the sacrifice, and the surrounding soft tissue was cleaned off. Undecalcified specimens were fixed in 70% ethanol, and dehydrated in xylene at 4°C before being embedded in methyl methacrylate. Sections (5 μm) were cut with a Leica microtome (SM2500S), mounted, and then stained with either naphthol ASTR phosphate and counterstained with toluidine blue (for TRAP activity detection), or with aniline blue (for the static parameters). Thick sections (12 μm) were also used to measure the formation dynamics parameters. Histomorphometry was conducted to quantify bone parameters as defined by Parfitt et al. [1987]. The trabecular bone volume (BV/TV), the trabecular number (Tr.Nb), the trabecular separation (Tr.Sp) and the trabecular thickness (Tb.Th) were measured using a software package developed for bone histomorphometry (Morphométrie osseuse; Biocom, Les Ulis, France). The surface area and number of osteoclasts were determined on TRAP-stained sections using a Leitz integrateplatte II eyepiece at 128× magnification. The dynamic parameters were measured in 12-μm-thick, unstained sections that were examined under UV light. The mineral apposition rate (MAR, mean inter-label thickness divided by the time between the two labelling periods, i.e., 4 days for 6-month-old mice) was measured using the same image analyser with a semi-automatic method. The mineralising surfaces were measured in the same area using the Leitz integrateplatte II eyepiece. The bone formation rate (BFR) was calculated in accordance with the ASBMR nomenclature [Parfitt et al., 1987].

Culture of Primary Osteoblast Cells Isolated From Calvaria

Primary osteoblasts were isolated from new born wild-type and transgenic mouse calvaria (2- to 4-day old) after sequential digestion with type IV collagenase (Sigma). Cells were plated in T-75 flasks containing phenol red free alpha-MEM 10% FBS, 2 mM glutamine, 100 U/ml of penicillin and 100 mg/ml streptomycin.

At confluence, cells were harvested with trypsine digestion and seeded in 6-well plates (10^5 cells per well with 2 ml of medium) and in 100 mm culture dishes (10^6 cells per dish), cultured in the same medium supplemented with 50 μM ascorbic acid and 10 mM sodium

beta Glycerophosphate. As indicated in the Results Section, cells were cultured with or without 17β-estradiol (10^{-8}M), or with or without mouse recombinant TIMP-1 (rTIMP-1). Cultured cells were supplied with fresh medium every 3 days, and all cultures were maintained at 37°C in 5% CO_2 and 95% humidity.

To determine the proliferation of the osteoblastic cells, cells were cultured in 96-well-plates (5,000 cells/well) for 2 days in the presence of 50-μM ascorbic acid. Proliferation was determined during the treatment period. Bromodeoxyuridine (BrdU) incorporation assay was performed using the colorimetric BrdUrd cell proliferation kit (Amersham, Orsay, France).

For alkaline phosphatase activity (ALP) measurement, cell layers were washed twice with ice-cold phosphate buffer saline (PBS), 200 μl of deionised water added, and 6-well plates were stored at −20°C until analysis. Cells in deionised water were then scraped off, sonicated and centrifuged at 4°C for 15 min at 4,000*g*. ALP activity was evaluated using ADVIAr1650 (Bayer Diagnostics). Protein concentrations were determined by the BCA method (Pierce). Results were expressed in International Units, and normalised to protein content (IU/mg of protein).

For the TGFβ1 assay, primary osteoblast cell supernatants were collected, concentrated on centrifugal filter devices (Centricon, Millipore) and stored at −80°C until analysis for TGFβ1 by ELISA (R & D Systems). To determine the total (active + latent) TGFβ1 levels, the samples were acidified with 1 N HCl, and then reneutralised using NaOH before adding to the assay plate. Latent TGFβ1 values were determined by subtracting the active TGFβ1 measurements from the total TGFβ1.

For immunolabelling, the cells were seeded in 6-well plates on a cover slip (300,000 cells/well). At day 6, cells were rinsed with cold PBS, fixed with 4% paraformaldehyde, permeabilised with methanol at −20°C for 15 min. After washing and BSA saturation, cells were incubated for 3 h at room temperature with appropriate dilutions of rabbit polyclonal antibodies against anti-ER-α (1/50; Santa Cruz Biotechnology, Inc.). Incubation with BSA in PBS replaced primary antibodies, and served as negative controls. The cover slips were rinsed in PBS and incubated for 1 h at room temperature with CY-3-conjugated donkey anti-rabbit secondary antibodies (1/400 final dilution; The Jackson Laboratory, West Grove, PA). After rinsing, samples were mounted in fluorescent mounting medium (Dako, Montreal, Denmark), and observed by epi-fluorescence microscopy on a Zeiss Photomicroscope III (Nikon, Japan). For photography, equal exposure times were used for labelled cultures and control cultures.

Culture of Osteoblastic Precursor Cells Isolated From Bone Marrow

Marrow stromal cells were collected from the femurs and tibiae of 5-month-old female mice 1 month after ovariectomy. Bone marrow was flushed out from each bone with alpha-MEM supplemented with FCS (15%) using a syringe. After a short spin, the cell pellets were resuspended in the same culture medium and were further filtered through a 0.45-μm-pore diameter nylon filter (Millipore, Molsheim, France).

Cells were seeded in 6-well plates (105 cells per well with 2 ml of medium) and cultured in the differentiation medium (alpha-MEM supplemented with FCS (15%), 180 μM ascorbic acid and 10 mM sodium beta Glycerophosphate). For Western blotting experiments, 12 h before collecting of the supernatant, the differentiation medium was replaced by serum-free medium containing the inducers.

Osteoclast Differentiation Assay

Bone marrow cells were isolated from 5-month-old female wild-type or transgenic mice, 1 month after sham-operation, ovariectomy or ovariectomy treated subcutaneously with oestrogen (as described above). Briefly, mice tibia and femurs were removed aseptically, dissected free of adherent tissues, and placed in PBS. The marrow cavities were flushed with phenol-red free alpha-MEM containing 10% FBS, and collected marrow cells were washed with the same medium, and seeded in 8-well chamber slides at 2×10^6 cells per ml (8 wells Lab-Tek, Brand Products, Nunc). Cells were cultured for 21 days with RANKL (12 ng/well) and M-CSF (10 ng/well) to induce osteoclast differentiation. Cells were then washed once with PBS before being fixed with PFA 4% for 20 min, rinsed three times with PBS, and stained for TRAP activity. For the microscopic examination, the nuclei were counter stained with methyl green. All TRAP-positive cells were counted and the TRAP-positive multinucleated cells containing

three nuclei or more were counted as osteoclast-like cells.

RT-PCR and RT-QPCR Analysis

The RT-PCR analysis of gene expression was performed as previously described [Geoffroy et al., 2004]. Briefly, total RNAs from the long bones were isolated using trizol reagent (Invitrogen), cleaned using an RNAeasy mini-kit (Qiagen) according to the manufacturer's instructions and the RT reaction was performed using the Reverse-ITmax RTase Blend® (ABgene). PCR amplification was performed using standard protocols in a GeneAmp PCR 2700 (Applied Biosystem). HPRT was used as internal control. For the PCR amplification, the following set of primers were used: Transgene (forward: 5′-ATCTCTGGCATCTGGCATCC-3′ and reverse: 5′-TTTCAAGATGTGGCGAGATGC-3′); TIMP-1 (forward: 5′-ATCTCTGGCATCTGGCATCC-3′ and reverse: 5′-GGCAGGCAAGCAAAGTGACG-3′); HPRT (forward: 5′-AGCGATGATGAACCAGGTTA-3′ and reverse: 5′-GTTGAGAGATCATCTCCACC-3′). The conditions of amplification were optimised for each pair of primers, and were as follows: 1 min at 94°C, 1 min at 52°C (HPRT), 57°C (transgene), or 60°C (TIMP-1), and 1 min at 72°C. 30 amplification cycles were used, except for the transgene (35 cycles).

