

Estrogen-deficient osteoporosis enhances the recruitment and activity of osteoclasts by breast cancer cells

Francesca Salamanna¹, Stefania Pagani^{1,2}, Melania Maglio², Veronica Borsari¹, Gianluca Giavaresi^{1,2}, Alberto M. Martelli³, Francesca Buontempo³ and Milena Fini^{1,2}

¹Laboratory of Biocompatibility, Technological Innovations and Advanced Therapies, Rizzoli Research Innovation Technology Department, ²Laboratory of Preclinical and Surgical Studies, Rizzoli Orthopaedic Institute and ³Department of Biomedical and Neuromotor Sciences, University of Bologna, Bologna, Italy

Summary. To reduce the burden of bone metastases, the pathophysiology of the metastatic niche should be elucidated and targeted. The aim of the present study was to assess the effect of tumor cells on osteoclast (OC) recruitment and activity in the presence of altered bone remodelling. Peripheral blood mononuclear cells (PBMC) were isolated from healthy and ovariectomized (OVX) rats and co-cultured with MRMT-1 rat breast carcinoma cells or with their conditioned medium for 1 and 2 weeks. Alamar Blue viability test, synthesis of cathepsin K, transforming growth factor-beta 1 (TGF- β 1), tumor necrosis factor alpha (TNF- α), vascular endothelial growth factor (VEGF), metalloproteinase (MMP)-7, MMP-9, FITC-conjugate phalloidin staining and tartrate-resistant acid phosphatase (TRAP) staining were evaluated.

The results indicate that breast carcinoma cells induced different responses in PBMC derived from rats affected by estrogen deficiency osteoporosis (OP) in comparison with healthy ones, with a significant increase in proliferation rate, OC differentiation, synthesis of TNF- α , MMP-7 and MMP-9.

The data support the “proof of concept” that OP due to estrogen deficiency might offer a receptive site for cancer cells to form bone metastases.

Key words: Bone metastasis, Estrogen deficiency, Osteoporosis, Breast cancer, Osteoclasts

Introduction

Metastatic tumor in bone is the most influential factor on the survival time of breast cancer patients. Postmortem studies have shown that approximately 70% of patients die with evidence of osteolytic metastases to the skeleton (Papachristou et al., 2012).

Osteolytic metastases act mainly by up-regulating osteoclast (OC) activity (Akhtari et al., 2008). Once tumor cells invade bone, they secrete factors that initiate OC-mediated bone destruction, during which growth factors are released from bone matrix, further stimulating the proliferation of the tumor cells and the expression of more bone-resorbing factors, perpetuating the “vicious cycle” of bone metastasis (Mundy, 1997; Sterling et al., 2011). The main characterized factors are transforming growth factor-beta (TGF- β), vascular endothelial growth factor (VEGF), insulin-like growth factors (IGFs), bone morphogenic proteins (BMPs) and fibroblast-derived factors (FBFs). TGF- β stimulates both proliferation of breast cancer cells and their production of parathyroid hormone-related protein (PTHrP), which in turn acts on osteoblasts (OB) to induce recruitment of OC via receptor activator of nuclear factor k-B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) production. Bone growth factors, such as VEGF, IGFs, BMPs and FBFs, as well as calcium, are released into the microenvironment, stimulating tumor cells to proliferate and produce more growth factors,

Offprint requests to: Francesca Salamanna, PhD, Laboratory of Biocompatibility, Technological Innovations and Advanced Therapies, Rizzoli Research Innovation Technology Department, Rizzoli Orthopaedic Institute, Via di Barbiano, 1/10, 40136 Bologna, Italy. e-mail: francesca.salamanna@ior.it

DOI: 10.14670/HH-11-651

further perpetuating the vicious cycle of bone metastasis (Chen et al., 2010).

To reduce the burden of bone metastases, the pathophysiology of the metastatic niche should be elucidated and targeted. Estrogen-deficient osteoporosis (OP) is a high turnover skeletal disease, where OC-mediated bone resorption exceeds OB-mediated bone formation. Estrogen withdrawal is associated with an increased local and systemic production of pro-inflammatory cytokines such as RANKL, M-CSF and TNF α (Pfeilschifter et al., 2002; Mundy, 2007; Pacifici, 2008), which cause up-regulation of OC formation and stimulation of bone resorption with extracellular matrix (ECM) protein release. Thus, it can be hypothesized that the increased levels of osteoclastogenic cytokines and ECM remodeling in the OP microenvironment may provide a favorable condition for tumor cells growth, as also supposed by Wu and colleagues (Wu et al., 2010).

To date, few papers have described a connection between bone remodeling and breast tumors and no data are reported on the relationship between altered bone remodeling and bone metastases. Bayraktar et al. analyzed the bone mass density (BMD) of 158 postmenopausal women diagnosed with primary breast cancer: they found that patients with higher BMD were associated with low-grade malignancy (Bayraktar et al., 2013). In 2011, Kreamer et al. evaluated the BMD and the presence of disseminating tumor cells in the bone marrow of 70 premenopausal and 111 postmenopausal women affected by breast cancer: they found that smaller tumors correlated significantly with higher BMD and that 50% of osteoporotic patients had the presence of disseminating tumor cells in the bone marrow (Kraemer et al., 2011).

Clinical trials of early adjuvant therapeutic interventions with bone targeting agents, such as bisphosphonates, MMP inhibitors and Denosumab, have been undertaken in the attempt to alter the bone metastatic niche and, thus, to prevent the establishment of bone metastases (Saarto et al., 2004; Powles et al., 2006; Diel et al., 2008; Kristensen et al., 2008; Gnant et al., 2009; Coleman et al., 2012; Thamake et al., 2012). In addition, three clinical trials provided evidence that selective estrogen receptor modulators administered to postmenopausal and/or osteoporotic women gave a reduction in the incidence of invasive breast cancer and protection against breast cancer metastases (Cauley et al., 2001; Martino et al., 2004; Grady et al., 2008).

However, neither *in vitro* nor *in vivo* studies assessed the mechanisms that link bone remodeling rate and the development of bone metastases.

The hypothesis of this study is that, in the presence of altered bone turnover, some biological factors can elicit an enhancement of local aggressiveness of breast cancer cells through their influence on OC precursors recruitment and activity. As OP is the most recurrent disease of bone remodeling (Hernlund et al., 2013), an *in vitro* study was settled in which OC precursors, isolated from the peripheral blood of healthy and ovariectomized

(OVX) rats, were co-cultured with MRMT-1 rat breast carcinoma cells to evaluate OC metabolism and anabolic activity. Several culture conditions were tested in order to evaluate static and dynamic co-culture systems and the effect of exogenous differentiation.

Materials and methods

Conditioned media preparation

MRMT-1 rat breast carcinoma cells were purchased from Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer (Tohoku University 4-1, Seiryō, Aoba-ku, Sendai, Japan). Cells were cultured in RPMI 1640 medium (Sigma, MO, USA) supplemented with 10% fetal bovine serum (FBS, Lonza, Verviers, Belgium), 2 mM glutamine and antibiotics (100 U/ml penicillin, 100 μ g/ml streptomycin) (Gibco, INVITROGEN Corporation, Carlsbad, CA). The cultures at confluence were rinsed and serum-free RPMI 1640 was added (15 ml per T75 flask, $\sim 1 \times 10^6$ cells/cm²). Twenty-four hours later, the conditioned medium was collected, centrifuged and stored at -80°C.

Isolation of mononuclear cells and differentiation into OC

Osteoclasts (OC) were obtained from the peripheral blood mononuclear cells (PBMCs) of 6 healthy (SHAM) and 6 ovariectomized (OVX) Sprague-Dawley adult female rats (Charles River Calco Lecco, Italy) from an uncorrelated study approved by the Rizzoli Orthopedic Institute Ethical Committee. The estrogen-deficient osteoporotic condition in OVX animals and the healthy condition in SHAM animals were verified by histomorphometric analysis of the iliac crest biopsy, microtomography and quantitative bone ultrasound evaluations (data not shown).

