Dual function of ERRα in breast cancer and bone metastasis formation: implication of VEGF and osteoprotegerin.

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Abstract

Bone metastasis are a complication occurring in up to 70 percent of advanced breast cancer patients. The estrogen receptor related receptor alpha (ERRα) has been implicated in breast cancer and bone development, prompting us to examine whether ERRα may function in promoting the osteolytic growth of breast cancer cells in bone. In a mouse xenograft model of metastatic human breast cancer over-expression of wild-type ERRα reduced metastasis whereas overexpression of a dominant negative mutant promoted metastasis. Osteoclasts were directly affected and ERRα upregulated the osteoclastogenesis inhibitor, osteoprotegerin (OPG), providing a direct mechanistic basis for understanding how ERRα reduced breast cancer cell growth in bone. In contrast, ERRα overexpression increased breast cancer cell growth in the mammary gland. ERRα - overexpressing primary tumors were highly vascularized, consistent with an observed upregulation of angiogenic growth factor, the vascular endothelial growth factor (VEGF). In support of these findings, we documented that elevated expression of ERRα mRNA in breast carcinomas was associated with high expression of OPG and VEGF and with disease progression. In conclusion, our results demonstrate that ERRα plays a dual role in breast cancer progression in promoting the local growth of tumor cells, but decreasing metastatic growth of osteolytic lesions in bone.
Abbreviations used in this paper: ANOVA: analysis of variance; ERR: estrogen receptor-related receptor; OCs: osteoclasts; OPG: osteoprotegerin; OPN: osteopontin; RANKL: receptor activator of nuclear factor kB ligand; RT: reverse transcription; PCR: polymerase chain reaction; TRAP: tartrate-resistant acid phosphatase; VEGF: vascular endothelial growth factor
Introduction

Bone metastasis are a frequent complication of cancer, occurring in up to 70 percent of patients with advanced breast cancer. Bone metastasis are not a direct cause of death but are associated with significant morbidity (1). For cancer cells to grow in bone, malignant cells recruit and activate osteoclasts (bone resorbing cells) to resorb the bone matrix. Indeed, osteolytic breast cancer metastases are characterized by an increase in osteoclast number and activity at the bone metastatic site, where excessive bone destruction provides a permissive microenvironment for breast cancer cells to proliferate and expand (2, 3). Unfortunately, current treatments for bone metastasis that rely on anti-resorptive agents are only palliative, raising the need for a better understanding of the molecular mechanisms involved in this pathology so as to design potential alternative therapies (3, 4).

Nuclear steroid receptors are transcription factors that comprise both ligand-dependent molecules such as estrogen receptors (ERs) and a large number of so-called orphan receptors, for which no ligand have yet been determined (5). Three orphan receptors, estrogen receptor-related receptor alpha (ERRα), ERRβ and ERRγ, share structural similarities with ERα and ERβ (5), but they do not bind estrogen (6, 7). Sequence alignment of ERRα and the ERs reveals a high similarity (68%) in the 66 amino acids of the DNA binding domain, but only a moderate similarity (36%) in the ligand-binding domain, which may explain the fact that ERRα recognizes the same DNA binding elements as ERs but does not bind estrogen (8). Although ERRα activity is decreased by the synthetic molecule XCT790, no natural ligand has yet been found (9-11).

ERRα is known to regulate fatty acid oxidation and the adaptative bioenergetic response (12, 13). It is widely expressed in normal tissues but several RNA expression studies show its presence in a range of cancerous cells including breast, prostate, endometrial, colorectal and ovarian tumour tissues (14-20). ERRα was markedly increased in neoplastic versus normal tissues and ERRα-positive tumors were associated with more invasive disease and higher risk of recurrences (14, 15). On the other hand, ERα and ERβ were significantly lower in neoplastic versus normal tissue and were associated with better prognosis (14, 17, 18). ERRα is also highly expressed in skeletal tissues (21, 22) and has been reported to regulate osteoblast, osteoclast differentiation and bone formation in vitro (21, 22, 23, 24) and in vivo (25-27). Consistent with these observations, osteopontin...
(OPN) has been reported to be a direct target gene of ERRα in osteoblastic cell lines (28-30). The role of ERRα in bone metastasis formation is currently unknown.

