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Estrogen related receptor alpha in castration-resistant prostate cancer cells promotes tumor progression in bone

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ABSTRACT

Bone metastases are one of the main complications of prostate cancer and they are incurable. We investigated whether and how estrogen receptor-related receptor alpha (ERRα) is involved in bone tumor progression associated with advanced prostate cancer. By meta-analysis, we first found that ERRα expression is correlated with castration-resistant prostate cancer (CRPC), the hallmark of progressive disease. We then analyzed tumor cell progression and the associated signaling pathways in gain-of-function/loss-of-function CRPC models in vivo and in vitro. Increased levels of ERRα in tumor cells led to rapid tumor progression, with both bone destruction and formation, and direct impacts on osteoclasts and osteoblasts. VEGF-A, WNT5A and TGFβ1 were upregulated by ERRα in tumor cells and all of these factors also significantly and positively correlated with ERRα expression in CRPC patient specimens. Finally, high levels of ERRα in tumor cells stimulated the pro-metastatic factor periostin expression in the stroma, suggesting that ERRα regulates the tumor stromal cell microenvironment to enhance tumor progression. Taken together, our data demonstrate that ERRα is a regulator of CRPC cell progression in bone. Therefore, inhibiting ERRα may constitute a new therapeutic strategy for prostate cancer skeletal-related events.

INTRODUCTION

Bone metastases are a frequent complication of cancer occurring in up to 80% of patients with advanced prostate cancer (PCa) and castration-resistance (CRPC) ("castration-resistant prostate cancer") with associated poor five-year survival rate [1, 2]. They are not curable and result in impaired mobility and pathological fractures [3]. To grow in bone, tumor cells alter bone formation and resorption by secreting proteins that
directly affect osteoblasts (bone-forming cells) and osteoclasts (bone-resorbing cells) resulting in the development of mixed lesions [1, 4]. These signaling proteins may include RANKL (receptor activator of the NF-κB ligand) which stimulates osteoclast differentiation [1, 5] and osteoprotegerin (OPG) which acts as a decoy receptor for RANKL receptor and inhibits osteoclastogenesis [5]. Therefore, the balance between RANKL and OPG is critical in controlling osteoclast activity and osteolysis in bone metastases. PCA cells also express factors such as TGFβ (transforming growth factor beta), WNT family members such as Wnt5a and the pro-angiogenic factor VEGFA that promote an aggressive tumor phenotype and bone metastases by directly affecting osteoclast and osteoblast formation [6, 7]. The induction of stromal niche signals by tumor cells, for example expression of extracellular matrix proteins such as PERIOD1 (POSTN) in the tumor microenvironment, also contributes to the expansion of the metastatic niches [8–11].

Nuclear receptors are transcription factors that comprise ligand-dependent molecules, such as estrogen receptors (ERs), and a large number of so-called orphan receptors for which no ligand has yet been determined [12]. Estrogen receptor-related receptor alpha (ERRα) (NR3B1) shares structural similarities with ERα and ERβ (NR3A1/NR3A2) [13] but does not bind estrogen [14]. Since very recently, ERRα was considering as the oldest orphan receptor but Wei et al. just described the cholesterol as a potential ERRα agonist [15]. Synthetic molecules like the inverse agonist XCT-790 were also designed to block ERRα activity by preventing its interaction with the co-activators peroxisome proliferator-activated receptor gamma coactivator (PGC1) [16].

ERRα is expressed in a range of cancer cell types and ERRα-positive tumors (breast and prostate) are associated with more invasive disease and higher risk of recurrence [17, 18]. Indeed in prostate cancer, ERRα is significantly higher in cancerous lesions compared to benign foci and high level of ERRα correlates with Gleason score and poor survival [18]. Moreover, in androgen receptor (AR)-positive models, ERRα has been implicated in AR signaling pathways and shown to increase HIF-1 signaling and to promote hypoxic growth adaptation of prostate cancer cells [19, 20]. ERRα is also expressed in bone where it regulates differentiation and activity of osteoblasts and osteoclasts, both of which are implicated into the mixed osteolytic and osteoblastic lesions observed in advanced prostate cancer patients [15, 21]. Based on our previous data in bone metastases from breast cancer [22], and on the fact that bone metastases are the hallmark of progressive disease and CRPC, mainly characterized by AR alterations [23], we investigated whether and how ERRα is involved in bone progression of CRPC (AR-negative) models.

RESULTS

ERRα is more highly expressed in CRPC patients and their associated bone metastases than normal prostate and non-metastasizing PCa

To determine whether ERRα is involved in PCa bone lesions, we first assessed ERRα mRNA expression (ERRα) levels during disease progression by performing a meta-analysis of data from the gene expression omnibus (GEO; GSE69129, GSE21034 and GSE32269) (Figure 1A–IC, Supplementary Table S1)[24, 25, 26]. We found that ERRα expression was significantly higher in CRPC compared to normal prostate (P = 0.0172) (Figure 1A) and (P = < 0.05, n = 22 (normal) vs n = 41 (CRPC)) (Figure 1B). Higher ERRα expression was also observed in primary tumors from CRPC patients who had developed bone metastases compared to androgen-sensitive PCa patients (P < 0.005, (PCa) vs (CRPC bone Mets)) (Figure 1B) and (P = 0.0178, (PCa) vs (CRPC who all developed bone metastases)) (Figure 1C). In the dataset GSE21034, we also found that ERRα mRNA was significantly higher in primary cancerous prostate lesions from CRPC who developed bone metastatic lesions (n = 5) compared to patients with had developed other types of metastases (brain, lung, bladder, colon or lymph nodes) (n = 41) (P < 0.05. Figure 1B) suggesting that ERRα is associated with advanced prostate cancer and bone metastases. Immunohistochemistry also revealed that ERRα protein expression in human PCa cells was maintained in the associated bone metastases (Figure 1D), suggesting that ERRα is an overall poor prognostic factor for bone metastases from CRPC.