PBMCs were separated onto Ficoll-Histopaque gradient (Sigma-Aldrich, MO, USA), according to the following protocol. Peripheral blood was diluted 1:1 with PBS and carefully layered on Histopaque 1077 (ratio 2:1). Centrifugation (700g for 30 min) was used to separate the mononuclear cells; PBMCs were collected, washed twice with PBS, resuspended in an appropriate volume of basal medium - Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, MO, USA) + 10% fetal calf serum (FCS, Lonza, Verviers, Belgium) + 2 mM glutamine + antibiotics (100 U/ml penicillin, 100 μ g/ml streptomycin) - and counted in a Neubauer chamber after a brief incubation with Turk solution (to lyse the contaminating red cells).

PBMCs derived from SHAM (PBMC_{SHAM}) and OVX (PBMC_{OVX}) rats were both seeded at the density of 1.5×10^6 /cm² in basal medium. Four culture days later, the non-adherent cells were removed, while the adherent cells were reseeded with different media, representing alternative cell culture conditions:

- Basal medium (CTR): basal medium only;

Synergy between osteoporosis and cancer

- Differentiation medium (CTR⁺): basal medium supplemented with differentiating factors towards the OC phenotype (30 ng/ml of RANKL, 25 ng/ml of M-CSF and 10⁻⁷ M PTH) (Peprotech, Rocky Hill, NJ);
- Basal conditioned medium (CM⁻): 50% basal medium +50% conditioned medium derived from MRMT-1 cells;
- Differentiation conditioned medium (CM⁺): 50% basal medium supplemented with differentiating factors towards OC phenotype (30 ng/ml of RANKL, 25 ng/ml of M-CSF and 10⁻⁷ M PTH) (Peprotech, Rocky Hill, NJ) +50% conditioned medium from MRMT-1 cells.
- Co-culture system (TW) set up by using transwell inserts (Millicell 0.4 μm pore-size, Millipore, Ireland): the upper inserts contained 1x10³/cm² MRMT-1 breast cancer cells in 500 μl of RPMI 1640, while the lower chambers contained 1.5x10⁶/cm² PBMC_{SHAM} or PBMC_{Ovx} in 1 ml of basal medium only.

MRMT-1 breast cancer cells were cultured also in transwell inserts alone (1x10³ cells with no PBMCs in the lower chamber).

Media were changed twice a week and then collected, centrifuged and stored at -80°C for assays at each experimental time (1 and 2 weeks).

PBMC viability

Alamar blue test (Serotec, Oxford, UK) was used to evaluate cell viability. It was added to each culture well (1:10 v/v) for 4 h at 37°C. The absorbance of supernatants was read spectrophotometrically at 570 and 600 nm wavelengths by means of a microplate reader (BioRad, CA, USA). The results were expressed as reduction percentage, following the manufacturer's instructions.

OC differentiation

OC differentiation was evaluated by tartrate-resistant acid phosphatase (TRAP) histochemical staining, according to the manufacturer's instructions (387A-KT, Sigma-Aldrich, MO, USA). The large, multinucleated cells (4 or more nuclei) which developed a brown color were scored as positive cells. The ratio (TRAP RGB%) between the brown colored region and total image area was measured using an image analysis system (Leica QWIN, Leica Microsystems Ltd, UK). The brown color was defined on the red-green-blue (RGB) scale as [R=36±86; G=23±40; B=14±24]. Images were taken using a standard light microscope (Olympus IX71, Olympus Italia Srl, Italy) equipped with a digital camera (XCell, Olympus Italia Srl, Italy) at 40x magnification. To avoid biases because of subjectivity, the measurements were performed by two experienced and blinded investigators. The number of osteoclasts (with 4 or more nuclei), in 10 regions of interest (ROI), were also counted for each culture condition at 40x magnification.

Finally, two weeks after the seeding of cells each culture was pre-washed with PBS and fixed in a solution

of 4% formaldehyde in PBS for 10 min at 37°C. Then, the samples were permeabilized with 0.1% Triton X-100 for 15 min, washed in PBS and a FITC-conjugate phalloidin solution (1:100 in PBS) was added for 30 min at 37°C. After washing with PBS, samples were examined by fluorescence microscope (Olympus IX 71) and the images acquired by a digital image capture system (20x objective and an Olympus XC camera).

OC synthetic activity

Supernatants from each culture condition were collected and centrifuged to remove particulates. Aliquots were assayed for total protein content (Total Protein Kit, Micro Lowry method, Petterson's Modification, Sigma-Aldrich, MO, USA), for cathepsin K (CTSK), transforming growth factor-beta 1 (TGF-β1), tumor necrosis factor alpha matrix (TNF-α), vascular endothelial growth factor (VEGF), matrix metalloproteinase-7 (MMP-7) and matrix metalloproteinase-9 (MMP-9) (Enzyme-linked Immunosorbent Assay Kits, Usbn Life Science Inc., Wuhan, China). The amount of each factor (CTSK, TGF-β1, TNF-α, VEGF, MMP-7 and MMP-9) for CM⁺, CM⁻ and TW culture conditions was normalized by subtracting those measured in MRMT-1 alone.

Statistical analysis

Statistical analysis was performed using the SPSS v.12.1 software (SPSS Inc., IL, USA). Data are reported as mean ± SD at a significance level of p<0.05. The Kolmogorov Smirnov test was performed to test normality of the variables. The General Linear Model (GLM) with adjusted Sidak's multiple comparison test with 'culture type' (CTR⁻, CTR⁺, CM⁺, CM⁻, TW) and 'cell type' (SHAM and OVX) as fixed effects was performed to assess the differences between factors on PBMC viability, OC differentiation and synthetic activity within each experimental time. In particular, the following comparisons were taken into account:

- within each 'cell type': (A) SHAM versus OVX;
 - within each 'culture type': (1) CTR⁺ versus CTR⁻; (2) CM⁺ versus CTR⁺; (3) CM⁻ and TW versus CTR⁻;
- Firstly to evaluate the influence of osteoporotic condition on OC behavior within each 'cell type' and second to understand if exogenous factors present in culture medium (CTR⁺) and the different 'culture type' might have an influence on OC response.

Results

A significantly higher cell viability, was observed in the osteoporotic condition when comparing PBMC_{Ovx} with PBMC_{SHAM} at both experimental times (Fig. 1). The osteoporotic condition also influenced the TRAP staining when compared to cultures of PBMC_{SHAM}, revealing, already at one week, significantly higher value in CTR⁻, CM⁺ and CM⁻ cultures (Fig. 2). At two

Synergy between osteoporosis and cancer

weeks, significantly higher values of TRAP staining were highlighted in CTR⁻, CM⁻ and TW conditions of PBMC_{OVX} than those of PBMC_{SHAM} (Fig. 2). At one week also the osteoclast count revealed significantly higher values in CTR⁻ and CTR⁺ cultures of PBMC_{OVX} than in PBMC_{SHAM} cultures. At 2 weeks the osteoporotic condition influenced also the osteoclast number in TW cultures when compared to TW of PBMC_{SHAM} (Fig. 3). Additionally, osteoclast analysis was evaluated by labeling the F-actin cytoskeleton with phalloidin. CTR⁺, CM⁺, CM⁻ and TW culture of PBMC_{SHAM} and PBMC_{OVX} showed the typical organization of the actin network in mature and functional multinucleated osteoclasts (Fig. 4). The main difference between PBMC_{SHAM} and PBMC_{OVX} was detected in CTR⁻ culture where no osteoclasts were present in PBMC_{SHAM}.