The real-time PCR analysis of gene expression was performed as previously described [Merciris et al., 2007]. Total RNA was isolated from a primary osteoblastic cell lysate using NucleoSpin® (Macherey-Nagel) according to the manufacturer's instructions and the RT reaction was performed using the Reverse-ITmax RTase Blend® (ABgene). Quantitative real-time PCR expression analysis was performed using a light cycler Roche, ABsolute SYBR® Green Capillary Mix (ABgene). 18S was used for normalisation. The sets of primers used for real-time PCR are: 18S (forward: 5′-CGGCTACCACATCCAAGGAA-3′; reverse: 5′-GCTGGAATTACCGCGGCT-3′), RANKL (forward: 5′-GGCCACAGCGCTTCTCAG-3′; reverse: 5′-GAGTGAC TTTATGGGAACCCGAT-3′), OPG (forward: 5′-AGTCCG- TGAAGCAGGAGTG-3′; reverse: 5′-CCATCTGGACATTTTTTGCAAA-3′).

Western Blotting

For Western blotting experiments, supernatant of osteoblastic cells deriving from bone marrow progenitors were used (see above). Serum-free supernatants of osteoblasts were harvested after 5 days of culture, and concentrated using microconcentrator (microcon, Millipore). Proteins were quantified with BCA protein assay. Ten or 30 μg of proteins in 1X Laemli buffer were denatured for 5 min at 95°C, and separated on SDS–PAGE under denaturing conditions before being transferred onto a PVDF membrane (Hybond-P; Amersham). The studies used two different antibodies raised against MMP-13 (rabbit polyclonal antibody, Chemicon International) and MMP-3 (Ab-1; mouse monoclonal antibody, Oncogene), which were incubated overnight at 4°C in 5% blocking solution at 1:2,500 and 1:100 dilutions respectively. The blots were then incubated with horseradish peroxidase-conjugated secondary antibodies (dilution 1:1,000), washed and incubated in chemoluminescence luminol reagent. Biomax MR films were used to visualise the chemoluminescence.

Statistical Analysis

Statistical analysis was performed using the StatView 4.5 software. Statistical differences between experimental groups were assessed using analysis of variance (ANOVA). The significance threshold was set at $P < 0.05$. All values are shown as mean ± SEM.

RESULTS

Overexpression of TIMP-1 in Osteoblastic Cells Prevents Ovariectomy (OVX)-Induced Bone Loss

In our previous publications describing TIMP-1-overexpressing mice, we reported a phenotype only in females and no significant changes in BMD after 2.5 months of age [Geoffroy et al., 2004]. In the present study, we used 4-month-old transgenic female mice. At baseline, transgenic mice displayed the same BMD than wild-type mice at the femur and the caudal vertebrae (Fig. 1).

We subject the 4-month-old wild-type and transgenic mice to ovariectomy (OVX). The effectiveness of ovariectomy was confirmed at necroscopy by severe atrophy of the uterus, as compared to the Sham control animals. The uteruses were weighed at the end of the experiments. The reductions in uterus weight after OVX were similar in wild-type and transgenic mice and oestrogen treatment (15 μg/kg/day) after ovariectomy restored uterus weight to a

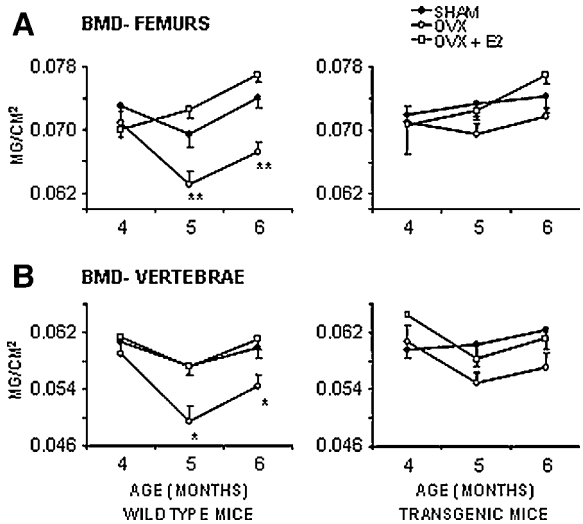

Fig. 1. Reduced effects of ovariectomy on bone in transgenic mice overexpressing TIMP-1. Four-month-old wild-type and transgenic TIMP-1 overexpressing female mice were either sham-operated (SHAM, black circle), ovariectomised (OVX, open circle) and treated with 10^{-8}M 17beta-estradiol (OVX + E2, open square). In a first set of experiment, the longitudinal evaluation of the bone mineral density (BMD) was realised by DXA. BMD was measured before, 1 and 2 months after surgery at two representative sites, the femur (**A**) and the caudal vertebrae (**B**). (n = 7–8 in the SHAM groups, n = 8–10 in OVX groups, n = 4–5 in the OVX + E2 groups.) $^{*}P < 0.05$ and $^{**}P < 0.005$ versus SHAM.

level comparable to that observed in sham-operated mice (data not shown). As expected we observed 1 and 2 months after surgery a significant decrease in BMD in wild-type mice at the femurs and the vertebrae (Fig. 1). In contrast, ovariectomy had no significant effect on BMD at the femur and the vertebrae in transgenic mice at either 1 or 2 months after surgery. Wild-type and transgenic OVX mice treated with 17-beta-oestradiol (E2) were not different from the Sham mice at any site or any time. For the rest of study, we decided to analyse the effect of transgene overexpression 1 month after surgery, when the bone loss induced by OVX is most pronounced.

One Month After Ovariectomy, Overexpression of TIMP-1 in Osteoblastic Cells Prevents Ovariectomy-Induced Trabecular Bone Loss and Increases in Bone Resorption Markers

To evaluate more precisely the effect of TIMP-1 overexpression on bone density and architecture, a new set of 4-month-old female mice were subjected to ovariectomy. One month after ovariectomy, wild-type mice had lost 9% and 14.5% of their initial BMD at the femur and the caudal vertebrae respectively (Fig. 2A,B). At that time, the transgenic OVX mice had no significant bone loss. Histological analysis confirmed the less severe phenotype in transgenic compared to wild-type mice (Fig. 2C). As expected, we showed that urinary deoxypyridinoline, a marker of bone resorption, was increased after ovariectomy only in wild-type mice (Fig. 2D).

Quantitative bone histomorphometric analysis of the vertebrae 1 month after surgery revealed greater trabecular bone volume (BV/TV) in Sham transgenic mice than in wild-type mice. This was associated with reduced trabecular spacing (Tb.Sp), suggesting decreased resorption in the transgenic mice as previously described in younger mice (Table I) [Geoffroy et al., 2004]. Before ovariectomy, BV/TV, trabecular number (Tr.N) and trabecular thickness (Tr.Th) all fell in wild-type mice, whereas Tb.Sp increased. These changes were abolished by E2 treatment (OVX + E2) in the wild-type mice. The changes in structural parameters induced by ovariectomy in wild-type mice did not occurred in the transgenic mice. These findings indicate that transgenic mice were less affected by estrogens deficiency than wild-type mice, thus demonstrating that osteoblastic MMPs are important in the response of bone cells to estrogens.

The Oestrogen Status Does Not Affect Osteoblastic Expression of the Transgene and MMP-3 and -13

PCR analysis performed on RNA isolated on wild-type and transgenic long bones, indicated that the transgene and the total TIMP-1 expression (endogenous TIMP-1 plus transgene) was not affected by oestrogen status 1 month after ovariectomy (Fig. 3A). Furthermore, we showed using Western blot analysis that ovariectomy has no effect on the expression of MMP-13 but increases MMP-3 expression in osteoblastic cells of both genotypes (Fig. 3B). The expression of MMP-13 and MMP-3 were the same in wild-type and transgenic cells supernatant indicating that modification in bone volume and structure observed after ovariectomy in wild-type mice cannot be explained by modifications of the TIMP-1 or MMP-13 and -3 expression. But an differential effect of the MMPs activity in these modifications cannot be completely excluded.