ERRα in PCA cells promotes tumor cells progression in vivo in bone microenvironment

To address ERRα function in PCA bone progression, we used three CRPC pre-clinical models, two human models (PC3 and PC3c) and one canine model (ACE-1). Specifically, a full-length ERRα cDNA was stably transfected into PC3 cells, which are known for their capacity to form osteolytic lesions in vivo [27]. Three independent PC3-ERRα clones (overexpressing ERRα) and three PC3-CT clones (harboring empty vector) were generated (Figure 1E, IF). In parallel, to validate further the human PC3 model, human PC3c and canine ACE-1 PCa cells that both induce mixed bone lesions (with both osteolysis and osteoformation) were stably transfected with full-length ERRα cDNA (Figure 1H, I) [28][29]. ACE-1 cells were also transfected with cDNA containing a truncated form of ERRα lacking the co-activator binding domain AF2 (AF2) (Figure 1J) [22]. Western blotting confirmed higher ERRα expression in PC3-ERRα, PC3c-ERRα and ACE-1-ERRα than in their respective control
cells (Figure 1E, 1H, 1J). The presence of a slightly lower molecular weight band in AF2 in ACE-1 cells expressing the ERRα-AF2 deletion mutant corresponded well with its expected smaller size (AF2; Figure 1J) [22]. As expected, expression of mRNA for VEGF-A, a known ERRα target gene [30] was higher in all of the ERRα overexpressing clones (ERRα, PC3, PC3e and ACE-1) but not in the AF2 ACE-1 clone, confirming the increased activity and the dominant negative functions of both wild-type ERRα and the truncated ERRα-AF2 constructs respectively (Figure 1G, 1H, 1K). To assess whether and how levels of ERRα in tumor cells affected progression of bone lesions, PC3, PC3e and ACE-1 clones were inoculated via intratibial injections into SCID male mice (Figure 2). Three weeks (for PC3 (pool of the 3 clones for CT and ERRα respectively) and ACE-1 clones (Figure 2 PC3 (A–E), ACE-1 (K–Q)) and six weeks (for PC3e clones) (Figure 2 PC3e (F–J)) after tumor cell injections, radiographs revealed that animals bearing ERRα overexpressing tumors had increased bone lesion surfaces whereas ACE-2F2 tumors had decreased bone lesion surface compared to CT tumors (Figure 2 -PC3 (A–B), (Mann-Whitney, P = 0.011) (bone lesion surface mm²)(E), -PC3e (F–G), (Mann-Whitney, P = 0.0175)(D) -ACE-1 (K–M) (Mann-Whitney, P = 0.007, P = 0.0304) (Q)). The stimulatory effect of ERRα on PCa-induced bone lesion surface was confirmed by three-dimensional micro-computed tomographic reconstruction (9%BV/TV) (cortical and