At each experimental time a significant increase of CTSK synthesis was detected in PBMC_{OVX} in

comparison to PBMC_{SHAM} for all cultures (Fig. 5). Regarding TGF- β 1 synthesis, CTR⁻ cultures, at both experimental times, and TW cultures, at two weeks, revealed a significant increase in PBMC_{OVX} in comparison with PBMC_{SHAM} (Fig. 6). On the contrary, in the other culture conditions, TGF- β 1 synthesis did not show significant differences between PBMC_{OVX} and PBMC_{SHAM} (Fig. 6). The TNF- α synthesis revealed significantly higher values when PBMC_{OVX} was present in comparison with PBMC_{SHAM} at both experimental times, except for TW cultures (Fig. 7). Regarding VEGF synthesis, with the exception of CTR⁻ in PBMC_{SHAM}, where no viable cells were detected, it can be appreciated an increasing trend in PBMC_{SHAM} compared to PBMC_{OVX} at both experimental times (Fig. 8). Significant differences in VEGF values between

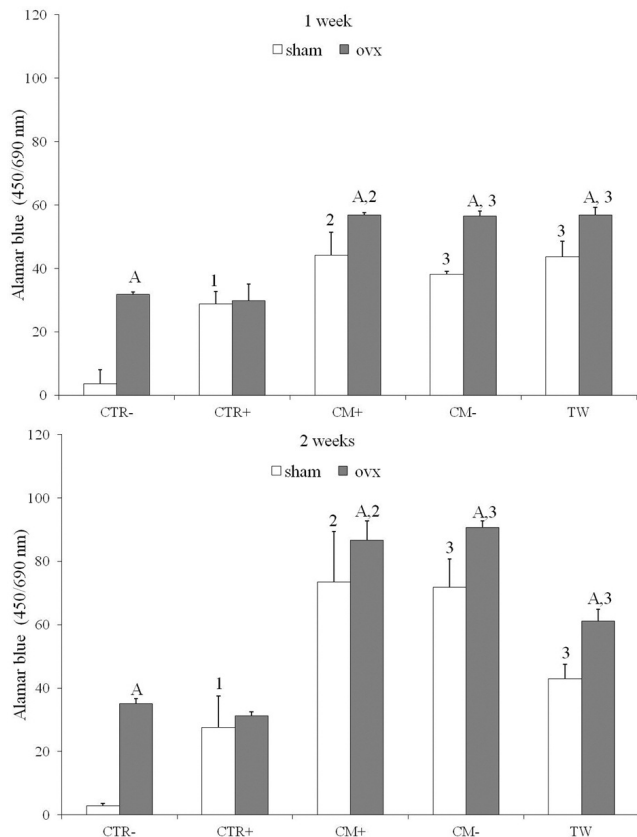


Fig. 1. Cell viability in terms of Alamar blue reduction percentage after 1 (a) and 2 weeks (b) of PBMC culture (Mean \pm SD, n=4 triplicates). Interaction of 'cell type' and 'culture type' at 1 and 2 weeks. Sidak test ($p < 0.05$): A, PMNC_{OVX} versus PMNC_{SHAM}; 1, CTR⁺ versus CTR⁻; 2, CM⁺ versus CTR⁺; 3, CM⁻ and TW versus CTR⁻. CM⁻: 50% basal culture medium +50% conditioned medium from MRMT-1 breast cancer cells; CM⁺: 50% differentiation medium +50% conditioned medium from MRMT-1 breast cancer cells; TW: co-culture.

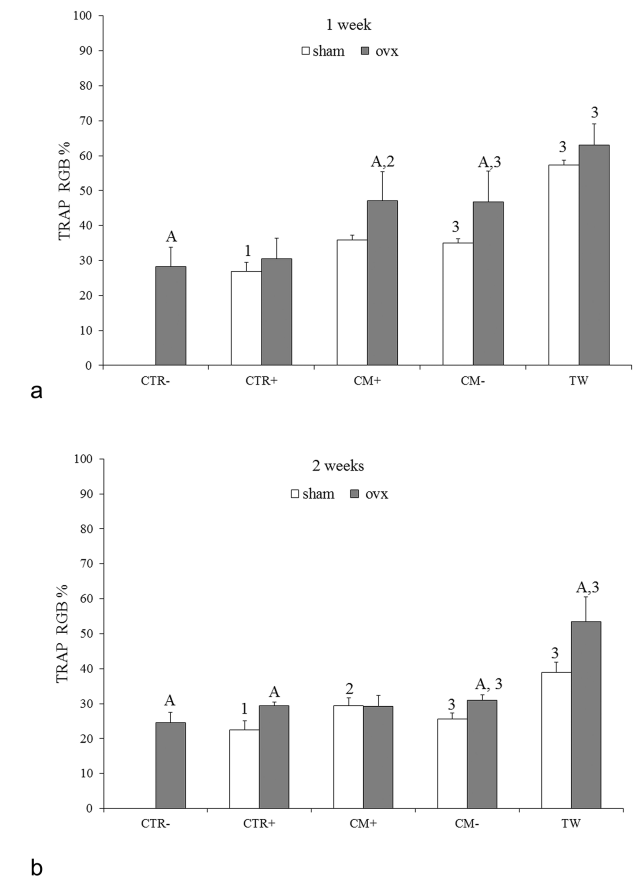


Fig. 2. TRAP staining and RGB evaluation at 1 (a) and 2 weeks (b) after PBMC seeding (Mean \pm SD, n=4 triplicates). Interaction of 'cell type' and 'culture type' at 1 and 2 weeks. Sidak test ($p < 0.05$): A, PMNC_{OVX} versus PMNC_{SHAM}; 1, CTR⁺ versus CTR⁻; 2, CM⁺ versus CTR⁺; 3, CM⁻ and TW versus CTR⁻. CM⁻: 50% basal culture medium +50% conditioned medium from MRMT-1 breast cancer cells; CM⁺: 50% differentiation medium +50% conditioned medium from MRMT-1 breast cancer cells; TW: co-culture.

Synergy between osteoporosis and cancer

PBMC_{SHAM} and PBMC_{OVX} resulted at 1 week in CM⁺ and TW and at 2 weeks in CM⁺ (Fig. 6). At each experimental time also a significant increase of MMP-7 synthesis was detected in PBMC_{OVX} in comparison to PBMC_{SHAM} for all culture conditions (Fig. 9). Finally, at 1 week also MMP-9 showed significantly higher values in PBMC_{OVX} in comparison to PBMC_{SHAM} for all cultures, while at 2 weeks significantly higher values were observed in CM⁺ and CM⁻ (Fig. 10).

To understand whether differentiating factors present in culture medium (CTR⁺) and the different 'culture type' might have an influence on OC response, we evaluated specifically the effect of differentiation factors on PBMC cells (CTR⁺ versus CTR⁻); the effect of MRMT-1 conditioned medium in the presence of differentiation medium (CM⁺ versus CTR⁺) and the effect of MRMT-1 conditioned medium and MRMT-1

co-culture system in the presence of basal medium (CM⁻ and TW versus CTR⁻).

Concerning the effect of differentiation factors on PBMCs, at each experimental time, CTR⁻ condition of PBMC_{SHAM} revealed a significantly lower value of cell vitality, TRAP staining, osteoclast number, CTSK, TGF- β 1, TNF- α and MMP-7 synthesis in comparison to CTR⁺ (Figs. 1-5, 9). In contrast, PBMC_{OVX} did not show significant differences in cell viability, TRAP staining, osteoclast number, CTSK, TGF- β 1, TNF- α (at 2 weeks), MMP-7 and MMP-9 between CTR⁻ and CTR⁺, revealing a spontaneous osteoclast formation in CTR⁻ condition (Figs. 1-3, 5-7, 9-10).

In cultures with differentiation medium, the presence of MRMT-1 conditioned medium (CM⁺) significantly increased cell viability of both PBMC_{SHAM} and PBMC_{OVX} at each experimental time, as well as CTSK, TNF- α and VEGF production (Figs. 1, 5, 7, 8), while TGF- β significantly increased at 2 weeks (Fig. 6). As regarding PBMC_{OVX}, the cultures with MRMT-1 conditioned medium also significantly enhanced the TRAP staining, osteoclast number and MMP-9 synthesis, while no differences were observed for PBMC_{SHAM} (Figs. 2-3, 10). Finally, the synthesis of MMP-7 was not affected by the presence of differentiation medium in CM conditions (Fig. 9).