In the light of these findings, we asked here whether ERRα is involved in breast cancer bone metastasis formation and progression, and whether modulating its activity abrogates bone destruction.
Materials and Methods

Ethics statement

BALB/c and NMRI mice were purchased from Charles River laboratories (Wilmington). All procedures involving animals, including housing and care, the method by which they were killed, and experimental protocols were conducted in accordance with a code of practice established by the local ethical committee (CREEA: comite Regionale d’Ethique pour l’Expérimentation Animale). Studies involving human primary breast tumors were performed according to the principles embodied in the Declaration of Helsinki. Patients were included anonymously in this study. All human experiments were approved by the Experimental Review Board from the Laennec School of Medicine.

Breast cancer tissue specimens

The autopsy files of the Department of Pathology (Pr. J. Boniver, Centre Hospitalier Universitaire of Liège, Belgium) were searched for diagnosis of disseminated breast cancer with histologically-proven bone metastasis during the period from 1991 to 1998. Slides were retrieved, and clinical history was obtained. Two breast cancer patients who died with disseminated disease, including bone metastasis, were selected for immunohistochemistry. Soft tissue metastasis were fixed with formalin, dehydrated, and paraffin-embedded.

Breast cancer cohort of patients

In the cohort, patients (n=251) were selected according to the following criteria: primary breast tumor without inflammatory features, no previous treatment (31). Breast cancer tissue biopsies were obtained by surgery, selected by the pathologist and immediately stored in liquid nitrogen until processing. The biopsies were pulverized using a MikroDismembrator (B.Braun Biotech International) and total RNA was extracted using TRI Reagent (Sigma). RNA quality was verified using an Agilent Bioanalyser 2100 (Agilent Technologies). Real-time RT-PCR was performed.

Cell lines and transfection

MDA-BO2-FRT (BO2) cells and stably transfected clonal derivatives were cultured in complete DMEM (Invitrogen), 10% fetal bovine serum (FBS, Perbio) and 1% penicillin/streptomycin (Invitrogen) at 37°C in a 5% CO2 incubator. Characteristics of MDA-MB-231/BO2-FRT (BO2) breast cancer cells were previously
described (32). To avoid potential effects of different insertion sites, a pcDNA5/FRT vector (Invitrogen) was used to obtain the stable BO2-ERRαWT, BO2-ERRαΔAF2, and BO2 (CT) cell lines. Human ERRα cDNA (WT and ΔAF2-AD) was obtained from mRNA extracted from BO2-FRT cells, by using RT-PCR with specific primers ((NM_004451.3): ERRα upstream (177bp): GGG AAG CTT AGC GCC ATG TCC AGC CAG; ERRα downstream (WT) (177-1461bp): GGG GGA TCC CCA CCC CTG TCA GTC C; ERRα downstream (ΔAF2-AD): GGG GGA TCC TCA TGT CTG GCG GAG GAG (177-1350bp; helix11-12 deletion (32 amino acids)). Amplimers were sequenced for verification. The pcDNA5/FRT/ERRα-WT and pcDNA5/FRT/ERRα-ΔAF2-AD constructs were co-transfected with the plasmid POG44 (Invitrogen) conferring the specific integration into the FRT site present in the BO2 cells. For clonal selection, cells were cultured for 4 weeks in the presence of hygromycin (20mg/ml) (Invitrogen). Conditioned medium from all clones and from BO2 treated with the inverse-agonist XCT-790 at 5.10⁻⁷M (Sigma) were obtained after 48h in α-MEM supplemented with 0.5% of serum, then filter sterilized and proteins quantified in order to use equal concentration of proteins for each conditions (25μg).

Animal studies

Tumor fat pad experiments were performed using BO2-ERRαWT-1, BO2-ERRαΔAF2 (pool of AF2-1, 2 and 3 clones) and BO2 (CT1/2) cell lines (10⁶ cells in 50μl of PBS) injected into the fat pad of the 4th mammary gland of female 4-week-old NMRI nude mice (Charles River). Tumor progression was followed by bioluminescence (NightOwl, Berthold), then tumor size and weight were determined after sacrifice at 66 days. Bone metastasis experiments using the same pool of clones were performed in 4-week-old BALB/c nude mice as previously described (33). Cells were suspended at a density of 5X10⁵ in 100μl of PBS and inoculated intravenously into animals. Radiographs (LifeRay HM Plus, Ferrania) of animals were taken at 35 days after inoculation using X-ray (MX-20; Faxitron X-ray Corporation). Animals were sacrificed, hind limbs were collected for histology and histomorphometrics analyses. Tibiae were scanned using microcomputed tomography (Skyscan1076, Skyscan, Belgium) with an 8.8 voxel size and three-dimensional reconstructions were performed with a dedicated visualization software (Amira 5.2, Visage Imaging Inc., Australia). The area
of osteolytic lesions was measured using the computerized image analysis system MorphoExpert (Exploranova). The extent of bone destruction for each animal was expressed in mm².