Figure 1: ERRα expression and CRPC from PCa patients. (A) Meta-analysis using public datasets showed that ERRα mRNA expression is higher in CRPC patients in GSE6919 (Student’s t-test P = 0.0172). (B) ERRα was also found to be higher in CRPC compared to androgen-sensitive PCa, as well as in primary tumors from CRPC patients that developed metastases to bone compared to other sites or normal prostate tissues in GSE21034 (One way ANOVA, bonferroni post-hoc test : P < 0.05, normal (n = 22) versus CRPC (n = 41); P < 0.0005, normal (n = 22) versus CRPC bone mets (n = 5); P < 0.005, PCa (n = 104) versus CRPC bone mets (n = 5)); and (C) PCa versus CRPC (that all had developed bone metastases) in GSE32269 (Student’s t-test P = 0.0178); *P < 0.05, **P < 0.005, ***P < 0.0005. (D) Visualization of ERRα protein expression by IHC on sections of prostate primary tumor (a) and the associated bone metastatic lesions (b) from the same patient. (E) Assessment of ERRα expression by Western blotting and (F) real-time RT-PCR on triplicate samples and normalized against the ribosomal protein gene L32 (ANOVA, Student’s t-tests P < 0.0001) in PC3 control (CT-1-3) and PC3-ERRα (ERRα-1-3) overexpressing ERRα clones. (G) Increased expression of VEGFA mRNA in PC3-ERRα (ANOVA, Student’s t-tests P < 0.0001). (H) Increase of ERRα protein expression in PC3e-ERRα (ERRα) overexpressing ERRα shown by Western blot and (I) by real-time RT-PCR for VEGF-A expression (Student’s t-tests P < 0.0001). (J) Assessment of ERRα expression by Western blotting in an ACE-1 empty-vector CT clone, an ACE-ERRα and a clone overexpressing the dominant negative ERRα with AF2 domain deletion (AF2). (K) VEGF-A mRNA expression was also increased in ACE-ERRα cells (Student’s t-tests P = 0.0001). Bar = 200 μm, T: Tumor, Ost: osteocytes, BM: Bone Matrix.
trabecular bone), with a decrease in bone volume in animals bearing PC3-ERRα and ACE-1-ERRα tumors (%B/VTV, Mann-Whitney, PC3 P = 0.022 and, ACE-1 P = 0.0411) suggesting an increase in bone destruction in both ERRα overexpression models (Figure 2E and 2Q, %B/VTV). The stimulatory effect of ERRα on PC3-induced bone lesion surface was also evident by histology (Figure 2C(D)) and histomorphometric analysis (TB/STV) with an increase of skeletal tumor burden (Figure 2E and 2Q). Since the osteoblastic region is highly stimulated in the PC3c model (Figure 2H, 2I) (see the increased of the %B/VTV; (Mann-Whitney, P = 0.022)), the surface of the tumor (TB/STV) decreased in animals bearing PC3c-ERRα (Figure 2J (Mann-Whitney, P = 0.0023)) (asterisks showing bone formation). Similarly, 70% of mice bearing PC3c-ERRα tumors exhibited small new bone formation compared to mice bearing PC3-CT tumors (Figure 2E). New bone formation was also seen in animals bearing ACE-1-ERRα versus ACE-1-CT tumors (Figure 2Q: extra-bone new spicules surface formation tissue volume; Figure 2 (N-P) (asterisks mark extra-new spicules bone formation). The bone lesion surface and bone volume-new

Figure 2: Over-expression of ERRα in prostate cancer cells induced bone lesions development. Radiography revealed larger lesions in mice injected with (A, B) PC3-ERRα versus PC3-CT, and (F, G) with PC3c-ERRα versus PC3c-CT. Histology after Goldner’s trichrome staining confirmed these radiography results in mice injected with (C, D) PC3-ERRα versus PC3-CT (H, I) with PC3c-ERRα versus PC3c-CT (bone matrix in green). (E) Induction of larger bone lesions surface in mice injected with PC3-ERRα (ERRα) (Mann-Whitney, P = 0.011), of a decrease in %B/VTV (Mann-Whitney, P = 0.022) and an increase of %TB/STV (Mann-Whitney, P = 0.008) compared with mice injected with PC3-CT (CT). Bone formation incidence show that 70% of mice injected with PC3-ERRα (ERRα) developed some bone formation as opposed to 10% of mice injected with PC3-CT (CT). (J) Increased bone lesions surface in mice injected with PC3c-ERRα (Mann-Whitney, P = 0.0175), of the decrease in %B/VTV (Mann-Whitney, P = 0.022) and of decrease of the %TB/STV (Mann-Whitney, P = 0.0023) compared with mice injected with PC3c-CT (CT). Radiography (K-M) and 3D microtomographic reconstructions (N-P) showed larger bone lesions in mice injected with ACE-ERRα versus ACE-CT with an abrogation of the bone lesion effects seen with ERRα overexpression in tumors bearing the dominant negative AF2-truncated ERRα. (Q) After 3 weeks post inoculation of ACE-ERRα, ACE-CT and ACE-AF2 cells, radiography revealed larger and smaller bone lesions surface in mice injected with ACE-ERRα and ACE-AF2 respectively compared to CT (Mann-Whitney, P = 0.0079, P = 0.0304) and microtomographic reconstructions of tissue show a decrease in mice injected with ACE-ERRα compared to CT (%B/VTV; Mann-Whitney, P = 0.0411), an increase in %TB/STV (Wilcoxon, P = 0.034) compared to CT, and an increase in % new bone formation/TV (extra-bone spicules formation): (Mann-Whitney, P = 0.0025). The increase in bone lesion surface (Mann-Whitney, P = 0.0011), in the %TB/STV (Mann-Whitney, P = 0.0052), in the extra-bone spicules formation (Mann-Whitney, P = 0.0012) and the decrease in %B/VTV (Wilcoxon, P = 0.0273) effects seen with ERRα overexpression were markedly abrogated in tumors bearing the dominant negative AF2-truncated ERRα. * P < 0.05; ** P < 0.001; *** P < 0.0001. T: Tumor; * bone formation; Arrow: bone degradation.
bone formation effects seen with ERRα overexpression were markedly abrogated in tumors bearing the dominant negative AF2-truncated ERRα (Figure 2K, 2M and 2N, 2P, 2Q). Taken together, our results indicate that overexpression of ERRα in PCs cells stimulates both new bone formation and destruction suggesting that it may be associated with mechanisms mediating mixed lesions in vivo.