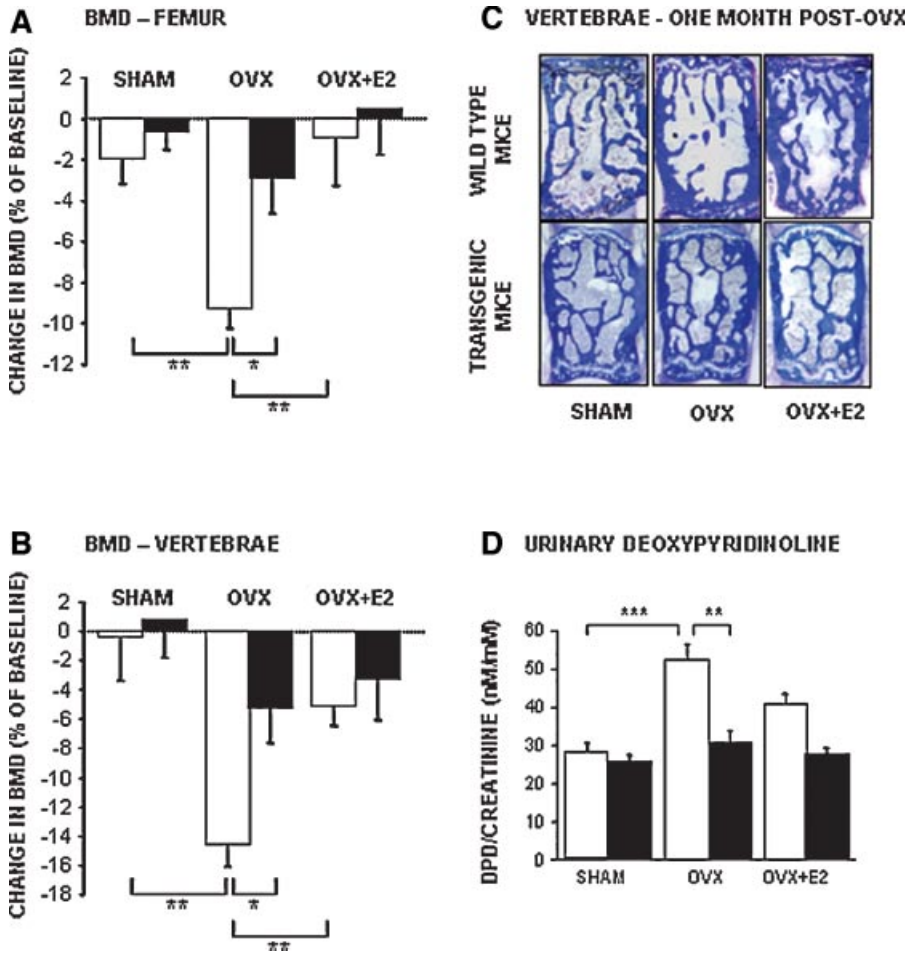

Fig. 2. Overexpression of TIMP-1 in osteoblasts prevents the ovariectomy-induced bone loss evaluated 1 month after surgery. **A**,**B**: In a second set of experiment, BMD of the whole femora and lumbar vertebrae of wild-type (open bars) and transgenic (black bars) sham-operated (SHAM), ovariectomised (OVX) and ovariectomised treated (OVX + E2) mice was measured before and 1 month after surgery using DXA. Data are represented as the percent of change from the baseline. (n = 7 in the SHAM groups, n = 7–10 in OVX groups, n = 5 in the OVX + E2 groups.) $^{*}P < 0.05$; $^{**}P < 0.005$. **C**: Toluidine blue stain of histological sections of lumbar vertebrae 4 weeks after ovariectomy shows that ovariectomy induced a deterioration of the microarchitecture of trabecular bone in the wild-type but not in the transgenic vertebrae. **D**: Urinary deoxypyridinoline (DPD) levels were determined 1 month after surgery in all groups. Data were normalised to the urinary creatinine concentration. $^{**}P < 0.005$ and $^{***}P < 0.0005$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Bone Formation and Osteoblasts Are Differently Affected by the Oestrogen Status in Wild-Type and Transgenic Mice In Vivo and Ex Vivo

We examine the alkaline phosphatase activity as marker of osteoblastic differentiation in differentiating primary osteoblast isolated from wild-type and transgenic new born calvaria. We confirmed first that after 15 days of culture, the alkaline phosphatase activity was the same in both genotypes as previously reported [Geoffroy et al., 2004]. But we showed that alkaline phosphatase activity was lower in transgenic than in wild-type osteoblasts from days 3 to 6 of culture (Fig. 4A). A reduced alkaline phosphatase activity was also observed after 3 days when wild-type primary osteoblasts were treated with recombinant TIMP-1 (Fig. 4B). Addition of 17beta-estradiol for a 3-day period enhanced alkaline phosphatase activity in the wild-type primary cells but not in the transgenic cells (Fig. 4C) indicating that TIMP-1 overexpression presumably affects osteoblastic differentiation signalling. Moreover, we showed that after 3 days in culture, transgenic osteoblasts had also a lower rate of proliferation than wild-type osteoblasts (Fig. 4D) and adding recombinant TIMP-1 to wild-type osteoblasts

TABLE I. Cancellous Bone Histomorphometry of 6-Month-Old Wild-Type and Transgenic, Sham and Ovariectomised Mice

	Wild-type mice			Transgenic mice		
Parameters[a]	SHAM	OVX	OVX + E2	SHAM	OVX	OVX + E2
Bone structure						
Bone volume/tissue volume (BV/TV; %)	31.5 ± 0.8	20.6 ± 1.4[c]	27.2 ± 1.5[b,d]	36.4 ± 1.3[c]	32.6 ± 1.4	32.9 ± 0.3
Trabecular number (Tr.Nb; 1/mm)	4.48 ± 0.13	3.50 ± 0.16[b]	4.19 ± 0.15[d]	4.88 ± 0.24	4.48 ± 0.20	4.26 ± 0.35
Trabecular separation (Tr.Sp; μm)	154 ± 5	241 ± 17[c]	177 ± 9[d]	133 ± 8[b]	153 ± 7	163 ± 13
Trabecular thickness (Tr.Th; μm)	70.8 ± 2.6	58.9 ± 2.6[b]	65.1 ± 3.0	75.6 ± 4.8	73.8 ± 5.1	79.2 ± 5.8
Bone resorption						
Osteoclast surface/bone surface (Oc.S/BS; %)	4.38 ± 1.17	7.75 ± 1.16[b]	10.90 ± 1.60	7.61 ± 2.52	4.08 ± 1.09	11.44 ± 1.41
Osteoclast number/bone area (N.Oc/BS; 1/mm^2)	28.9 ± 7.2	36.83 ± 6.3	19.0 ± 1.3	41.3 ± 8.6	25.1 ± 4.1	21.7 ± 3.4
Bone dynamics						
Mineralising surface/bone surface (MS/BS; %)	27.6 ± 3.2	17.3 ± 1.7[c]	32.3 ± 1.9[d]	16.7 ± 2.6[b]	21.4 ± 3.5	25.7 ± 1.3
Mineralising apposition rate (MAR; μm/day)	1.70 ± 0.19	2.05 ± 0.21	1.93 ± 0.27	1.15 ± 0.13[b]	1.27 ± 0.21	1.45 ± 0.18
Bone formation rate (BFR/BS; μm^3/μm^2/day)	0.49 ± 0.10	0.25 ± 0.06	0.54 ± 0.10	0.18 ± 0.05[b]	0.28 ± 0.08	0.37 ± 0.05

Bone histomorphometric parameters have been evaluated on calcified tissue after sham-operation (SHAM), ovariectomy (OVX) or ovariectomy followed by a 1 month oestrogen treatment period (OVX + E2). Dose of E2 was 15 μg/kg of weight/day.
[a]Values are shown as mean ± SEM.
[b]Significantly different from WT SHAM, $P < 0.05$.
[c]Significantly different from WT SHAM, $P < 0.005$.
[d]Significantly different from WT OVX, $P < 0.005$.

during a 3-day period also resulted in decreased proliferation (Fig. 4E). Addition of 17beta-estradiol induced an increase in cell proliferation in wild-type and transgenic cells but the proliferation rate was still significantly lower in transgenic than in wild-type cells (Fig. 4D).