In cultures with basal medium, the presence of MRMT-1 conditioned medium (CM⁻) and MRMT-1 co-culture system (TW) both in PBMC_{SHAM} and PBMC_{OVX} showed a significantly higher vitality, TRAP staining, osteoclast number (only at 1 week for CM⁻ in PBMC_{OVX}), CTSK, TGF- β 1 (only at 1 week for PBMC_{OVX}), TNF α and VEGF synthesis in comparison to those cultured in CTR⁻ only (Figs. 1-3, 6-8). As for MMP-7 synthesis, only PBMC_{SHAM} showed a

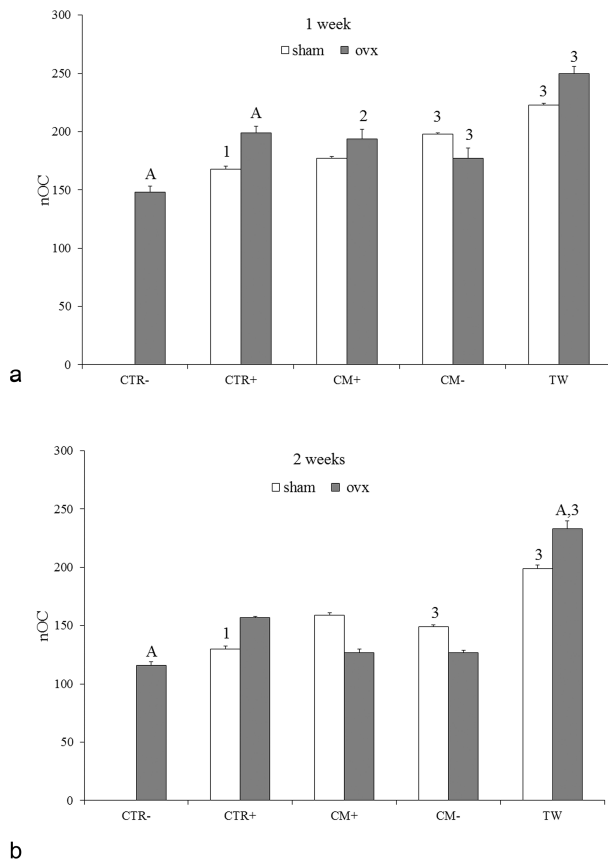


Fig. 3. Osteoclasts count at 1 (a) and 2 weeks (b) after PBMC seeding (Mean \pm SD, n=4 triplicates). Interaction of 'cell type' and 'culture type' at 1 and 2 weeks. Sidak test ($p < 0.05$): A, PBMC_{OVX} versus PBMC_{SHAM}; 1, CTR⁺ versus CTR⁻; 2, CM⁺ versus CTR⁺; 3, CM⁻ and TW versus CTR⁻. CM⁻: 50% basal culture medium +50% conditioned medium from MRMT-1 breast cancer cells; CM⁺: 50% differentiation medium +50% conditioned medium from MRMT-1 breast cancer cells; TW: co-culture.

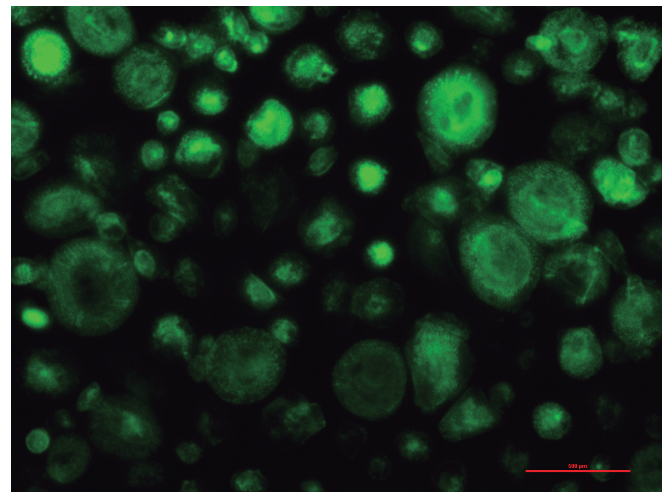


Fig. 4. Representative field of TW culture condition of PBMC_{OVX} stained with phalloidin (shown in green) to reveal cell shape through definition of the F-actin cytoskeleton. x 10

significantly higher amount when co-cultured with MRMT-1 cells or cultured with CM⁻ (Fig. 9). On the contrary, PMNC_{OVX} showed a significant increase in the synthesis of MMP-9 in the presence of MRMT-1 cells or their conditioned medium in comparison to those cultured with CTR⁻ (Fig. 10).

Discussion

Research on the mechanisms underlying bone metastasis spread has lead to consider diseases affecting bone remodeling, e.g. osteoporosis, as potentially involved in the development of metastatic progression. In order to assess this possible relationship, the effect of MRMT-1 rat breast carcinoma cells on the recruitment and activity of rat OC in the presence of estrogen-deficiency OP was evaluated by using various *in vitro* co-cultured models.

As expected, the production of a variety of GFs and other signal molecules by tumor cells enhanced OC

function (Karaplis and Gottzman, 2000; Kang et al., 2003; Nicolin et al., 2008; Edwards and Mundy, 2011) but, for the first time, in this study it was shown that bone resorbing cells derived from PBMCs of osteoporotic rats are more responsive to these factors than healthy cells. Thus, in the active crosstalk between tumor cells and bone microenvironment, the altered bone remodeling due to estrogen deficiency could provide support for tumor cell growth. The consequent enhanced osteoclastogenesis, leading to an enrichment of the microenvironment with bone matrix degradation products, cytokines and growth factors, could represent a trophic signal able to activate tumor cells from a dormant to a proliferative state, supporting their growth and the development of metastatic lesions, as reported in the literature (Wu et al., 2010; Coleman et al., 2012).

In detail, the comparison within each 'cell type' (SHAM versus OVX) showed increased cell vitality and differentiation in all culture conditions of PBMCs from

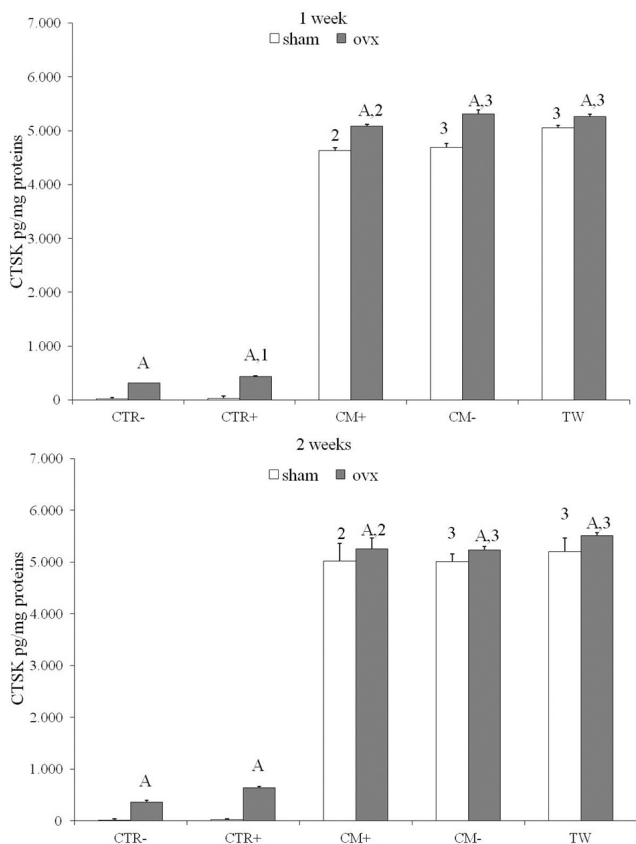


Fig. 5. CTSK related to total protein content after 1 (a) and 2 weeks (b) of PBMC culture (Mean \pm SD, n=4 triplicates). Interaction of 'cell type' and 'culture type' at 1 and 2 weeks. Sidak test ($p < 0.05$): ^A, PMNC_{OVX} versus PMNC_{SHAM}; ², CM⁺ versus CTR⁺; ³, CM⁻ and TW versus CTR⁻. CM⁻: 50% basal culture medium +50% conditioned medium from MRMT-1 breast cancer cells; CM⁺: 50% differentiation medium +50% conditioned medium from MRMT-1 breast cancer cells; TW: co-culture.