Modulation of ERRα expression in cancer cells affects the bone microenvironment

Since our in vivo data suggested an impact of ERRα expression levels on PCa-induced bone destruction and formation, we next assessed whether PCa overexpressing ERRα cells affected osteoclasts (bone-resorbing cells) and osteoblasts (bone-forming cells). A 0% increase in TRAP-positive osteoclast surface (% Ocs/BS) was seen at the bone-tumor cell interface in PC3-ERRα tumors (Figure 3A). Consistent with these in vivo data, the number of TRAP-positive cells (Figure 3B) and the expression of osteoclast markers (trap, ck, call and rank) (Figure 3C) were higher in co-cultures of primary mouse bone marrow cells with PC3-ERRα cells compared to PC3-CT cells [5]. Moreover, treatment of bone marrow cells by the conditioned medium obtained from parental PC3 cells treated with the inverse agonist XCT-790, which blocks ERRα activity, inhibited osteoclast formation (Figure 3D). Similarly, PC3c- ERRα cells co-cultured with primary mouse bone marrow cells also stimulated osteoclast formation compared to PC3c-CT cells (Figure 3E), as did ACE-1-ERRα compared to ACE-1-CT cells while ACE-1-AF2 inhibited osteoclast formation compared to ACE-1-ERRα (Figure 3F) suggesting that cancerous cells expressing ERRα increase osteoclastogenesis.

The increased bone formation observed in vivo suggests that changes in ERRα expression in PCa cells also alters differentiation of osteoclasts. Consistent with this hypothesis, a higher number of bone nodules formed in primary mouse calvaria cells cultured with PC3-ERRα versus PC3-CT conditioned medium (Figure 4A). Similarly, the expression of the osteoclastic markers alkaline phosphatase (alp), bone sialoprotein (bsp) and osteocalcin (ocn) increased (Figure 4C) in co-cultures of MC3T3-E1 and PC3-ERRα cells (ERRα) (Figure 4C) [31]. The pro-osteoclastic factors rankl but not nog, was increased in MC3T3-E1 cells co-cultured with PC3-ERRα.

Figure 3: ERRα overexpression in PCa-ERRα cells modified bone-resorbing cells. (A) Increase in osteoclast (Oc) surface in bone lesions induced by PC3-ERRα cells in vivo (% Oc/BS: Mann-Whitney, P = 0.0062; n = 7 (CT) and n = 10 (ERRα)). (B) PC3-ERRα cells increase the number of TRAP+ osteoclasts in vitro (Oc number/well: paired t-test, P = 0.0275). (C) mRNA was extracted from co-cultures on day 7. The expression of trap (Tartrate Resistant Acid Phosphatase), ck (Cathepsin K), rank and call (Carbonic Anhydrase II) was assessed by real-time RT-PCR on replicate samples; all markers were higher in Oc/PC3-ERRα (ERRα) versus Oc/PC3-CT (Student’s t-tests, P = 0.0026; P = 0.0055, P = 0.0057; P = 0.008). (D) Conditioned medium obtained from parental PC3 cells treated with the inverse agonist XCT-790 decreased Oc formation, confirming the results obtained with PC3-ERRα (Student’s t-tests, P = 0.0023). (E-F) PC3c-ERRα (E) and ACE-ERRα (F) increased the number of TRAP+ osteoclasts in vitro compared to the respective controls (Oc number/well: paired t-test, P = 0.022 (PC3c-ERRα versus PC3c-CT) and ANOVA p = 0.0048, P < 0.05 (ACE-1-ERRα versus ACE-1-CT) while ACE-1-AF2 inhibited Oc formation P < 0.005 (ACE-1-AF2 versus ACE-1-ERRα). Oc results are representative of two independent experiments, each performed in triplicate samples.
cells (Figure 4D). Consistent with this, we observed a trend towards an increase in the number of bone nodules formed in primary mouse calvaria cells cultured with PC3c-ERRα conditioned medium (Figure 4B) and an increase in osteoblastic markers bsp and ocn expression in co-cultures of MC3T3-E1 and ACE-1-ERRα cells (ERRα) (Figure 4E). In contrast, no change in expression of either osteoblastic markers was detected in co-cultures of MC3T3-E1 with ACE-1-AF2 cells (Figure 4E). Also no regulation of rankl and opg expression was noticed (Figure 4F). Taken together, our results suggest that ERRα expression in PCa cells induces changes in osteoblast lineage cells.

Identification of two factors regulated in PCa cells that couple bone resorption and formation: VEGFA and Wnt5a

Since both osteoclasts and osteoblasts are stimulated by PCa cells overexpressing ERRα, we next sought to identify factors secreted by PCa cells that may stimulate both lineages. As shown earlier, PC3, PC3c and ACE-1 cells overexpressing ERRα stimulate expression of VEGF-A (Figure 1G, I, K), a known ERRα target and stimulator of osteoblasts and osteoclasts [32, 33]. By immunohistochemistry, ERRα (Figure 5A, 5B) and VEGF-A (Figure 5C, 5D) expression was observed in PCa cells and higher in PC3-ERRα-induced bone lesions in vivo (Figure 5B and 5D respectively) compared to PC3-CT lesions (Figure 5A and 5C respectively). Regulation of VEGF-A by ERRα was confirmed in vitro in PC3 cells transfected with a pool of three ERRα-siRNA sequences (Si) compared to control sequences (Sc) (Figure 5E–5G). Blocking ERRα activity by treating PC3-ERRα cells with the ERRα inverse agonist XCT-790 also demonstrated a statistical down-regulation of VEGF-A (Figure 5H). That ERRα regulates VEGF-A in both non-bone and bone microenvironments was determined by assessing subcutaneous (SC) tumors obtained by injecting PC3-ERRα and PC3-CT clones in male SCID mice. VEGF-A expression was higher in PC3-ERRα (ERRα)-tumors compared to PC3-CT (CT)-induced SC tumors in vivo (Figure 5I–5J). The correlation of increased ERRα and increased VEGF-A expression was confirmed in PC3c-ERRα-induced bone