These results were in accordance with the significant decrease in bone formation parameters (mineralising surfaces, and rates of mineral apposition and bone formation) in the vertebrae of transgenic mice relative to wild-type Sham mice (Table I). After ovariectomy a reduction of bone formation was observed especially in wild-type mice. Alkaline phosphatase activity measured ex vivo, the mineralising surfaces and the bone formation rate were all reduced in wild-type mice in absence of oestrogen. But the mineralising surfaces and the bone formation rate were restored when wild-type OVX mice were treated with E2. In transgenic mice, no change in mineralising surfaces occurred in transgenic mice after either OVX or OVX + E2 treatment (Table I).

Although 17beta-estradiol increased osteoblast proliferation in transgenic cells, in vivo and in vitro data suggest a decreased osteoblast proliferation in transgenic mice, resulting in a lower number of active osteoblasts and bone formation rate. Our results indicate also that overexpression of TIMP-1 blunted the effects of oestrogen on osteoblasts in vitro and ex vivo since neither E2 depletion nor E2 treatment had

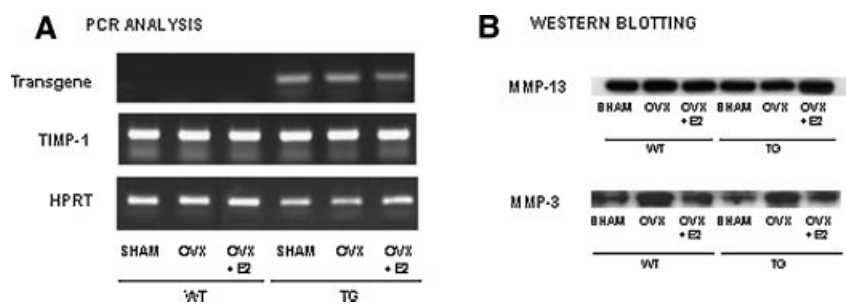

Fig. 3. Effect of estrogens on TIMP-1, MMP-13 and MMP-3 expression ex vivo. **A**: Transgene and global TIMP-1 expression was evaluated by RT-PCR. The RT reaction was performed on total RNA extracted from long bones of 5-month-old wild-type and transgenic that were sham-operated (SHAM), ovariectomised (OVX) and ovariectomised treated (OVX + E2). HPRT expression was used as internal control. The gels presented are representative of two independent RNA extractions. **B**: Bone marrow progenitor cells from the same mice we cultured in osteoblastic differentiation medium for 5 days. MMP-3 and MMP-13 expression was evaluated in the serum-free cell supernatants by immunoblotting with mouse anti-MMP-13 and anti-MMP-3 antibodies. The blots presented are representative of three independent experiments.

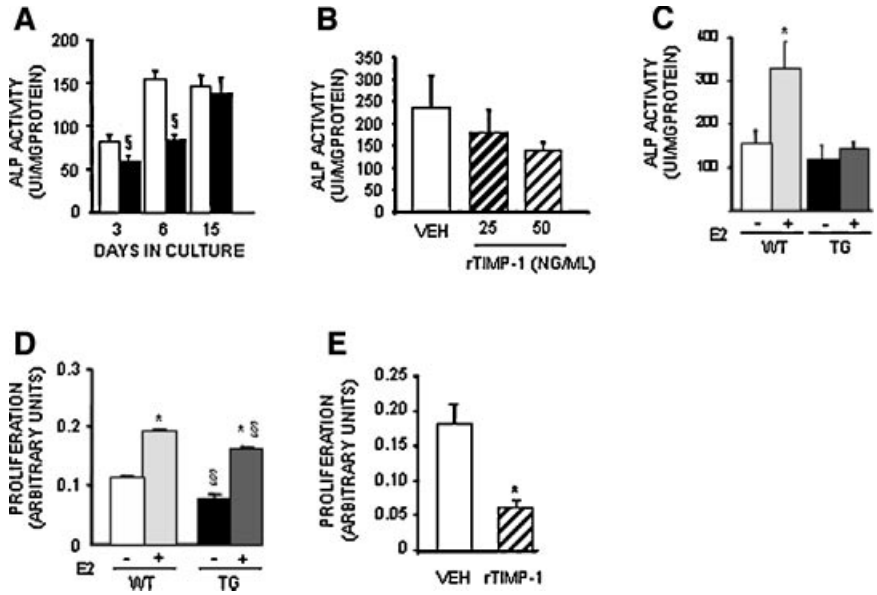

Fig. 4. TIMP-1 overexpression affects the osteoblast differentiation and proliferation in vitro. **A**–**C**: Alkaline phosphatase activity was evaluated on wild-type (open bars) and transgenic (black bars) calvaria-derived osteoblasts after 3, 6 and 15 days of culture in presence of 50 μM ascorbic acid alone (A) or 3 days in presence or absence 10^{-8}M 17beta-estradiol (C). Wild-type osteoblasts were also cultured 3 days in presence or absence of recombinant TIMP-1 (rTIMP-1) at the indicated concentration (B). **D**,**E**: Proliferation was assessed on primary osteoblasts from new born mice calvaria after a 2-day culture period by BrdU incorporation. Proliferation was evaluated in wild-type and transgenic primary osteoblasts in presence or absence of 10^{-8}M 17beta-estradiol (D) or in wild-type cells in presence or absence of recombinant TIMP-1 (50 ng/ml; E). The results are presented as mean of 3–6 individual determination ± SEM. $^{*}P < 0.05$ versus untreated cells; $^{\S}P < 0.05$ versus wild-type cells receiving the same treatment.

any effect on differentiated transgenic osteoblasts.

TIMP-1 Overexpression Blunts the Osteoclastogenesis Induced by Ovariectomy

Structural parameters suggested that overexpression of TIMP-1 in osteoblastic cells reduces bone resorption, but the osteoclastic surfaces and osteoclast number measured were identical in Sham transgenic and wild-type mice although there was a trend for higher osteoclast in transgenic mice (Table I), suggesting that MMPs inhibition by TIMP-1 decreases osteoclast resorbing activity. Because osteoclast differentiation depends on the balance between OPG and RANKL expression, we assessed the expression of OPG and RANKL by real-time PCR, and calculated the OPG/RANKL ratio in the long bones of the different experimental groups (Fig. 5). The levels of expression of both

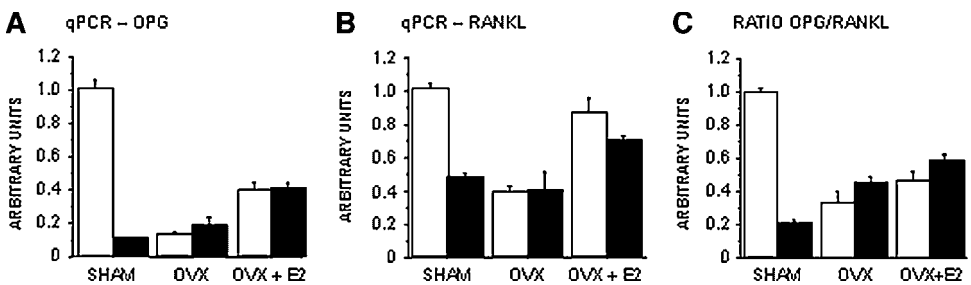

Fig. 5. Ovariectomy does not affect RANKL and OPG expression in transgenic mice. Total RNA were extracted from tibiae of 5-month-old sham-operated (SHAM), ovariectomised (OVX) and treated with 10^{-8}M 17beta-estradiol (OVX + E2) wild-type and transgenic mice. Gene expression was evaluated by real-time PCR analysis (qPCR). The analyses were repeated twice, using samples from two independent RNA extractions. The expression of OPG (**A**), and RANKL (**B**) were normalised versus 18S expression. The OPG/RANKL ratio (**C**) was calculated. The data were reported as mean ± SEM of two independent experiments.