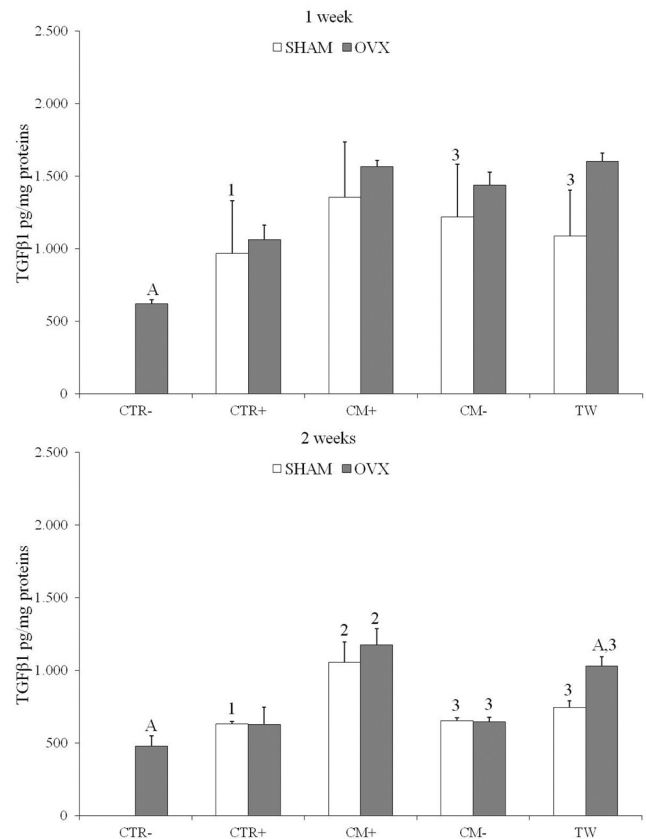


Fig. 6. TGFβ1 related to total protein content after 1 (a) and 2 weeks (b) of PBMC culture (Mean \pm SD, n=4 triplicates). Interaction of 'cell type' and 'culture type' at 1 and 2 weeks. Sidak test ($p < 0.05$): ^A, PMNC_{OVX} versus PMNC_{SHAM}; ¹, CTR⁺ versus CTR⁻; ², CM⁺ versus CTR⁺; ³, CM⁻ and TW versus CTR⁻. CM⁻: 50% basal culture medium +50% conditioned medium from MRMT-1 breast cancer cells; CM⁺: 50% differentiation medium +50% conditioned medium from MRMT-1 breast cancer cells; TW: co-culture.

Synergy between osteoporosis and cancer

estrogen deficient rats, with the exception of CTR⁺, because the presence of differentiation factors in the medium stand above the PBMC_{OVX} influence. The presence of OB or marrow stromal cells and at least two factors expressed by the accessory cells, M-CSF and RANKL, is essential to promote osteoclastogenesis. Nevertheless, our results, in agreement with D'Amelio et al. (2008), showed that cells isolated from estrogen-deficient rats do not require additional cell signaling to form OC, reflecting the finding that osteoclastogenesis is increased by estrogen deficiency and indicating that the effect of MRMT-1 cells is more remarkable on PBMCs in the case of altered bone remodeling, even in the absence of other mediators (D'Amelio et al., 2008). The powerful, synergic action of both osteoporotic "microenvironment" and cancer cells is confirmed by viability and differentiation results already at 1 week, in different culture conditions (CM⁻, CM⁺ and TW) of PBMC_{OVX} and also by the increase in cathepsin K

production, critical for normal bone resorption and remodeling (Troen, 2004; Le Gall et al., 2007; Fuller et al., 2008; Sturge et al., 2011).

Obviously, also the production by the tumor cells of a variety of osteotropic cytokines, GFs and other signal molecules, such as TGF- β 1, TNF- α , VEGF and MMPs, enhances OC function leading to bone degradation but, for the first time, it was shown that in the case of estrogen-deficiency osteoporosis bone resorbing cells might be more responsive to these factors than normal cells.

In our study, TGF- β 1, despite its role in bone resorption (Quinn et al., 2001; Itonaga et al., 2004; Buijs et al., 2012), did not show significantly higher levels in PBMC_{OVX}, but it could be hypothesized that the elevated production of TGF- β in tumoral culture might make less evident the differences between osteoporotic and healthy condition, except for the TW cultures at the longest experimental time.

Instead, as regarding TNF- α , (Bingham, 2002;

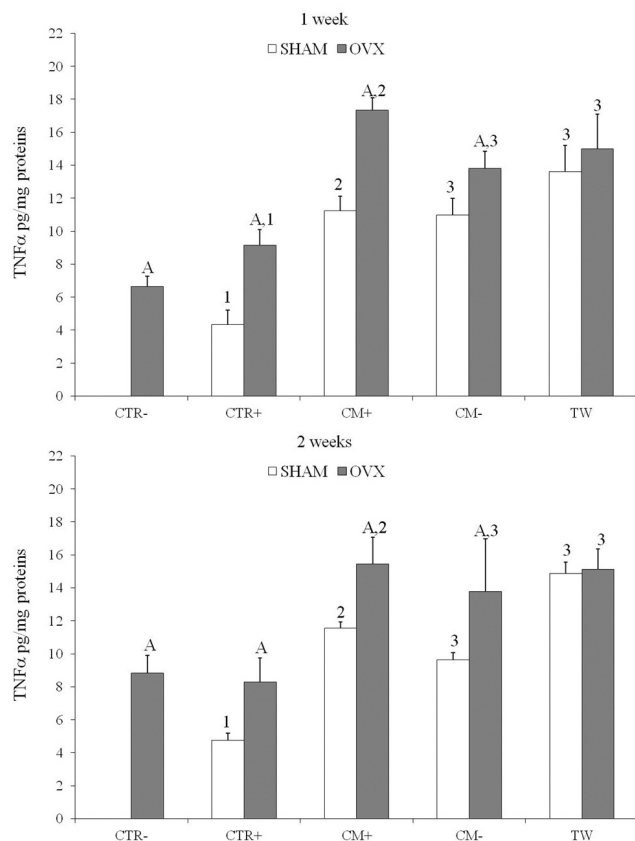


Fig. 7. TNF α related to total protein content after 1 (a) and 2 weeks (b) of PBMC culture (Mean \pm SD, n=4 triplicates). Interaction of 'cell type' and 'culture type' at 1 and 2 weeks. Sidak test ($p < 0.05$): A, PMNC_{OVX} versus PMNC_{SHAM}; 1, CTR⁺ versus CTR⁻; 2, CM⁺ versus CTR⁺; 3, CM⁻ and TW versus CTR⁻. CM⁻: 50% basal culture medium +50% conditioned medium from MRMT-1 breast cancer cells; CM⁺: 50% differentiation medium +50% conditioned medium from MRMT-1 breast cancer cells; TW: co-culture.