Figure 4: ERRα overexpression in PCa-ERRα cells stimulates bone-forming cells. (A) Osteoblasts (Ob) from primary mouse calvaria cell cultures were treated with conditioned medium of PC3-ERRα (ERRα) or PC3-CT (CT) cells. Bone nodule number was higher in co-cultures with PC3-overexpressing ERRα (Ob-ERRα) (Mann-Whitney, p = 0.0118). (B) When treated with conditioned medium of PC3c-ERRα (ERRα), bone nodule number was increased but not significantly compared to PC3c-CT (CT). (C) Confirming mouse calvaria results, co-cultures of PC3-ERRα (ERRα) or PC3-CT (CT) cells with the MC3T3-E1 osteoblastic cell line for 2 weeks stimulated expression of osteoblastic markers alp (Alcalin Phosphatase), bsp (bone sialoprotein) and ocn (osteocalcin) (ANOVA, P < 0.0001; Student’s t-tests, P = 0.0031, P = 0.0098; P = 0.0017) (D) Expression of rankl mRNA was also higher (ANOVA, P < 0.0001, Student’s t-tests, P = 0.0279) while opg expression was not altered. (E) Co-cultures of ACE-1-ERRα (ERRα) or ACE-1-CT (CT) cells with the MC3T3-E1 osteoblastic cell line for 2 weeks also stimulated bsp and ocn mRNA expression (ANOVA bsp, p = 0.004; Student’s t-tests, p = 0.0003 (ACE-1-CT versus ACE-1-ERRα), ANOVA ocn, p = 0.0069; Student’s t-tests, p = 0.0067 (ACE-1-CT versus ACE-1-ERRα) while no regulation was observed with ACE-1-AF2 cells (AF2). (F) Expression of rankl and opg mRNA was not regulated. Ob results are representative of two independent experiments, each performed in triplicate samples.
lesions in vivo (Figure 5L, 5N) compared to PC3c-CT lesions (Figure 5K, 5M). It is interesting to mention that beside tumor mass, **vegfa** was also highly expressed in peri-tumoral cells in PC3c-CT and PC3c-ERRα (Figure 5M, 5N) compared to PC3-CT and PC3-ERRα-induced bone lesions in vivo (Figure 5C, 5D). This peri-tumoral vegfa-expression that probably contributes to the formation of the mixed phenotype observed in PC3c cells, was not modulated in PC3-ERRα-induced bone lesions in vivo compared to CT.

Finally, in line with our pre-clinical models, we found that **VEGF-A** positively correlated with **ERRα** expression levels in primary CRPC tumor samples from combined microarray datasets GSE10344, GSE6919 and GSE2269 (n = 140, r = 0.364, P < 0.0001) and in bone metastases (GSE29650, GSE41619 and GSE74685) [34–36] (n = 64, r = 0.646, P < 0.0001) (Table 1, Supplementary Table S1).

The second factor that was found upregulated by **ERRα** in our human preclinical models is **WNT5A**, a factor also known to stimulate osteoblasts and osteoclasts [37] and previously linked to **ERRα** in osteoblasts [38]. Thus, **WNT5A** expression was higher in **ERRα**-overexpressing PC3 cells (PC3-ERRα) compared to PC3-CT clones (Figure 6A), and down-regulating **ERRα** levels in PC3 cells with **ERRα-SiRNA** (pool of three sequences) decreased **WNT5A** expression compared to control sequences (Sc, Figure 6B). **WNT5A** expression was also upregulated in PC3-ERRα- versus PC3-CT-induced bone lesions in vivo (Figure 6C, a versus b). XCT-790 treated PC3-ERRα cells also demonstrated a down-regulation of **WNT5A** expression (Figure 6D).

Stimulation of **WNT5A** expression by **ERRα** was also confirmed in PC3-ERRα- versus PC3-CT-induced SC tumors in vivo (Figure 6E), and in PC3c-ERRα-versus PC3c-CT clones in vitro (Figure 6F) as well as in PC3c-ERRα versus PC3c-CT-induced bone lesions in vivo (Figure 6G, a versus b). Notably, **WNT5A** expression also positively correlated with **ERRα** expression levels in bone metastases microarray datasets (GSE29650, GSE41619).