genes were reduced in transgenic mice compared to wild-type mice and as expected the OPG/RANKL ratio was lower in transgenic than wild-type Sham animals. After OVX, there was a marked decrease in RANKL, OPG and OPG/RANKL ratio in wild-type mice that was not observed in transgenic mice. Accordingly, wild-type mice showed an increase in bone resorption after ovariectomy, as illustrated by the increased of osteoclastic surface (Oc.S/BS) and of the number of osteoclasts per bone area (N.Oc/BS; Table I) and increased excretion of deoxypyridinoline as mentioned above (Fig. 2D). In OVX transgenic mice, these parameters remained unchanged. Treatment with E2 of OVX mice did not result in any significant change in the OPG/RANKL ratio for either genotype also expression of both RANKL and OPG was increased (Fig. 5A).

We also performed ex vivo osteoclast differentiation of bone marrow progenitor cells isolated from Sham, OVX and OVX + E2 mice. These experiments showed that the number of TRAP-positive cells (Fig. 6A) and multinuclear TRAP-positive osteoclasts-like cells (Fig. 6B) were identical between wild-type and transgenic sham-operated mice. An increased of total TRAP-positive cells and of multinuclear TRAP-positive osteoclasts-like cells was observed after OVX and a reduction of these by in vivo E2 treatment only when the bone marrow progenitors had been obtained from wild-type mice. No increase of TRAP-positive and multinuclear osteoclasts-like cells was observed after OVX in transgenic cells culture. These results indicated that estrogens depletion increased osteoclastic differentiation from wild-type, but not from transgenic bone marrow precursor cells.

TIMP-1 Overexpression Affects Bone Formation In Vitro and Modifies Osteoblastic Response to Oestrogen Treatment

In order to identify one molecular mechanism that could lead to these in vivo changes, we cultured primary calvaria osteoblasts from wild-type and transgenic mice. In order to shed light on the molecular mechanisms that could explain this effect, we analysed first the expression of the oestrogen receptor (ER) alpha. Both wild-type and transgenic cells expressed ER mRNA during the osteoblastic differentiation with no marked difference according to the genotype (data not shown). Furthermore, immunocytochemistry revealed no difference in ER alpha staining in osteoblasts from wild-type and transgenic mice (Fig. 7A).

TGFβ1 levels are known to be modulated by estrogens status in bone, and TGFβ1 is known to be able to modulate osteoblast and osteoclast differentiation and/or function, and so we further investigated TGFβ1 release in the supernatant of calvaria primary osteoblastic cells. Ex vivo experiments revealed that levels of total TGFβ1 in cell supernatant were not dependent on genotype, but that the total TGFβ1 level was increased after estradiol treatment in wild-type cells only. In the absence of estradiol, active TGFβ1 was markedly lower in the supernatant of the osteoblasts from transgenic mice (Fig. 7B). Although the stimulatory effect of oestrogen on TGFβ1 activation was observed in both

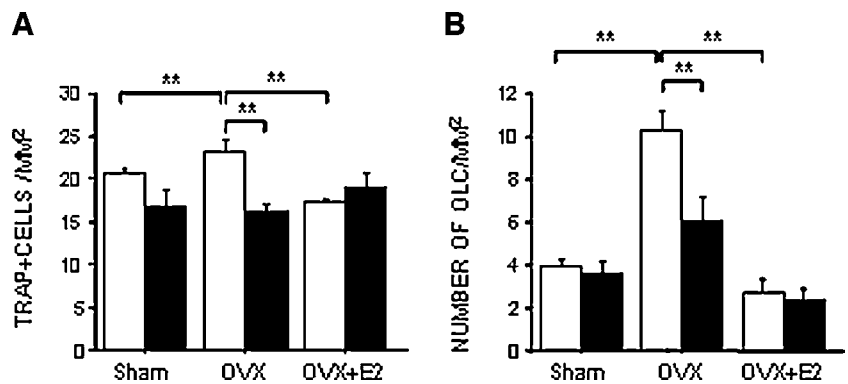

Fig. 6. TIMP-1 overexpression blunts the increase in osteoclastic differentiation induced by ovariectomy ex vivo. Thirty days after surgery, osteoclastic bone marrow progenitors from sham-operated (Sham), ovariectomised (OVX) and estrogen-treated ovariectomised (OVX + E2) wild-type and transgenic mice were cultured for 21 days in the presence of RANKL (12 ng/well) and M-CSF (10 ng/well). All TRAP-positive cells (**A**) and the multinucleated TRAP-positive osteoclast-like cells (OLC; **B**) were counted, and expressed per mm^2. OLC were TRAP+ cells with at least three nuclei. The results are presented as mean ± SEM. $^{**}P < 0.005$.

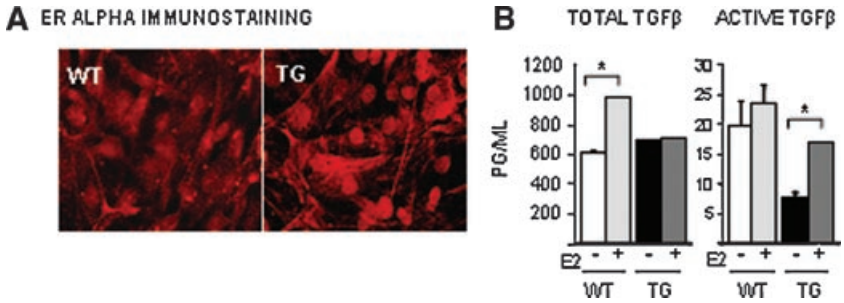

Fig. 7. TIMP-1 overexpression affects the response of osteoblastic cells to oestrogen treatment. **A**: Detection of Estrogen receptor alpha by immunolabelling on 6-day cultures of wild-type and transgenic calvaria-derived primary osteoblast. **B**: TGFβ1 assays were performed in cells supernatants from wild-type and transgenic calvaria-derived primary osteoblasts. Total and active levels of TGFβ1 were measured after 3 days in presence or absence of 10^{-8}M 17beta-estradiol. The results presented are representative of two independent experiments. $^{*}P < 0.05$ versus untreated cells.

genotypes, active TGFβ1 remained higher in wild-type cells than in transgenic cells in presence of oestrogen. In summary, TGFβ1 expression was less affected by estrogens deprivation in transgenic osteoblast, and the active form was lower in transgenic than in wild-type cells both in the presence and absence of oestrogen.

DISCUSSION

In this study, we presented evidence that TIMP-1 overexpression prevented the increase in estrogen-deficiency dependent bone resorption, and therefore also prevented post-ovariectomy bone loss.

Bone loss after OVX in mice depends on the strain [Bouxsein et al., 2005; Iwaniec et al., 2006] and is often mild. It has been also well documented by Iwaniec et al. [2006] that some mouse strain commonly used for transgenic mice generation are not ideal model to study the effects of oestrogen deficiency that mimics postmenopausal bone loss. This article present evidence that a lack of increase of the bone formation parameters is sometime observed following oestrogen deficiency in certain mouse strain. But because we observed in our model a rapid cancellous bone loss induced by high bone resorption exceeding bone formation, we can conclude that our transgenic model is an appropriate model to evaluate the effect of TIMP-1 overexpression on bone resorption. The effect of TIMP-1 overexpression on bone formation might to be analysing carefully.