Bone histomorphometry and histology

Hind limbs from animals were fixed, and embedded in paraffin. Five mm sections were stained with Goldner’s Trichrome and processed for histomorphometric analyses to calculate the BV/TV ratio (bone volume/tissue volume) and the TB/TV ratio (tumor burden/tissue volume). The in situ detection of OC was carried out on sections of bone tissue with metastasis using the tartarte-resistant acid phosphatase (TRAP) activity kit assay (Sigma). The resorption surface (Oc.S/BS) was calculated as the ratio of TRAP-positive trabecular bone surface (Oc.S) to the total bone surface (BS) using the computerized image analysis system MorphoExpert (Exploranova).

Osteoclastogenesis assay

Bone marrow cells from 6-week-old OF1 male mice were cultured for 7 days in differentiation medium: α-MEM medium containing 10% fetal calf serum (Invitrogen), 20 ng/mL of M-CSF (R&D Systems) and 200 ng/mL of soluble recombinant RANKL (34). Cells were continuously (day 1 to day 7) exposed to conditioned medium extracted (25μg proteins for each conditions) from BO2 clones. After 7 days, mature multinucleated OC were stained for TRAP activity (Sigma-Aldrich), and counted as OC when containing three or more nuclei.

Immunofluorescence

BO2 cultures were fixed in culture wells with 3.7% paraformaldehyde (Sigma) in PBS for 10 min and permeabilized with 0.2% Triton X-100 in PBS. Immunodetection was performed using a goat polyclonal antibody against human ERRα (Santa Cruz, Tebu) and the secondary antibody (FITC-conjugated donkey anti-goat) (Rockland, Tebu-bio). The distribution of F-actin was visualized using phalloidin (Molecular Probes)(14). Cells were observed using a LMS510 laser scanning confocal microscope (Zeiss, Le Pecq, France) with a 63X (numerical aperture 1.4) Plan Neo Fluor objective.

Immunoblotting
Cell proteins were extracted, separated in 4-12% SDS-PAGE (Invitrogen), then transferred to nitrocellulose membranes (Millipore) using a semidry system. For ERRα and α-tubulin detection, the same goat polyclonal antibody ERRα (Santa Cruz) and a mouse polyclonal antibody against human α-tubulin (Sigma-Aldrich) were used. Membrane was incubated with secondary antibody HRP-conjugated donkey anti-goat (Santa Cruz) and anti-mouse (Amersham) respectively. An ECL kit (PerkinElmer) was used for detection.

**Immunocytochemistry**

Five μm sections were subjected to immunohistochemistry using the same goat polyclonal antibody ERRα (Santa Cruz) and a rabbit polyclonal antibody against human OPG (Abbiotec). Sections were incubated with secondary antibody HRP-conjugated donkey anti-goat and anti-rabbit respectively (Amersham) (dilution 1/300) for 1 hour. After washing, the sections were revealed by 3,3′-diaminobenzidine (Dako).

**Real time RT-PCR**

Total RNA was extracted with Trizol reagent (Sigma) from cancer cells and OCs. Real-time RT-PCR was performed on a Roche Lightcycler Module (Roche) with specific primers see Table S2. Real-time RT-PCR was carried out by using (SYBR Green; Qiagen,) on the LightCycler system on (Roche) according to the manufacturer’s instructions. Amplimers were all normalized to corresponding L32 values. Data analysis was carried out using the comparative C_T method.

Real-time RT-PCR on breast cancer tissue biopsie mRNA was performed using primers specific for human L32 (101bp): 5'-CAAGGAGCTGGAAGTGCTGC-3', 5'-CAGCTTTCACGATGGCT-3'; TBP (138pb) 5'-TGTTGTGCACAGGACAGCAAG-3', 5'-TTACATCAGCTCCCCAC-3'; ERRα (101bp): 5'-ACCGAGAGATTGTGGTGCACAGCAAG-3', 5'-CATCCACACGCTCTTGAGTACT-3'and OPG (Table S2) and SYBR green (Invitrogen) in 96-well plates on a Mastercycler®EP system (Realplex2, Eppendorf) according to the manufacturer’s instructions with an initial step for 10 min at 95°C followed by 40 cycles of 20 sec at 95°C, 15 sec at Tm (L32: 62°C; TBP: 67°C; ERRα: 59°C) and 10 sec at 72°C. ERRα and OPG expression were normalized with the average of the genes expression encoding the ribosomal protein L32 and the TATA-box binding protein TBP.
Cell invasion assay

Invasion assays were carried out using Bio-Coat migration chambers (Becton Dickinson) with 8μm filters coated with Matrigel as described previously (35). BO2 cells (5x10^4) were plated in the upper chambers and the chemoattractant (10% FBS) in the lower chambers. After 24h at 37°C in 5% CO2 incubator, cells that had migrated through the filters were fixed and stained. Cells were counted (200X magnification). All experiments were run in triplicate and invasion was expressed in cells/mm².