Figure 5: Stimulation of **VEGF-A** expression in PCa cells by **ERRα**. (A–B) Visualization of the overexpression of **ERRα** protein expression in tumor by IHC on bone lesions in vivo induced by PC3-ERRα (ERRα)(B) compared PC3-CT (CT)(A). (C–D) Paralleling the overexpression of **ERRα**, **VEGF-A** expression in tumor was also stimulated in vivo in bone lesions induced by PC3-ERRα (ERRα) (D) compared PC3-CT (CT) (C). (E) Decreased **ERRα** protein expression by transfection of three pooled siRNA sequences in parental PC3 cells shown by Western blot and (F–G) by real-time RT-PCR for **ERRα** and **VEGF-A** expression (Student’s t-tests P = 0.0002; P = 0.0027). (H) Decreased **VEGF-A** mRNA expression was also observed after XCT-790 treatment at 10 μM for 48 h in PC3-ERRα cells (Student’s t-tests P = 0.006). (I–J) Real-time RT-PCR was performed on triplicate samples and normalized against the ribosomal protein gene L32 to evaluate **ERRα** (P = 0.0001) and **VEGF-A** (P = 0.0001) expression within subcutaneous (SC) tumors in vivo (pool of n = 3 for each condition). (K–N) Similarly, to the PC3 model, IHC revealed that **ERRα** and **VEGF-A** protein levels in tumors were increased in mixed lesions induced by PC3c-ERRα (ERRα)(L and N concomitantly) compared to PC3c-CT (CT) (K and M concomitantly) in vivo. Bar = 200 μm, T: Tumor; OB: osteoblasts; BM: Bone Matrix.

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and GSE74685) \((n = 64, r = 0.342, P = 0.00572)\). Contrary to bone metastases specimens, a negative correlation with a weak Pearson \(R\) value was observed in primary CRPC tumor samples from combined microarray datasets GSE1034, GSE6919 and GSE2269 (\(n = 140, r = -0.178, P < 0.0001\)) (Table 1; Supplementary Table S1).

**Stimulation of stromal POSTN expression by tumor cells overexpressing ER\(\alpha\).**

Given that WNT signaling is known to be recruited by the extracellular matrix protein PERIOSTINE (POSTN) in the stroma of at least certain tumor types and to participate in the steps required for cancer cell colonization and tumor progression [8], we next analyzed postn expression in our tumor models. By real-time PCR, we found mouse postn expression up-regulated in PC3-ERR\(\alpha\) versus PC3-CT SC tumors (Figure 7A). Immunostaining confirmed the stimulation of postn in peri-tumoral infiltrating cells in PC3-ERR\(\alpha\) bone lesions (Figure 7B, a versus b), in PC3-ERR\(\alpha\) bone lesions (Figure 7C, a versus b) and in peri-tumoral cells in PC3-ERR\(\alpha\) SC tumor versus PC3-CT SC tumors (Supplementary Figure S1A a versus b). Vimentin expression was also addressed to show the stromal phenotype of the peri-tumoral cells expressing postn (Figure 7Cc). Importantly, ER\(\alpha\) and postn expression levels were significantly positively correlated in bone metastases specimens (\(n = 44, r = 0.528, P = 0.00023\)) (Table 1; Supplementary Table S1). A positive correlation was also found in adjacent normal tissue of primary CRPC tumors (\(n = 184, r = 0.401, P < 0.0001\)) confirming the stimulation of POSTN in peri-tumoral cells (Table1; Supplementary Table S1).

Postn has been shown to be stimulated in infiltrating stromal cells by factors secreted by breast cancer cells such as TGF\(\beta\) [8, 9]. By real-time PCR, we found higher human TGF\(\beta\) expression in PC3-ERR\(\alpha\) clones compared to PC3-CT (Figure 7D). Regulation of TGF\(\beta\) expression by ERR\(\alpha\) in PCa was also confirmed by the lower levels of TGF\(\beta\) expression in ERR\(\alpha\)-SiRNA versus control sequences-transfected PC3 cells (Si versus Sc, Figure 7E), in XCT-790 treated PC3-ERR\(\alpha\) cells versus DMSO (Figure 7F) and by its stimulation in PC3-ERR\(\alpha\) versus PC3-CT SC tumors in vivo (Figure 7G). Immunohistochemistry also confirmed higher TGF\(\beta\) staining in tumor cells in PC3-ERR\(\alpha\) (Figure 7Hb) compared to PC3-CT-induced (Figure 7Ha) bone lesions in vivo and in PC3-ERR\(\alpha\) (Supplementary Figure S1Bb) compared to PC3-CT-induced bone lesions in vivo (Supplementary Figure S1Ba). Finally, ERR\(\alpha\) and TGF\(\beta\) expression levels were significantly positively correlated in primary CRPC tumor samples (\(n = 140, r = 0.684\),

![Figure 6: Stimulation of WNT5A expression in PCa cells by ER\(\alpha\).](image-url)