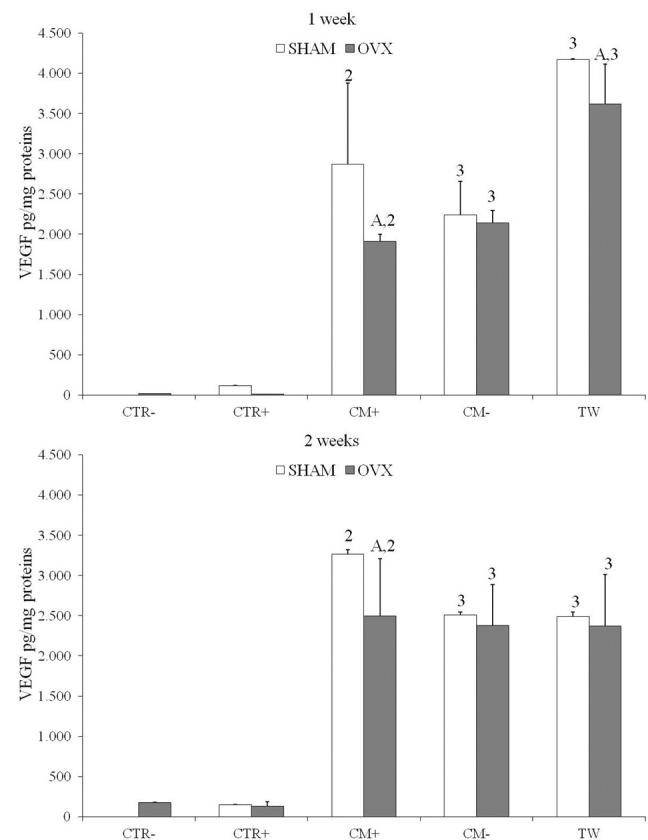


Fig. 8. VEGF related to total protein content after 1 (a) and 2 weeks (b) of PBMC culture (Mean \pm SD, n=4 triplicates). Interaction of 'cell type' and 'culture type' at 1 and 2 weeks. Sidak test ($p < 0.05$): A, PMNC_{OVX} versus PMNC_{SHAM}; 2, CM⁺ versus CTR⁺; 3, CM⁻ and TW versus CTR⁻. CM⁻: 50% basal culture medium +50% conditioned medium from MRMT-1 breast cancer cells; CM⁺: 50% differentiation medium +50% conditioned medium from MRMT-1 breast cancer cells; TW: co-culture.

Kwan Tat et al., 2004), monocytes derived from estrogen deficient rats were found to express more TNF- α than healthy ones in all culture conditions and in particular in co-culture with breast cancer tumor cells, confirming that it is involved in the autocrine regulation of bone resorption (Hagemann et al., 2004).

As previously mentioned, once tumor cells invade bone, they secrete factors that initiate OC-mediated bone destruction, among which the main characterized is VEGF. Whereas VEGF is critical in the development of tumor angiogenesis, its role during osteoporosis has not been clearly established yet and the data currently present in the literature are controversial (Pufe et al., 2003; Costa et al., 2009; Cebi et al., 2010; Senel et al., 2013). Our results revealed an increasing trend in VEGF synthesis in $\text{PBMC}_{\text{SHAM}}$, with the exception of those cultivated in basal medium only (CTR^-), where no cell viability was detected, in comparison to PBMC_{OVX} , in particular in the presence of MRMT-1 or their conditioned medium. These data are in agreement with a

recent study where VEGF concentrations were found to be decreased in post-menopausal patients than in control subjects (Senel et al., 2013). In addition, our study confirmed the implication of VEGF in breast cancer development (Perrot-Appianat and Di Benedetto, 2012) also in the osteoporotic microenvironment.

There is considerable evidence in the literature that different MMPs are present in the tumor bone microenvironment, as essential for tumor growth, invasion and metastasis; in particular, MMP-7 and MMP-9 were found to be expressed by OC of human bone metastasis from breast cancers (Winding et al., 2002; Thiolloy et al., 2009). In fact, it has been demonstrated that particularly MMP-7 was an important mediator of cell-tumor communications by processing soluble factors, such as RANKL, which in turn stimulate osteoclastogenesis (Thiolloy et al., 2009; Sleeman, 2012). Our data indicate that in the presence of tumor cells, the synthesis of MMP-7 by monocytes derived from estrogen deficient rats is significantly higher than

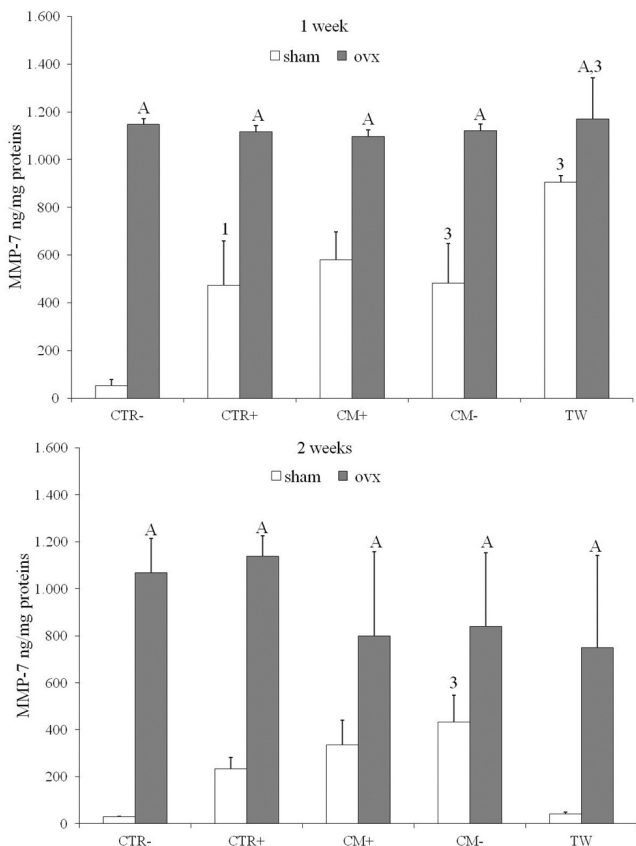


Fig. 9. MMP-7 related to total protein content after 1 (a) and 2 weeks (b) of PBMC culture (Mean \pm SD, n=4 triplicates). Interaction of 'cell type' and 'culture type' at 1 and 2 weeks. Sidak test ($p < 0.05$): A, PMNC_{OVX} versus $\text{PMNC}_{\text{SHAM}}$; 3, CM- and TW versus CTR-. CM-: 50% basal culture medium +50% conditioned medium from MRMT-1 breast cancer cells; CM+: 50% differentiation medium +50% conditioned medium from MRMT-1 breast cancer cells; TW: co-culture.

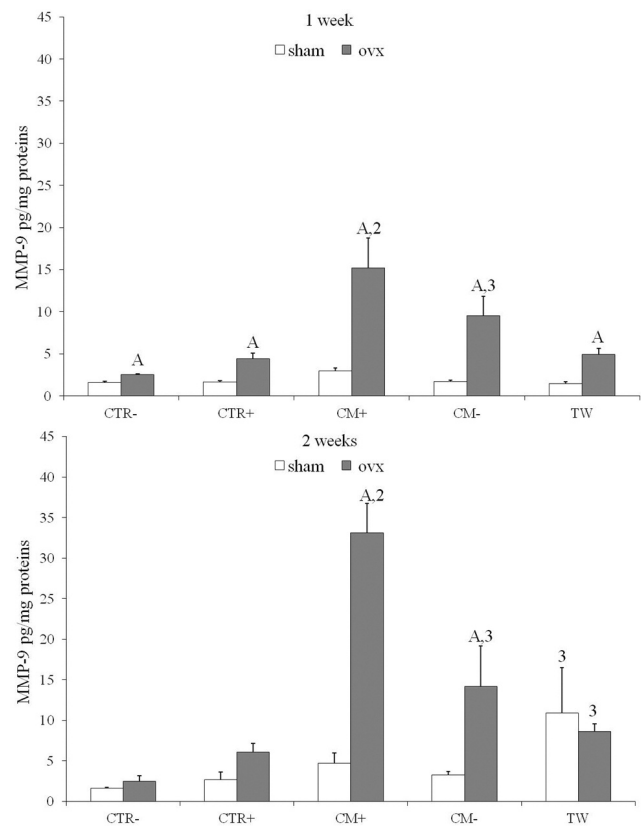


Fig. 10. MMP-9 related to total protein content after 1 (a) and 2 weeks (b) of PBMC culture (Mean \pm SD, n=4 triplicates). Interaction of 'cell type' and 'culture type' at 1 and 2 weeks. Sidak test ($p < 0.05$): A, PMNC_{OVX} versus $\text{PMNC}_{\text{SHAM}}$; 2, CM+ versus CTR+; 3, CM- and TW versus CTR-. CM-: 50% basal culture medium +50% conditioned medium from MRMT-1 breast cancer cells; CM+: 50% differentiation medium +50% conditioned medium from MRMT-1 breast cancer cells; TW: co-culture.