OPG ELISA

Conditioned medium obtained from BO2-CT(1/2), BO2-ERRα-WT-1 and BO2-FRT-ERRαΔAF2 (pool of AF2-1, 2 and 3 clones) were diluted following the manufacturer's instructions and OPG concentration was evaluated using the ELISA kit (RayBiotech).

Statistical analysis

Data were analyzed statistically by one way analysis of variance (ANOVA) followed by post hoc t-tests to assess the differences between groups for in vitro and in vivo studies. Concerning the cohort, the median follow-up at the time of analysis was 54 months. The criterion for statistical analyses was the metastasis free survival (MFS), i.e. the delay between the time of primary surgery and the first event: nodal or distant metastasis, or death. Analysis of the distribution of ERRα expression in relation to usual prognostic parameters was performed using the Mann & Whitney or Kruskall Wallis test. Survival probabilities were estimated using Kaplan Meier estimators and were compared using the log-rank test. Univariate analysis was performed using the Cox proportional hazard model. Results of p<0.05 were considered significant.
Results and Discussion

ERRα mRNA and protein expression in human primary breast tumors and bone metastasis

We analyzed ERRα mRNA expression by real-time RT-PCR in a cohort of 251 breast tumor biopsies (Suppl Table S1) (31). As reported previously by others (14, 15, 17, 18), a statistically significant association was detected in all patients analyzed between ERRα expression and histological type, node status, and ERs (radioligand method) (p=0.026, p<0.001, p<0.001) (Table 1). The Kaplan Meier curve was constructed after segmentation into two groups on the basis of the median value for ERRα expression (Fig.1 A-D). It was observed that high levels of ERRα mRNA expression were related to a decrease in metastasis free survival (N=251, P=0.034) (Fig. 1A). Sixty-two percent of patients (35/56) with high ERRα expression levels exhibited liver, lung, bone and soft tissue metastasis (TM) compared to 38% of patients (21/56) having low ERRα levels (Fig. 1A, see frame). This paralleled the frequencies seen in patients (n = 22) who had developed “only” bone metastases (BM), i.e. 64% (high ERRα) and 36% (low ERRα) (Fig. 1A) suggesting that ERRα is an overall bad prognostic factor that is not a determinant of metastasis location of breast cancer cells. Moreover, high ERRα expression correlated with a higher risk of recurrence at an early stage of the disease in the ER-positive group (N=209), the pN0 subset and in the pN<3 lymph-node positive subset (P=0.04; P=0.029 and P=0.009; log-rank test), when compared to low ERRα (Fig. 1B,C,D) suggesting that ERRα may be a very useful early prognostic marker in breast cancer. Finally, as previously described (15), ERRα protein was present in situ and in invasive breast carcinoma cells (Suppl Fig S1B, C respectively) but not in normal breast epithelial cells (Suppl Fig S1A). ERRα was also clearly present in breast cancer cells that metastasized to bone (Suppl Fig S1H see T). As previously reported by us (21), ERRα was also detected in osteocytes embedded in the bone matrix.

ERRα expression in breast cancer cells reduces their ability to induce osteolytic lesions in vivo

To assess whether ERRα is involved in bone metastasis formation, we used MDA-BO2-FRT (BO2) cells, a subpopulation of the human MDA-231 breast cancer cell line, that was selected for the high efficiency with which it metastasizes to bone (32). ERRα protein was seen in the nucleus and cytoplasm of BO2 cells in vitro
(Fig. 2A) and in situ in bone metastasis from legs of animals, 30 days after intravenous tumor cell inoculation (Fig. 2B).