(A) Assessment of WNT5A expression by real-time RT-PCR on triplicate samples and normalized against the ribosomal protein gene L32. WNT5A (Student's t-tests \(P = 0.0305\)) was higher in PC3-ERR\(\alpha\) versus PC3-CT (CT) cells in vitro. (B) WNT5A (Student's t-tests \(P = 0.0007\)) mRNA expression was decreased in Sc-ERR\(\alpha\) (pool of three SiRNA sequences) transfected cells compared to control-transfected (Sc) cells. (C) Paralleling the overexpression of ER\(\alpha\), WNT5A protein level expression was also stimulated in tumor in vivo in bone lesions induced by PC3-ERR\(\alpha\) (ERR\(\alpha\)) compared to PC3-CT (CT) cells (a). (D) Decreased WNT5A mRNA expression was also observed after XCT-790 treatment at 10\(^{\text{th}}\) M for 48 h in PC3-ERR\(\alpha\) cells (Student's t-tests \(P = 0.0077\)). (E) By real-time RT-PCR, we also found WNT5A mRNA expression (Student's t-tests \(P = 0.0001\)) increased in PC3-ERR\(\alpha\) SC tumors in vivo compared to PC3-CT SC tumors (F) WNT5A (Student's t-tests \(P = 0.0026\)) mRNA expression was also upregulated in the PC3-ERR\(\alpha\) (ERR\(\alpha\)) clone compared to the PC3-CT (CT) in vitro and (G) in tumor in vivo in bone lesions induced by PC3-ERR\(\alpha\) (ERR\(\alpha\)) compared to PC3-CT (CT) cells (a). Bar = 200 \(\mu\) m, T: Tumor, OB: osteoblasts, BM: Bone Matrix.
Table 1: Meta-analysis using public datasets showing correlation between ERRα and VEGFA, WNT5A, POSTN and TGFβ1 expression levels in bone metastases (GSE29650, GSE41619 and GSE74685) and/or in primary CRPC tumor (GSE1034, GSE6919 and GSE2269) and/or in primary samples (N)

| Genes names | Database | Correlations | Primary tumour | Bone Metastases |
|-------------|----------|--------------|----------------|-----------------|
| VEGFA/ERRα | GSE21034/ GSE6919/ GSE32269 | Pearson R = 0.364, P = 0.000001 | 140 CRPC | N/A |
|            | GSE29650/ GSE41619/GSE74685 | Pearson R = 0.646, P = 0.000001 | N/A | 64 |
|            | GSE21034/ GSE6919/ GSE32269 | Pearson R = -0.178, P = 0.035 | 140 CRPC | N/A |
| WNT5A/ERRα | GSE29650/ GSE41619/GSE74685 | Pearson R = 0.342, P = 0.00572 | N/A | 64 |
|            | GSE21034/ GSE6919/ GSE32269 | Pearson R = 0.401, P = 0.000001 | 184 in adjacent normal tissue | N/A |
| POSTN/ERRα | GSE29650/ GSE41619 | Pearson R = 0.528, P = 0.00023 | N/A | 44 |
|            | GSE21034/ GSE6919/ GSE32269 | Pearson R = 0.684, P = 0.000001 | 140 CRPC | N/A |
| TGFβ1/ERRα | GSE29650/ GSE41619 | Pearson R = -0.365, NS | N/A | 14 |

To obtain z-scores, normalized data were log₂ transformed and calculated by subtracting the population mean from individual expression values for each gene and then dividing the difference by the population standard deviation. Correlation scores were calculated using the Pearson correlation coefficient. P-values less than 0.05 were considered statistically significant.

\[ P < 0.00001 \] (Table 1; Supplementary Table S1). On the contrary, no significant correlation was observed in bone metastases (Table 1; Supplementary Table S1). The data suggest that the upregulation of stromal cell POSTN may be the initial step through which ERRα induces rapid bone lesion progression.

**DISCUSSION**

Our findings indicate that ERRα is a stimulator of prostate cancer cell progression in bone. In addition to confirming the regulation of the previously identified ERRα-regulated gene, VEGF-A [30], we identified WNT5A and TGFβ1 as new ERRα-regulated genes. Together with our observation that ERRα expression is higher in CRPC patients with bone metastases than without, and that ERRα in PCa alters molecular signaling in the stroma via the regulation of POSTN expression in infiltrating fibroblasts [10], our data provide new mechanistic insights into ERRα-regulated pathways mediating PCa progression in bone.

ERRα-positive prostate tumors have been associated with more invasive disease and a higher risk of recurrence [18]. For example, ERRα immunoreactivity score has been linked with Gleason scores [18]. ERRα was also recently associated with hypoxia adaptation in PCa cells (AR+ (LNCaP) and AR-(PC3) pre-clinical models), suggesting that ERRα might be involved in advanced progression of PCa [19]. Advanced PCa has a propensity of 90% to metastasize to bone, of 25% to metastasize to liver and to develop CRPC, all of which are associated with poor five-year survival rate [2].

Since we had shown that ERRα may be involved in bone metastases from breast cancer [22] that prompted us to determine if and how ERRα could contribute to malignant growth of PCa in bone. Interestingly, ERRα stimulation of PCa-induced bone lesion progression is different from what we observed in breast cancer [22]. ERRα plays a dual role in the progression of breast cancer -by promoting local tumor growth but by decreasing osteolytic lesions in bone, the latter due to the inhibition of osteoclast differentiation. The opposite activity of ERRα in PCa versus breast cancer on bone-resorbing-cells may explain the differences in tumor progression in bone between breast and prostate cancers [22] and suggests distinct clinical management of prostate versus breast cancer patients with bone metastases.

ERRα expression levels in primary tumors and bone metastases associated with PCa patients correlated well with our results in three CRPC preclinical mouse models. At the cellular level, we found that ERRα directly stimulates both osteoclasts and osteoblasts, as well as factors in PCa cells thought to stimulate osteoclasts and osteoclasts, i.e., VEGF-A [33] [39], WNT5A [37, 40, 41] and TGFβ1 [1, 6, 42] (Figure 8, pathways 1 and 2). Notably, all these factors were also found to correlate
with ERRα expression in our meta-analysis studies of human PCa datasets.