In the hybrid B6CBA wild-type mice that were used in this study, we observed bone loss at 30 days that was higher at the vertebrae than at the femora (14.5% vs. 9%), and that had partially recovered 60 days after OVX. At baseline, transgenic mice displayed the same BMD than wild-type mice. It is only when the transgenic mice were challenged by OVX that a marked difference between wild-type and transgenic mice were observed. After OVX, transgenic mice showed no change in bone density or trabecular bone volume and bone resorption increased in wild-type, but not in transgenic mice. Resorption parameters including D-pyridinoline excretion, osteoclast surfaces assessed by bone morphometry, and TRAP cells differentiated from bone marrow were increased 30 days after OVX only in wild-type mice. Because high resorption is one of the major mechanism of OVX bone loss, this explains why bone mass was maintained in transgenic mice after OVX.

It has been reported that bone loss after OVX depends on the increased cytokine-driven osteoclastogenesis [Riggs et al., 2002; Weitzmann and Pacifici, 2006]. This increased osteoclastogenesis relies on a complex interplay between osteoblasts, inflammatory cells and osteoclast precursors. Cell–cell communication depends on soluble or membrane bound cytokines. We observed an unexpected lack of increase in osteoclast differentiation after OVX in transgenic mice. Osteoclastic differentiation depends on several soluble and trans-membrane factors, but the terminal effector of osteoclast differentiation is the OPG/RANKL ratio. RANKL shedding is an important process that regulates local osteoclastogenesis. It has been shown that MMP-14 which is not inhibited by TIMP-1 [Fernandez-Catalan et al., 1998] has a strong RANKL shedding activity and that suppression of MMP-14 reduces the shedding of RANKL from the osteoblast membrane and promotes

osteoclastogenesis [Hikita et al., 2006]. It is therefore unlikely that the data we observed in transgenic mice could be explained by modification of the local soluble RANKL production by osteoblasts. However, we observed that, as expected, the OPG/RANKL mRNA ratio was reduced in wild-type mice after OVX [Lindberg et al., 2006]. In contrast, in transgenic mice, this ratio was unaffected by OVX. We therefore extrapolated that a cytokine or growth factor modulating the OPG/RANKL ratio could be responsible for the difference between transgenic and wild-type mice post-OVX. Our hypothesis was that osteoblastic metalloproteinase could influence the shedding or activation of estrogen-dependent cytokines. Estrogen regulation of bone resorption involved many factors including proinflammatory cytokines (IL-1, IL-6, TNF-α, M-CSF, PGE2) that are mainly down regulated and TGFβ that is up regulated by estrogen (for review see Riggs [2000]). TGFβ is one of the most abundant growth factors in bone. Its release from the extra-cellular matrix and dissociation from the TGFβ-binding protein-1 [Dallas et al., 2002], and its activation by proteolysis [Karsdal et al., 2002; Selvamurugan et al., 2004] depends on MMPs. MMP induced the release of TGFβ from a synthetic matrix, triggering changes in osteoblast morphology, and in osteoclastic resorption [Karsdal et al., 2001]. Furthermore, TGFβ transcription is increased by estradiol [Lindberg et al., 2002]. The level of TGFβ is reduced in the bone of OVX rats [Finkelman et al., 1992]. TGFβ prevents OVX-induced hyperresorption either through direct intra-osseous injection [Beaudreuil et al., 1995] or gene transfer [Gao et al., 2004]. Low TGFβ levels had also been shown to stimulate osteoclast differentiation by impacting the RANKL/OPG ratio [Karst et al., 2004]. But it is also established that, in presence of RANKL, TGFβ reduces osteoclastic differentiation [Fox et al., 2003; Karsdal et al., 2003]. TGFβ is one of the mediators of increased OPG mRNA after estradiol treatment [Hofbauer et al., 1999]. Therefore, the reduced activation of TGFβ that was observed in transgenic osteoblast supernatant ex vivo could be part of the explanation for the lack of increase osteoclastic differentiation that has been observed in vivo and ex vivo.

The 5-month-old Sham TIMP-1 female mice had the same bone density as their wild-type counterparts. However, their trabecular bone volume was higher, and their trabecular spacing lower, suggesting lower osteoclastic resorption associated with a reduced remodelling rate. Most of the in vivo and ex vivo resorption parameters that we measured were the same in Sham wild-type and transgenic mice, with the exception of the resorption surfaces, which were higher in transgenic mice possibly because of their lower osteoclast function [Karsdal et al., 2007]. These subtle changes are consistent with our previous findings in younger female mice. The role of different proteinases in osteoclastic resorption is still debated [Everts et al., 1998]. But it has been already described that osteoclastic MMPs are involved in matrix degradation when cathepsin K is absent [Kiviranta et al., 2005; Everts et al., 2006]. It is also widely accepted that osteoblastic metalloproteinases, which were inhibited by TIMP in our model, allow osteoclasts to gain access to the calcified matrix and to start resorbing [Chambers and Fuller, 1985; Holliday et al., 1997]. TIMP-1 is the predominant form of TIMP. We showed that there was no change of the transgene expression and TIMP-1 after OVX. There is indeed no report presenting evidence for a regulation of the 2.3 kb alpha-1 type-1 collagen gene promoter by estradiol. TIMP-1 is able to inhibit MMP-2, MMP-3 and MMP-13. MMP-13 is the major osteoblastic MMP, and its invalidation induces an increase bone mass [Inada et al., 2004; Stickens et al., 2004]. MMP-13 is regulated by various bone-resorbing agents [Uchida et al., 2000] but has not been shown to be dependent on estradiol. Indeed, there was no change in MMP-13 after OVX. In contrast, we observed an increased level of MMP-3 in transgenic and wild-type OVX mice. This parallels the previous observation by Breckon et al. [1999] that more osteoblasts synthesise MMP-3 after OVX. It is therefore possible that TIMP-1 overexpression could induce changes in bone remodelling, particularly after OVX, partly as a result of reduction of MMP-3 OVX induced-activity.

At baseline the bone formation rate was lower in Sham transgenic than in Sham wild-type mice. After OVX, we observed a significant decrease in mineralising surfaces in wild-type mice that was not observed in TIMP-1 mice. In order to quantify osteoblast proliferation and differentiation, we used homogeneous population of progenitors originating from the calvaria. These in vitro studies of calvaria primary osteoblasts from wild-type and transgenic new born mice confirmed the lower rate of prolife-

ration, and the delayed differentiation in cells of transgenic mice or in wild-type cells treated with recombinant TIMP-1. To date, there are only few convincing studies to provide mechanistic insight into the role of MMPs in osteoblast differentiation. A recent study, using primary cells isolated from MMP-$2^{-/-}$ mice indicated a negative effect of MMP-$2^{-/-}$ on both osteoblast proliferation and differentiation [Mosig et al., 2007]. But this cannot explain the effect observed in TIMP-1 transgenic mice since MMP-2 is not inhibited by TIMP-1. It was also suggested that MMPs inhibition could affect osteoblastic motility that are important for cell–cell contacts achievement and osteoblastic differentiation [De Becker et al., 2007]. MMPs are involved in important signalling pathways regulating osteoblast differentiation that can be affected when TIMP-1 is overexpressed [Fratzl-Zelman et al., 2003; Nakashima and Tamura, 2006].

We showed that the reduction of proliferation of osteoblastic cells overexpressing TIMP-1 is not dependant on the oestrogen status. It has been reported that estradiol can induce an increase of osteoblast alkaline phosphatase in vitro [Qu et al., 1998]. Interestingly, when osteoblasts from transgenic mice were treated with estradiol, we did not observe the increase in alkaline phosphatase occurring in wild-type osteoblasts. Most of our findings are consistent with a lack of sensitivity to estradiol depletion or administration in transgenic mice. However, there was no major decrease in estrogen receptor alpha expression in transgenic osteoblasts. It has been demonstrated that TGFβ is one of the growth factors responsible for the positive effect of estradiol on osteoblasts [Riggs, 2000; Gao et al., 2004]. Therefore, TGFβ could be responsible for the different levels of alkaline phosphatase activity in wild-type and transgenic osteoblasts in the presence of estradiol.