To establish a functional role for ERRα in bone metastasis development, we next transfected B02 cells with a full-length (wild type; WT) ERRα or a truncated version of ERRα lacking the co-activator binding domain AF2, ERRαΔAF2, which acts as a dominant negative form (22, 23, 36) (Fig. 2C). Constructs of human ERRαWT and ERRαΔAF2 were stably transfected into the genomic FRT site present in the BO2 cells. Three independent BO2-ERRαΔAF2 (1, 2, 3), one BO2-ERRαWT and two BO2-CT (empty vector) clones were obtained, named AF2-1, AF2-2, AF2-3, WT-1, CT-1 and CT-2, respectively. As judged by real-time PCR, total ERRα mRNA expression was increased when compared to CT-1/2 clones (Fig. 2C). Western blotting detected a band of approximately 50kD for ERRα protein in CT1-2 and WT-1 that was increased in WT-1 cells and in AF2-1, AF2-2, and AF2-3 cells. The presence of a band with a slightly lower molecular weight in AF2-1, AF2-2, AF2-3 cells corresponded well with the expected size for truncation of the AF2 domain (42 aa) (Fig. 2D). mRNA expression levels of the ERRα target genes VEGF and OPN were statistically significantly increased in WT-1 cells compared to CT-1/2 cells (Fig. 2E). By contrast, VEGF and OPN mRNA levels remained reduced or unchanged in AF2 clones (Fig. 2E), confirming the increased activity and the dominant-negative functions of the WT and truncated ERRαΔAF2 constructs, respectively.

To assess the involvement of ERRα in bone metastasis formation, CT (pool of CT-1 and 2 clones), WT-1 and AF2 (pool of AF2-1, -2 and -3 clones) cells were inoculated intravenously into female BALB/c nude mice. Thirty-five days after tumor cell injection, radiographic analysis revealed that animals bearing WT-1 tumors had osteolytic lesions that were 40% smaller than those of mice bearing CT tumors (Fig. 3A, B, J). By contrast, there was a 3-fold increase in the extent of osteolytic lesions in animals bearing AF2 tumors, when compared to control (Fig. 3A, C, J). The inhibitory effect of ERRα on cancer-induced bone destruction was confirmed using three-dimensional microCT reconstruction (Fig. 3D-F), histology (Fig. 3G-I) and histomorphometric analyses of tibiae (bone volume (BV/TV); skeletal tumor burden (TB/STV)) (Fig. 3J). Taken together, our results indicated that over-expression of ERRα in breast cancer cells reduced the formation of osteolytic lesions.
**Regulation of osteoclast formation by ERRα-expressing BO2 cells**

Given these data, we next asked whether modulation of ERRα in breast cancer cells could alter osteoclasts (OCs), the bone resorbing cells. Tartrate-resistant acid phosphatase (TRAP) staining of tibial sections of metastatic legs from animals bearing WT-1 and AF2 tumors showed a 43% decrease and a 143% increase of TRAP-positive OC surface (Oc.S/BS) at the bone/tumor cell interface, respectively, when compared to CT tumors (Fig. 4A; Fig. 3J; Suppl Fig S2). Consistent with these *in vivo* data, the treatment of primary mouse bone marrow cell cultures with RANKL and macrophage colony-stimulating factors (M-CSF) together with the conditioned medium of WT-1 cells inhibited the formation of TRAP-positive multinucleated OCs compared to that observed with the conditioned medium of CT cells (Fig. 4B, C). By contrast, the conditioned medium from AF2 cells stimulated OC formation (Fig. 4B, C). In addition, the conditioned medium from parental BO2 cells treated with the inverse-agonist XCT-790, which blocks ERRα activity, increased OC formation compared to control (DMSO) (Fig. 4D), confirming our osteoclastogenesis data obtained with the conditioned medium of AF2 cells.

**ERRα regulates OPG expression in breast cancer cells**

We showed that B02 breast cancer cells over-expressing wild-type ERRα markedly inhibited osteolysis *in vivo* (Fig. 3J), reduced OC formation *in vitro* (Fig. 4). We quantified several markers involved in osteoblasts and OC differentiation and we found that the osteoprotegerin (OPG) a soluble decoy receptor for RANKL that inhibits osteoclastogenesis was regulated by ERRα (Fig. 5A)(37). By immunohistochemistry, we show that OPG expression was higher in skeletal WT-1 tumors compared to that observed in AF2 and CT tumors (Fig. 5B). In addition, as judged by ELISA, WT-1 cells secreted higher amounts of OPG compared to CT-1/2 and AF2 cells (pool of AF2-1, -2 and -3 clones) (Fig. 5C). OPG mRNA expression was also quantified by real time RT-PCR in the cohort of 251 patients. OPG levels were statistically significantly higher in ERRα-positive tumors compared to ERRα-negative tumors (Fig. 5D; p=0.013). Moreover, there was a positive correlation between high mRNA expression levels of both ERRα and OPG (ERRα+/OPG+) and a decrease
in relapse-free survival (p=0.028, log-rank test) (Fig. 5E). All together, the significant correlation between high ERRα and OPG in patients and the regulation of OPG by ERRα in BO2 cells provide a mechanistic basis for the reduction of osteoclastogenesis in vitro and in vivo. Interestingly, OPG in our preclinical data suggest that, alone it had no prognostic value in breast carcinomas (Fig. 5F) while in association with high ERRα mRNA levels, a correlation with a poor clinical outcome in patients was found (Fig. 5E). OPG is not only an osteoclastogenesis inhibitor, but also a survival factor for human breast cancer cells (38, 39). It also promotes angiogenesis (40) and its over-expression in human MCF-7 breast cancer cells enhances tumor growth following orthotopic inoculation in animals (41). ERRα has been implicated in tumor progression and the positive association between high ERRα/OPG mRNA levels and increased risk of recurrences in patients (Fig. 5E) suggested that ERRα could play a role on primary tumor expansion.