Synergy between osteoporosis and cancer

in healthy ones. Regarding MMP-9, it was shown that PBMCs produced more MMP-9 than healthy cells in the presence of estrogen deficient-induced OP.

Since our data indicated that estrogen deficient-induced OP effectively induced an increased OC differentiation and the synthesis of TNF- α , MMP-7 and MMP-9, thus it can be supposed that OP may provide a more favorable microenvironment for tumor cell growth in comparison with healthy bone.

From a methodological point of view, the simultaneous comparison of the different "culture type", to understand if differentiating factors present in culture medium and the different culture conditions might have an influence on OC response, confirmed the spontaneous ability of PBMC_{OVX} as compared with PBMC_{SHAM}. Additionally, the present study highlighted that rat MRMT-1 cells or their conditioned medium determined a trophic and chemotactic action on PBMC recruitment and activity even in CM⁺ and in TW conditions, where no differentiating factors were present, indicating that the tumor cells are able to promote osteoclastogenesis. This aspect is probably due to the activation of different pathways in which MRMT-1 can synthesize various osteoclastogenic cytokines and GFs. Cytokines produced by tumor cells set up what has been described as a "vicious cycle" of bone breakdown (Guise and Mundy, 1998), in which factors released from the bone matrix fuel breast cancer proliferation, leading to yet more bone loss.

Promising results were thus obtained in this study, indicating that breast carcinoma cells can strengthen responses of PBMCs derived from rats affected by estrogen deficient OP in comparison with healthy ones, although some limitations of the study should be considered. First of all, the use of primary rat-derived cells instead of human cells. The choice of non-human cells, isolated from animals homogeneous for age, strain, estrogen deficient length and bone mineral density, aimed to reduce intra-donor variability. Thus, a rat breast cancer cell line was selected, considering that allogenic *in vitro* models are to be preferred to xenogenic ones. Second, limitations derive from the lack of bidirectional cell signaling information: in fact, the effects of tumor cells on PBMC behavior in the presence of estrogen-deficient OP were investigated without analyzing the reversal effects of PBMCs on tumor cells. Further studies on cell signaling, molecular gene expression and activated pathways, evaluating the effects of tumor cells on OC and vice versa, might lead to an understanding of the causes at the base of the different behavior that was observed in cells derived from healthy and osteoporotic rats. Moreover, 3D and tissue culture models of bone metastases have been developed *in vitro* and are surely more representative of the clinical situation and better mimic complex interactions between cancer cells and bone (Curtin et al., 2012; Krishnan et al., 2014).

To sum up, understanding the cellular mechanisms involved in bone metastasis would be essential for the development of targeted therapies and especially

preventive strategies. It is assumed that targeting the OC-mediated disruption of bone occurring during the rapid phase of estrogen deficient OP might emerge as an important strategy to counteract the microenvironment for the potential development of bone metastases. The present results, confirmed by different culture conditions, may be considered as preliminary "proof of concept" that the rapid phase of estrogen deficiency OP might offer a receptive site for cancer cells to form bone metastases.

Acknowledgements. This work was supported by Rizzoli RIT - Research, Innovation & Technology, Regione Emilia-Romagna and by FIRB 2012-2015 project grant "Idrogeli nanocompositi ibridi contenenti nanoparticelle ferromagnetiche per il trattamento di tumori ossei primitive e secondari".

References

- Akhtri M., Mansuri J., Newman K.A., Guise T.M. and Seth P. (2008). Biology of breast cancer bone metastasis. *Cancer Biol. Ther.* 7, 3-9.
- Bayraktar S., Avery T., Altundag K., Broglio K. and Arun B.K. (2013). Association between tumor characteristics and bone mineral density in postmenopausal breast cancer patients. *Breast J.* 19, 431-434.
- Bingham C.O. 3rd (2002). The pathogenesis of rheumatoid arthritis: pivotal cytokines involved in bone degradation and inflammation. *J. Rheumatol. Suppl.* 65, 3-9.
- Buijs J.T., Stayrook K.R. and Guise T.A. (2012). The role of TGF- β in bone metastasis: novel therapeutic perspectives. *Bonekey Rep.* 1, 96.
- Cauley J.A., Norton L., Lippman M.E., Eckert S., Krueger K.A., Purdie D.W., Farrerons J., Karasik A., Mellstrom D., Ng K.W., Stepan J.J., Powles T.J., Morrow M., Costa A., Silfen S.L., Walls E.L., Schmitt H., Muchmore D.B., Jordan V.C. and Ste-Marie L.G. (2001). Continued breast cancer risk reduction in postmenopausal women treated with raloxifene: 4-year results from the MORE trial. Multiple Outcomes of Raloxifene Evaluation. *Breast Cancer. Res. Treat.* 65, 125-134.
- Cebi H., Akşahin E., Yüksel H.Y., Celebi L., Aktekin C.N., Hapa O., Muratli H.H. and Biçimoğlu A. (2010). Plasma vascular endothelial growth factor levels are similar in subjects with and without osteoporosis. *Eklemler Hastalıkları Cerrahisi.* 21, 91-7.
- Chen Y.C., Sosnoski D.M., Mastro A.M. and Chen Y.C. (2010). Breast cancer metastasis to the bone: mechanisms of bone loss. *Breast Cancer Res.* 12, 215.
- Coleman R., Gnani M., Morgan G. and Clezardin P. (2012). Effects of bone-targeted agents on cancer progression and mortality. *J. Natl. Cancer Inst.* 104, 1059-1067.
- Costa N., Paramanathan S., Mac Donald D., Wierzbicki A.S. and Hampson G. (2009). Factors regulating circulating vascular endothelial growth factor (VEGF): association with bone mineral density (BMD) in post-menopausal osteoporosis. *Cytokine* 46, 376-81.
- Curtin P., Youm H. and Salih E. (2012). Three-dimensional cancer-bone metastasis model using ex-vivo co-cultures of live calvarial bones and cancer cells. *Biomaterials* 33, 1065-1078.
- D'Amelio P., Grimaldi A., Di Bella S., Brianza S.Z., Cristofaro M.A., Tamone C., Giribaldi G., Ulliers D., Pescarmona G.P. and Isaia G.