In this study, we show that in transgenic mice OVX induces reduction of bone resorption with sustained osteoclast number and bone formation rate. In normal mice, bone formation is coupled to bone resorption in a tight equilibrium. Mouse mutations or pathological situations leading to ablation/reduction of osteoclasts lead to negative effects on bone formation indicating a central role for osteoclasts, and not necessarily their resorptive activity, in the control of bone formation [Karsdal et al., 2007]. Our results indicated that osteoblastic MMPs could be mediators of the production/activation of signalling factors by the osteoclast that induce bone formation.

In conclusion, our data show that inhibiting MMP abolishes or prevents bone loss after ovariectomy. TIMP-1 overexpression reduced osteoclast resorption and altered the response of osteoblast to oestrogen resulting in a decreased expression/activation of local regulators of osteoclastogenesis. TIMP-1 acts either directly by inhibiting the MMPs activity or indirectly by regulating growth factor release and activation leading to reduction of bone resorption. MMPs are therefore among the major regulators of bone remodelling especially in situations in which bone remodelling is accelerated. TGFβ1, a major mediator of OVX bone loss [Finkelman et al., 1992], might participate to this effect.

A number of studies (for review see Varghese [2006]), indicated that the bisphosphonates used for the treatment of different bone disorders including osteoporosis, are potent inhibitors of MMPs. Thus, the effectiveness of bisphosphonates in the treatment of osteoporosis could be partly due to their ability to inhibit MMP activity in bone. The pharmacological inhibition of the MMPs deserves investigation in the prevention of estradiol-dependent osteopenia.

REFERENCES

Andersen TL, Del Carmen Ovejero M, Kirkegaard T, Lenhard T, Foged NT, Delaisse JM. 2004. A scrutiny of matrix metalloproteinases in osteoclasts: Evidence for heterogeneity and for the presence of MMPs synthesized by other cells. Bone 35:1107–1119.

Beaudreuil J, Mbalaviele G, Cohen-Solal M, Morieux C, De Vernejoul MC, Orcel P. 1995. Short-term local injections of transforming growth factor-beta 1 decrease ovariectomy-stimulated osteoclastic resorption in vivo in rats. J Bone Miner Res 10:971–977.

Bouxsein ML, Myers KS, Shultz KL, Donahue LR, Rosen CJ, Beamer WG. 2005. Ovariectomy-induced bone loss varies among inbred strains of mice. J Bone Miner Res 20:1085–1092.

Breckon JJ, Papaioannou S, Kon LW, Tumber A, Hembry RM, Murphy G, Reynolds JJ, Meikle MC. 1999. Stromelysin (MMP-3) synthesis is up-regulated in estrogen-deficient mouse osteoblasts in vivo and in vitro. J Bone Miner Res 14:1880–1890.

Burrage PS, Mix KS, Brinckerhoff CE. 2006. Matrix metalloproteinases: Role in arthritis. Front Biosci 11: 529–543.

Chambers TJ, Fuller K. 1985. Bone cells predispose bone surfaces to resorption by exposure of mineral to osteoclastic contact. J Cell Sci 76:155–165.

Dallas SL, Rosser JL, Mundy GR, Bonewald LF. 2002. Proteolysis of latent transforming growth factor-beta

(TGF-beta)-binding protein-1 by osteoclasts. A cellular mechanism for release of TGF-beta from bone matrix. J Biol Chem 277:21352–21360.

De Becker A, Van Hummelen P, Bakkus M, Vande Broek I, De Wever J, De Waele M, Van Riet I. 2007. Migration of culture-expanded human mesenchymal stem cells through bone marrow endothelium is regulated by matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-3. Haematologica 92:440–449.

Delaisse JM, Engsig MT, Everts V, del Carmen Ovejero M, Ferreras M, Lund L, Vu TH, Werb Z, Winding B, Lochter A, Karsdal MA, Troen T, Kirkegaard T, Lenhard T, Heegaard AM, Neff L, Baron R, Foged NT. 2000. Proteinases in bone resorption: Obvious and less obvious roles. Clin Chim Acta 291:223–234.

Everts V, Delaisse JM, Korper W, Beertsen W. 1998. Cysteine proteinases and matrix metalloproteinases play distinct roles in the subosteoclastic resorption zone. J Bone Miner Res 13:1420–1430.

Everts VKW, Hoeben KA, Jansen ID, Bromme D, Cleutjens KB, Heeneman S, Peters C, Reinheckel T, Saftig P, Beertsen W. 2006. Osteoclastic bone degradation and the role of different cysteine proteinases and matrix metalloproteinases: Differences between calvaria and long bone. J Bone Miner Res 21: 1399–1408.

Fernandez-Catalan C, Bode W, Huber R, Turk D, Calvete JJ, Lichte A, Tschesche H, Maskos K. 1998. Crystal structure of the complex formed by the membrane type 1-matrix metalloproteinase with the tissue inhibitor of metalloproteinases-2, the soluble progelatinase A receptor. EMBO J 17:5238–5248.

Finkelman RD, Bell NH, Strong DD, Demers LM, Baylink DJ. 1992. Ovariectomy selectively reduces the concentration of transforming growth factor beta in rat bone: Implications for estrogen deficiency-associated bone loss. Proc Natl Acad Sci USA 89:12190–12193.

Fox SW, Haque SJ, Lovibond AC, Chambers TJ. 2003. The possible role of TGF-beta-induced suppressors of cytokine signaling expression in osteoclast/macrophage lineage commitment in vitro. J Immunol 170:3679–3687.

Fratzl-Zelman N, Glantschnig H, Rumpler M, Nader A, Ellinger A, Varga F. 2003. The expression of matrix metalloproteinase-13 and osteocalcin in mouse osteoblasts is related to osteoblastic differentiation and is modulated by 1,25-dihydroxyvitamin D3 and thyroid hormones. Cell Biol Int 27:459–468.

Gao YQW, Dark K, Toraldo G, Lin AS, Guldberg RE, Flavell RA, Weitzmann MN, Pacifici R. 2004. Estrogen prevents bone loss through transforming growth factor beta signaling in T cells. Proc Natl Acad Sci USA 101: 16618–16623.

Geoffroy V, Le Goupil N, Clement-Lacroix P, Terraz C, Frain M, Roux S, Rossert J, de Vernejoul MC. 2004. In vivo inhibition of osteoblastic metalloproteinases leads to increased trabecular bone mass. J Bone Miner Res 19: 811–822.

Hikita A, Yana I, Wakeyama H, Nakamura M, Kadono Y, Oshima Y, Nakamura K, Seiki M, Tanaka S. 2006. Negative regulation of osteoclastogenesis by ectodomain shedding of receptor activator of NF-kappaB ligand. J Biol Chem 28:36846–36855.

Hill PA, Murphy G, Docherty AJ, Hembry RM, Millican TA, Reynolds JJ, Meikle MC. 1994. The effects of selective inhibitors of matrix metalloproteinases (MMPs) on bone resorption and the identification of MMPs and TIMP-1 in isolated osteoclasts. J Cell Sci 107:3055–3064.

Hofbauer LC, Khosla S, Dunstan CR, Lacey DL, Spelsberg TC, Riggs BL. 1999. Estrogen stimulates gene expression and protein production of osteoprotegerin in human osteoblastic cells. Endocrinology 140:4367–4370.

Holliday LS, Welgus HG, Fliszar CJ, Veith GM, Jeffrey JJ, Gluck SL. 1997. Initiation of osteoclast bone resorption by interstitial collagenase. J Biol Chem 272:22053–22058.

Holmbeck K, Bianco P, Yamada S, Birkedal-Hansen H. 2004. MT1-MMP: A tethered collagenase. J Cell Physiol 200:11–19.