**ERRα stimulates tumor growth and angiogenesis in vivo**

To address this hypothesis, orthotopic tumors were induced with CT (pool of CT-1 and -2 clones), WT-1 or AF2 (pool of AF2-1, -2 and -3 clones) cells upon inoculation within the mammary fat pad of NMRI nude female mice. Bioluminescence analysis from day 5 to day 66 revealed a dramatically greater tumor progression in WT-1 tumor-bearing animals compared to that observed with CT and AF2 tumor-bearing animals a modest increase in AF2 tumor burden was also observed at day 62 and 66 (Fig. 6A,B). Tumor weight/size at day 66 (Fig. 6C, D) correlated well with bioluminescence quantification (Fig. 6B,C). Interestingly, WT-1 tumors were highly vascularized compared to CT and AF2 tumors (Fig. 6E), an observation correlating with higher VEGF mRNA levels observed in WT-1 versus AF2 or CT tumors (Fig. 6E). Moreover, if these results are in agreement with previous data describing VEGF as a target gene for ERRα in breast cancer (42), we show for the first time a positive association between high levels of ERRα and VEGF in breast tumors from patients (P =0.002) (Table 1). Interestingly, OPG expression that can be stimulate by VEGF in endothelial cells, is also known to be a positive regulator of microvessel formation in vivo (43) and therefore can participate to the neovascularisation observed in WT-1 tumors. We also observed that ERRα promoted BO2 breast cancer cell invasion in vitro (Suppl Fig S3A) but has a slightly effect on proliferation.
(data not shown). Consistent with this we found matrix metalloproteinases MMP1 and MMP13 regulated by ERRα (Suppl Fig S3B). These results were in agreement with previous findings showing that the silencing of ERRα dramatically reduced the in vitro migratory capacity of breast cancer cell lines (44). Taken together, these results strongly suggested that ERRα promoted tumor growth, mainly through the stimulation of angiogenesis and invasion. Based on our results on high ERRα/OPG/VEGF in our preclinical study, we propose that OPG worked in concert with VEGF to stimulate tumor angiogenesis which, in turn, promoted the growth of BO2-ERRαWT cells. Conversely in bone metastasis although the angiogenic factor VEGF was overproduced in BO2-ERRαWT cells, tumor-derived VEGF had probably a low impact on progression of osteolytic lesions. Indeed, recent studies had shown that hypoxia was nonessential for bone metastasis while promoting angiogenesis in lung metastasis and primary tumor growth (45). Therefore modulating angiogenesis through VEGF and the pro-angiogenic role of OPG may have no impact on angiogenesis in bone, as bone is already extremely vascularized (46), but have dramatic impact on vascularization and progression of primary breast tumors or metastasis to non-bone sites. These data provide novel insights into how ERRα can be a bad prognostic factor in the primary tumor (angiogenesis via VEGF and OPG) but a favorable biomarker in the very special case of bone metastasis (inhibition of OC formation through OPG).

In conclusion, our results show for the first time that ERRα plays a dual role, promoting the progression and invasion of primary tumors, but decreasing osteolytic lesions in bone. Additionally, our data show that OPG is modulated by ERRα that probably contributes to the overall negative clinical outcome which is associated with the expression of ERRα in human breast carcinomas.
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Clinical, biological characteristics and ERα mRNA expression in breast cancer patients

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<td>93</td>
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<td>0.002</td>
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<tr>
<td>ND</td>
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* Histological grade defined only in ductal carcinoma. ** Low: < 50% quartile; High: ≥ 50% quartile

P-values correspond to Mann & Whitney test or Kruskall Wallis test (histological grade and node status)

Table 1
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Legends

Figure 1: ERRα is a bad prognostic marker. Kaplan-Meier curves show correlation between high expression of ERRα, categorized with median value, and metastasis free survival in patients in (A) the whole population (N=251), (B) the ER positive subset (N=209), (C-D) the pN0 subset patients (N=115) and the <3 lymph-node positive subset (N=198). Low ≤ 50% quartile; high ≥ 50%.