- (2008). Estrogen deficiency increases osteoclastogenesis up-regulating T cells activity: a key mechanism in osteoporosis. *Bone* 43, 92-100.
- Diel I.J., Jaschke A., Solomayer E.F., Gollan C., Bastert G., Sohn C. and Schuetz F. (2008). Adjuvant oral clodronate improves the overall survival of primary breast cancer patients with micrometastases to the bone marrow: a long-term follow-up. *Ann. Oncol.* 19, 2007-11.
- Edwards J.R. and Mundy G.R. (2011). Advances in osteoclast biology: old findings and new insights from mouse models. *Nat. Rev. Rheumatol.* 7, 235-243.
- Fuller K., Lawrence K.M., Ross J.L., Grabowska U.B., Shiroo M., Samuelsson B. and Chambers T.J. (2008). Cathepsin K inhibitors prevent matrix-derived growth factor degradation by human osteoclasts. *Bone* 42, 200-211.
- Gnant M., Mlineritsch B., Schippinger W., Luschin-Ebengreuth G., Pöstlberger S., Menzel C., Jakesz R., Seifert M., Hubalek M., Bjelic-Radisic V., Samonigg H., Tausch C., Eidtmann H., Steger G., Kwasny W., Dubsy P., Fridrik M., Fitzal F., Stierer M., Rücklinger E., Greil R. and Marth C. (2009). Endocrine therapy plus zoledronic acid in premenopausal breast cancer. *N. Engl. J. Med.* 360, 679-91.
- Grady D., Cauley J.A., Geiger M.J., Kornitzer M., Mosca L., Collins P., Wenger N.K., Song J., Mershon J. and Barrett-Connor E. (2008). Reduced incidence of invasive breast cancer with raloxifene among women at increased coronary risk. *Natl. Cancer. Inst.* 100, 854-61.
- Guisse T.A. and Mundy G.R. (1998). Cancer and bone. *Endocr. Rev.* 19, 18-54.
- Hagemann T., Robinson S.C., Schulz M., Trümper L., Balkwill F.R. and Binder C. (2004). Enhanced invasiveness of breast cancer cell lines upon co-cultivation with macrophages is due to TNF-alpha dependent up-regulation of matrix metalloproteases. *Carcinogenesis.* 25, 1543-1549.
- Hernlund E., Svedbom A., Ivergård M., Compston J., Cooper C., Stenmark J., McCloskey E.V., Jönsson B. and Kanis J.A. (2013). Osteoporosis in the European Union: medical management, epidemiology and economic burden. A report prepared in collaboration with the International Osteoporosis Foundation (IOF) and the European Federation of Pharmaceutical Industry Associations (EFPIA). *Arch. Osteoporos.* 8, 136.
- Itonaga I., Sabokbar A., Sun S.G., Kudo O., Danks L., Ferguson D., Fujikawa Y. and Athanasou N.A. (2004). Transforming growth factor-beta induces osteoclast formation in the absence of RANKL. *Bone* 34, 57-64.
- Kang Y., Siegel P.M., Shu W., Drobnjak M., Kakonen S.M., Cordon-Cardo C., Guise T.A. and Massagué J. (2003). Multigenic program mediating breast cancer metastasis to bone. *Cancer Cell* 3, 537-549.
- Karaplis A.C. and Goltzman D. (2000). PTH and PTHrP effects on the skeleton. *Rev. Endocr. Metab. Disord.* 1, 331-341.
- Kraemer B., Rothmund R., Banys M., Krawczyk N., Solomayer E.F., Mack C., Wallwiener D. and Fehm T. (2011). Impaired bone microenvironment: correlation between bone density and presence of disseminated tumor cells. *Anticancer Res.* 31, 4423-4428.
- Krishnan V., Vogler E.A., Sosnoski D.M. and Mastro A.M. (2014). *In vitro* mimics of bone remodeling and the vicious cycle of cancer in bone. *J. Cell Physiol.* 229(4), 453-62.
- Kristensen B., Ejlersten B., Mouridsen H.T., Jensen M.B., Andersen J., Bjerregaard B., Cold S., Edlund P., Ewertz M., Kamby C., Lindman H., Nordenskjöld B. and Bergh J. (2008). Bisphosphonate treatment in primary breast cancer: results from a randomized comparison of oral pamidronate primary breast cancer. *Acta Oncol.* 47, 740-746.
- Kwan Tat S., Padrines M., Théoleyre S., Heymann D. and Fortun Y. (2004). IL-6, RANKL, TNF-alpha/IL-1: interrelations in bone resorption pathophysiology. *Cytokine Growth Factor Rev.* 15, 49-60.
- Le Gall C., Bellahcène A., Bonnelye E., Gasser J.A., Castronovo V., Green J., Zimmermann J. and Clézardin P. (2007). Cathepsin K inhibitor reduces breast cancer induced osteolysis and skeletal tumor burden. *Cancer Res.* 67, 9894-9902.
- Martino S., Cauley J.A., Barrett-Connor E., Powles T.J., Mershon J., Disch D., Secret R.J. and Cummings S.R. (2004). Continuing Outcomes Relevant to Evista: breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene. *J. Natl. Cancer Inst.* 96, 1751-1761.
- Mundy G.R. (1997). Mechanisms of bone metastasis. *Cancer* 80, 1546-1556.
- Mundy G.R. (2007). Osteoporosis and inflammation. *Nutr. Rev.* 65, 147-151.
- Nicolin V., Bortol R., Bareggi R., Baldini G., Martinelli B. and Narducci P. (2008). Breast adenocarcinoma MCF-7 cell line induces spontaneous osteoclastogenesis via a RANK-ligand-dependent pathway. *Acta Histochem.* 110, 388-396.
- Pacifici R. (2008). Estrogen deficiency, T cells and bone loss. *Cell Immunol.* 252, 68-80.
- Papachristou D.J., Basdra E.K. and Papavassiliou A.G. (2012). Bone metastases: Molecular mechanisms and novel therapeutic interventions. *Med. Res. Rev.* 32, 611-36.
- Perrot-Applanat M. and Di Benedetto M. (2012). Autocrine functions of VEGF in breast tumor cells: adhesion, survival, migration and invasion. *Cell. Adh. Migr.* 6, 547-53.
- Pfeilschifter J., Koditz R., Pfohl M. and Schatz H. (2002). Changes in proinflammatory cytokine activity after menopause. *Endocr. Rev.* 23, 90-119.
- Powles T., Paterson A., McCloskey E., Schein P., Scheffler B., Tidy A., Ashley S., Smith I., Ottestad L. and Kanis J. (2006). Reduction in bone relapse and improved survival with oral clodronate for adjuvant treatment of operable breast cancer. *Breast Cancer Res.* 8, 13
- Pufe T., Scholz-Ahrens K.E., Franke A.T., Petersen W., Mentlein R., Varoga D., Tillmann B., Schrezenmeir J. and Glüer C.C. (2003). The role of vascular endothelial growth factor in glucocorticoid-induced bone loss: evaluation in a minipig model. *Bone* 33, 869-876.
- Quinn J.M., Itoh K., Udagawa N., Hausler K., Yasuda H., Shima N., Mizuno A., Higashio K., Takahashi N., Suda T., Martin T.J. and Gillespie M.T. (2001). Transforming growth factor beta affects osteoclast differentiation via direct and indirect actions. *J. Bone Miner. Res.* 16, 1787-1794.
- Saarto T., Vehmanen L., Virkkunen P. and Blomqvist C. (2004). Ten-year follow-up of a randomized controlled trial of adjuvant clodronate treatment in node-positive breast cancer patients. *Acta Oncol.* 43, 650-656.
- Senel K., Baykal T., Seferoglu B., Altas E.U., Baygülp F., Ugur M. and Kiziltunc A. (2013). Circulating vascular endothelial growth factor concentrations in patients with postmenopausal osteoporosis. *Arch. Med. Sci.* 9, 709-12.
- Sleeman J.P. (2012). The metastatic niche and stromal progression. *Cancer Metastasis Rev.* 31, 429-440.
- Sterling J.A., Edwards J.R., Martin T.J. and Mundy G.R. (2011). Advances in the biology of bone metastasis: how the skeleton affects tumor behavior. *Bone* 48, 6-15.

Synergy between osteoporosis and cancer

- Sturge J., Caley M.P. and Waxman J. (2011). Bone metastasis in prostate cancer: emerging therapeutic strategies. *Nat. Rev. Clin. Oncol.* 8, 357-368.
- Thamake S.I., Raut S.L., Gryczynski Z., Ranjan A.P. and Vishwanatha J.K. (2012). Alendronate coated poly-lactic-co-glycolic acid (PLGA) nanoparticles for active targeting of metastatic breast cancer. *Biomaterials* 33, 7164-7173.
- Thiolloy S., Halpern J., Holt G.E., Schwartz H.S., Mundy G.R., Matrisian L.M. and Lynch C.C. (2009). Osteoclast-derived matrix metalloproteinase-7, but not matrix metalloproteinase-9, contributes to tumor-induced osteolysis. *Cancer Res.* 69, 6747-6755.
- Troen B.R. (2004). The role of cathepsin K in normal bone resorption. *Drug News Perspect.* 17, 19-28.
- Winding B., NicAmhlaoibh R., Misander H., Høegh-Andersen P., Andersen T.L., Holst-Hansen C., Heegaard A.M., Foged N.T., Brünner N. and Delaissé J.M. (2002). Synthetic matrix metalloproteinase inhibitors inhibit growth of established breast cancer osteolytic lesions and prolong survival in mice. *Clin. Cancer Res.* 8, 1932-1939.
- Wu Y.P., Chen W.S., Xu S.J. and Zhang N. (2010). Osteoporosis as a potential contributor to the bone metastases. *Med Hypoth.* 75, 514-516.

Accepted August 7, 2015