Hou P, Troen T, Ovejero MC, Kirkegaard T, Andersen TL, Byrjalsen I, Ferreras M, Sato T, Shapiro SD, Foged NT, Delaisse JM. 2004. Matrix metalloproteinase-12 (MMP-12) in osteoclasts: New lesson on the involvement of MMPs in bone resorption. Bone 34:37–47.

Inada MWY, Byrne MH, Rahman MU, Miyaura C, Lopez-Otin C, Krane SM. 2004. Critical roles for collagenase-3 (Mmp13) in development of growth plate cartilage and in endochondral ossification. Proc Natl Acad Sci USA 101: 17192–17197.

Iwaniec UT, Yuan D, Power RA, Wronski TJ. 2006. Strain-dependent variations in the response of cancellous bone to ovariectomy in mice. J Bone Miner Res 21:1068–1074.

Kanis JA. 2002. Diagnosis of osteoporosis and assessment of fracture risk. Lancet 359:1929–1936.

Karsdal MA, Fjording MS, Foged NT, Delaisse JM, Lochter A. 2001. Transforming growth factor-beta-induced osteoblast elongation regulates osteoclastic bone resorption through a p38 mitogen-activated protein kinase- and matrix metalloproteinase-dependent pathway. J Biol Chem 276:39350–39358.

Karsdal MA, Larsen L, Engsig MT, Lou H, Ferreras M, Lochter A, Delaisse JM, Foged NT. 2002. Matrix metalloproteinase-dependent activation of latent transforming growth factor-beta controls the conversion of osteoblasts into osteocytes by blocking osteoblast apoptosis. J Biol Chem 277:44061–44067.

Karsdal MA, Hjorth P, Henriksen K, Kirkegaard T, Nielsen KL, Lou H, Delaisse JM, Foged NT. 2003. Transforming growth factor-beta controls human osteoclastogenesis through the p38 MAPK and regulation of RANK expression. J Biol Chem 278:44975–44987.

Karsdal MA, Martin TJ, Bollerslev J, Christiansen C, Henriksen K. 2007. Are nonresorbing osteoclasts sources of bone anabolic activity? J Bone Miner Res 22:487–494.

Karst M, Gorny G, Sells Galvin RJ, Oursler MJ. 2004. Roles of stromal cell RANKL, OPG, and M-CSF: Expression in biphasic tgf-b regulation of osteoclast differentiation. J Cell Physiol 200:99–106.

Kiviranta R, Morko J, Alatalo SL, NicAmhlaoibh R, Risteli J, Laitala-Leinonen T, Vuorio E. 2005. Impaired bone resorption in cathepsin K-deficient mice is partially compensated for by enhanced osteoclastogenesis and increased expression of other proteases via an increased RANKL/OPG ratio. Bone 36:159–172.

Koh AJ, Demiralp B, Neiva KG, Hooten J, Nohutcu RM, Shim H, Datta NS, Taichman RS, McCauley LK. 2005. Cells of the osteoclast lineage as mediators of the anabolic actions of parathyroid hormone in bone. Endocrinology 146:4584–4596.

Lindberg MK, Moverare S, Eriksson AL, Skrtic S, Gao H, Dahlman-Wright K, Gustafsson JA, Ohlsson C. 2002. Identification of estrogen-regulated genes of potential importance for the regulation of trabecular bone mineral density. J Bone Miner Res 17:2183–2195.

Lindberg MK, Svensson J, Venken K, Chavoshi T, Andersson N, Movérare Skrtic S, Isaksson O, Vanderschueren D, Carlsten H, Ohlsson C. 2006. Liver-derived IGF-I is permissive for ovariectomy-induced trabecular bone loss. Bone 38:85–92.

Malemud CJ. 2006. Matrix metalloproteinases: Role in skeletal development and growth plate disorders. Front Biosci 11:1702–1715.

Meikle MC, Bord S, Hembry RM, Compston J, Croucher PI, Reynolds JJ. 1992. Human osteoblasts in culture synthesize collagenase and other matrix metalloproteinases in response to osteotropic hormones and cytokines. J Cell Sci 103:1093–1099.

Merciris D, Schiltz C, Legoupil N, Marty-Morieux C, de Vernejoul MC, Geoffroy V. 2007. Over-expression of TIMP-1 in osteoblasts increases the anabolic response to PTH. Bone 40:75–83.

Mosig RA, Dowling O, DiFeo A, Ramirez MC, Parker IC, Abe E, Diouri J, Aqeel AA, Wylie JD, Oblander SA, Madri J, Bianco P, Apte SS, Zaidi M, Doty SB, Majeska RJ, Schaffler MB, Martignetti JA. 2007. Loss of MMP-2 disrupts skeletal and craniofacial development and results in decreased bone mineralization, joint erosion and defects in osteoblast and osteoclast growth. Hum Mol Genet 16:1113–1123.

Nakashima A, Tamura M. 2006. Regulation of matrix metalloproteinase-13 and tissue inhibitor of matrix metalloproteinase-1 gene expression by WNT3A and bone morphogenetic protein-2 in osteoblastic differentiation. Front Biosci 11:1667–1678.

Otsuka K, Sodek J, Limeback H. 1984. Synthesis of collagenase and collagenase inhibitors by osteoblast-like cells in culture. Eur J Biochem 145:123–129.

Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, Ott SM, Recker RR. 1987. Bone histomorphometry: Standardization of nomenclature, symbols, and units. J Bone Miner Res 2:595–610.

Qu Q, Perala-Heape M, Kapanen A, Dahllund J, Salo J, Vaananen HK, Harkonen P. 1998. Estrogen enhances differentiation of osteoblasts in mouse bone marrow culture. Bone 22:201–209.

Raisz LG. 2005. Pathogenesis of osteoporosis: Concepts, conflicts, and prospects. J Clin Invest 115:3318–3325.

Riggs BL. 2000. The mechanisms of estrogen regulation of bone resorption. J Clin Invest 106:1203–1204.

Riggs BL, Khosla S, Melton LJ III. 2002. Sex steroids and the construction and conservation of the adult skeleton. Endocr Rev 23:279–302.

Selvamurugan N, Kwok S, Alliston T, Reiss M, Partridge NC. 2004. Transforming growth factor-beta 1 regulation of collagenase-3 expression in osteoblastic cells by crosstalk between the Smad and MAPK signaling pathways and their components, Smad2 and Runx2. J Biol Chem 279:19327–19334.

Sternlicht MD, Werb Z. 2001. Matrix metalloproteinases regulate cell behavior. Annu Rev Cell Dev Biol 17:463–516.

Stickens D, Behonick DJ, Ortega N, Heyer B, Hartenstein B, Yu Y, Fosang AJ, Schorpp-Kistner M, Angel P, Werb Z. 2004. Altered endochondral bone development in matrix metalloproteinase 13-deficient mice. Development 131:5883–5895.

Uchida M, Shima M, Shimoaka T, Fujieda A, Obara K, Suzuki H, Nagai Y, Ikeda T, Yamato H, Kawaguchi H. 2000. Regulation of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) by bone resorptive factors in osteoblastic cells. J Cell Physiol 185:207–214.

VanSaun MN, Matrisian LM. 2006. Matrix metalloproteinases and cellular motility in development and disease. Birth Defects Res C Embryo Today 78:69–79.

Varghese S. 2006. Matrix metalloproteinases and their inhibitors in bone: An overview of regulation and functions. Front Biosci 11:2949–2966.

Weitzmann MN, Pacifici R. 2006. Estrogen deficiency and bone loss: An inflammatory tale. J Clin Invest 116:1186–1194.

Williams S, Barnes J, Wakisaka A, Ogasa H, Liang CT. 1999. Treatment of osteoporosis with MMP inhibitors. Ann NY Acad Sci 878:191–200.

Zhao W, Byrne MH, Wang Y, Krane SM. 2000. Osteocyte and osteoblast apoptosis and excessive bone deposition accompany failure of collagenase cleavage of collagen. J Clin Invest 106:941–949.