Figure 2: Modulation of ERRα in BO2 breast cancer cell line. (A) ERRα protein expression (nucleus and cytoplasm) in BO2 cells by immunofluorescence and confocal microscopy and (B) in vivo by immunohistochemistry in bone metastasis present 30 days after intravenous injection of BO2 cells. (C) Isolation after stable transfection of three independent BO2-ERRαΔAF2 clones (ERRα dominant-negative form), one clone BO2-ERRαWT and two controls (CT-1 and CT-2) BO2-CT (empty vector). ERRα expression was assessed by real-time PCR on triplicate samples and normalized against that of the ribosomal protein gene L32 (ANOVA, p<0.0001) and (D) by Western blotting. (E) VEGF and OPN expression was increased in BO2-ERRαWT and decreased or not regulated in BO2-ERRαΔAF2 (ANOVA, p<0.0001 for VEGF and OPN in WT-1 or ΔAF2 versus CT). (A) bar=20μm and (B) bar=200μm. T: Tumor; GP: growth plate

Figure 3: Overexpression of ERRα inhibits development of bone metastasis. (A-C) BO2-ERRαWT-1, BO2-ERRαΔAF2 (Pool) or BO2-CT (pool) cells were inoculated into BALB/c nude mice; 35 days post inoculation, radiography revealed smaller osteolytic lesions in mice injected with BO2-ERRαWT-1 cells and much larger lesions in mice injected with BO2-ERRαΔAF2 cells compared to mice injected with CT cells (see white arrows). (D-F) Three-dimensional microCT reconstructions of tibiae and (G-J) histology after Goldner’s Trichrome staining confirmed the radiography results. (J) Quantification of bone destruction T: Tumor.

Figure 4: ERRα expression in BO2 cells regulates OCs formation. (A) TRAP staining of OCs in sections of tibiae taken from mice injected with BO2-ERRαWT-1, BO2-ERRαΔAF2 (pool) or BO2-CT (pool) cells shows decreased and increased surface of OCs in BO2-ERRαWT-1 and BO2-ERRαΔAF2 respectively.
compared to CT (ANOVA, p<0.0001). (B-C) Primary mouse bone marrow cells were cultured in the presence of RANKL and M-CSF and treated or not with medium conditioned by BO2-ERRαWT-1, BO2-ERRαΔAF2 or BO2-CT cells. Fewer OCs formed in cultures treated with BO2-ERRαWT-1 conditioned medium, while more formed in cultures treated with BO2-ERRαΔAF2 conditioned medium, compared to CT (1, 2) conditioned medium (ANOVA, p<0.0001). (D) Conditioned medium obtained from parental BO2 cells treated with the ERRα inverse agonist XCT-790 increased OCs formation, mimicking the results obtained with BO2-ERRαΔAF2 conditioned medium (ANOVA, p<0.001). Bar=100μm

**Figure 5: Correlation of ERRα and OPG in BO2 cells and breast cancer patients.** (A) Real-time PCR performed on RNA extracted from BO2 clones showed increased expression of OPG by ERRα (ANOVA, p<0.0001). (B) Staining for OPG is higher in bone metastasis induced by BO2-ERRαWT cells compared to BO2-CT and BO2-ERRαΔAF2 cells; tissues collected 35 days post cell inoculation. (C) ELISA quantification confirmed the increased secretion of OPG by BO2-ERRαWT compared to BO2-CT (pool) and BO2-ERRαΔAF2 (pool) cells (ANOVA, p=0.0064; p<0.01 CT versus WT-1 and WT-1 versus AF-2). (D-E) A significant correlation was also found between levels of ERRα mRNA and median values of OPG mRNA in the cohort (ERRα 1st quartile and median OPG=2.03; ERRα 2nd-4th quartile and median OPG=3.45). Kaplan-Meier curves show that ERRα+/OPG+ expression was associated with a decrease in metastasis free survival. (F) OPG alone was not associated with metastasis free survival.

**Figure 6: Stimulation of tumor progression and angiogenesis by ERRα in vivo.** (A-C) BO2-ERRαWT, BO2-ERRαΔAF2 (pool) or BO2-CT (pool) cells were inoculated into the fat pad of NMRI nude mice. Tumor progression was followed by bioluminescence from day 5-66. Greater tumor expansion was observed in mice with BO2-ERRαWT-1 compared to BO2-ERRαΔAF2 (pool) or BO2-CT (pool) cells. (C-D) Weight and volume of tumors dissected at endpoint (E) and VEGF expression normalized against L32 (ANOVA, p<0.001) within tumors (pool of n=3 for each condition) correlated with greater tumor vascularization.
**Figure S1: ERRα is expressed in bone metastasis.** (B-C) Detection of ERRα by immunohistochemistry on *in situ* and invasive breast cancer cells; ERRα is not expressed in normal breast epithelium (A). (D) ERRα expression is maintained in breast cancer cells that metastasized to bone. Bar=200μm T: Tumor; Ost: osteocytes

**Figure S2: Visualization of OC in vivo.** TRAP (red) staining of OCs (black arrow) in sections of tibiae taken from mice injected with BO2-ERRαWT-1, BO2-ERRαΔAF2 (pool) or BO2-CT (pool) cells was realized to quantify TRAP-positive OC surface (Oc.S/BS) at the bone/tumor cell interface.

**Figure S3: ERRα implication in BO2 invasion.** (A) Cell invasion was increased in BO2-ERRαWT-1 cells and decreased in BO2-ERRαΔAF2 cells versus BO2-CT cells (pool) (ANOVA, p<0.0001). (B) Expression of MMP1 and MMP13 was regulated by ERRα level, as assessed by real-time PCR on triplicate samples; normalized against expression of the ribosomal protein gene L32 (ANOVA, p<0.0001).
A  All patients (MFS)

Cumulative survival

P = 0.034

Number of events
Low - 6 13 16 20
High - 12 22 29 33

Number at risk
Low 125 112 88 55 24
High 126 107 85 49 21

B  ER positive subset

Cumulative survival

P = 0.040

Number of events
Low - 4 9 12 16
High - 6 15 21 24

Number at risk
Low 112 101 82 52 23
High 97 85 67 40 18

C  pN0 subset

Cumulative survival

P = 0.029

Number of events
Low - 0 0 1 4
High - 2 5 6 6

Number at risk
Low 71 64 47 31 11
High 44 35 21 12 6

D  < 3 lymph-node positive subset

Cumulative survival

P = 0.009

Number of events
Low - 1 2 4 8
High - 3 10 16 17

Number at risk
Low 106 98 80 51 21
High 92 82 64 37 16

Figure 1
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BLACK AND WHITE
Figure 2
Fradet et al, 2011
BLACK AND WHITE
Table 3: Morphometric measurements of BO2-FRT Clones

<table>
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<th>BO2-FRT Clones</th>
<th>CT</th>
<th>WT</th>
<th>ΔAF</th>
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<tr>
<td></td>
<td>(mm²/animal)</td>
<td>(n=9)</td>
<td>(n=8)</td>
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<tr>
<td>Radiography</td>
<td>10.9±1.9</td>
<td>7.1±3.4*</td>
<td>30.7±7.2***</td>
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<td>Bone volume (BV/TV,%)</td>
<td>11.4±1.1</td>
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<td>6.4±0.4***</td>
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<td>Tumor burden (TB/STV,%)</td>
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<td>21±4.8*</td>
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<td>Osteolyse (Oc.S/BS,%)</td>
<td>49.3±3.4</td>
<td>21±5.6**</td>
<td>70±6***</td>
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Figure 3

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Figure 4
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**A**  
Relative Normalized Expression (fold change)  

![Bar graph showing relative normalized expression](image)

**B**  
CT- WT-1 AF-2-1 AF-2-2 AF-2-3  

![Image of tissue sections](image)

**C**  
OPG concentration (pg/ml)  

![Bar graph showing OPG concentration](image)

**D**  
Number at risk  

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<td>63</td>
<td>56</td>
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<tr>
<td>ERRα and/or OPG+</td>
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<td>170</td>
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**E**  
Cumulative Survival  

![Survival curve](image)

**F**  
Cumulative Survival  

![Survival curve](image)

**Number at risk**  

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<td>Low</td>
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<td>114</td>
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Figure 5  
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COLOR
**Figure 6**

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COLOR
Dual function of ERRα in breast cancer and bone metastasis formation: implication of VEGF and osteoprotegerin


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