Previously, WNT11 was shown to be regulated by ERRα in PCa cells and implicated in cancer cell invasion [43]. Our data on WNT5A and ERRα expression in both in vivo and in vitro experiments and meta-analysis of human PCa datasets reinforced the link between ERRα activity and Wnt signaling. Interestingly, analysis of circulating tumor cells (CTC) from patients had indicated that WNT5A is activated, in keeping with a role for WNT5A in cell polarization and cancer cell invasion [44, 45, 46]. Indeed, analysis of CTCs from patients progressing on AR inhibitor compared with untreated case indicates activation of WNT5A [46]. Moreover, Miyamoto D et al. show that ectopic expression of WNT5A inhibits the anti-proliferative effect of AR inhibition suggesting that it may participate in the acquisition of resistance to androgen deprivation and to treatment failure [46].

However, WNT5A has variously been described as a pro or an inhibitor of tumor growth in bone [47, 48]. Our results suggest that ERRα-mediated WNT5A is associated with advanced PCa metastatic disease in bone, but to fully understand the molecular mechanisms and determine direct versus indirect regulation by ERRα in tumor cells, additional studies are required. Nevertheless, the identification of WNT5A as an ERRα-regulated gene may represent implications for its potential use as a prognostic marker in prostate cancer.

With respect to PCa-induced osteolysis, we found that rankl expression was stimulated in MC3T3-E1 cells co-cultured with PC3-ERRα but not with the ACE-1 cell model, which is a mixed lesion model, whether or not ERRα levels were modified in the latter. These data suggest indirect regulatory effects on osteoclasts that participate in the resorption observed in bone lesions in animals bearing ERRα osteolytic tumors but not mixed

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**Figure 7: Regulation of stromal postn by ERRα.** (A) Assessment of the mouse postn expression by real-time RT-PCR on triplicate samples and normalized against the mouse ribosomal protein gene L32. Real-time RT-PCR showed increased postn mRNA expression (Student’s t-tests P = 0.0026) in PC3-ERRα-induced SC tumors in vivo. (B) Increased expression of postn in peri-tumoral and infiltrating fibroblasts was visualized by IHC on PC3-ERRα-induced bone lesions (b) compared to PC3-CT (a). (C) Similarly, postn protein was upregulated in the infiltrating fibroblasts in vivo in bone lesions induced by PC3-ERRα (ERRα)(b) compared PC3c-CT (CT) cells (a). Vimentin antibody was used to show the fibroblastic type of the postn expressing cells (c) (D) Real-time RT-PCR showed that TGFβ1 mRNA expression (Student’s t-tests P = 0.00246) was higher in PC3-ERRα versus PC3-CT (CT) cells in vitro while (E) TGFβ1 (Student’s t-tests P = 0.0022) mRNA expression was decreased in Sc-ERRα (pooled of three sequences) transfected cells compared to control-transfected (Sc) cells. (F) Real-time RT-PCR also showed decreased TGFβ1 mRNA expression (Student’s t-tests P = 0.0033) in XCT790 treated PC3-ERRα cells and (G) increased TGFβ1 mRNA expression (Student’s t-tests P = 0.0002) in PC3-ERRα-induced SC tumors in vivo. (H) TGFβ1 staining was higher in PC3-ERRα (b) compared to PC3-CT (a) in tumor-induced bone lesions in vivo. Bar = 200 μm. T: Tumor; OB: osteoblasts; BM: Bone Matrix; IF: infiltrating fibroblasts.
lesions (Figure 8, pathway 3).

Our data suggest that ERRα may also have an impact on the stromal microenvironment. The reciprocal interaction between cancer cells and tissue-specific stroma is known to be critical for primary and metastatic tumor growth progression and POSTN is one of the extracellular matrix molecules known to participate in tumor proliferation and invasion when secreted from stromal cells infiltrating tumors [8, 10, 11]. Here, we confirm that postn is not only expressed in the stromal cells infiltrating tumors of bone xenografts but also in orthotopic PCa xenografts [10]. However, we also show for the first time that mouse postn expression can be stimulated by PCa cells overexpressing ERRα in bone and in SC tumors in vivo. Our data also suggest that ERRα may participate in the initial expansion of cancer cells by regulating the extracellular matrix component postn in stromal cells infiltrating tumors at least in part through the regulation of TGFβ1 in prostate cancer cells (Figure 8, pathway 4) [8]. The link between ERRα/POSTN in the clinical models was confirmed by our meta-analyses of bone metastases and in tumor adjacent tissues in primary tumors, which suggests that POSTN may constitute a new prognostic marker in association with ERRα. The data also suggest that this is may be the initial step by which ERRα induces rapid bone lesion progression. Consequently, targeting the tumor microenvironment may also constitute a new therapeutic approach for prostate cancer patients with high levels of ERRα [49].

Very recently, the ERRα coactivator PGC1α has been described as a suppressor of PCa metastasis [50]. PGC1α is expressed at very low levels (i.e., CT: 33) in our PC3, PC3c models, suggesting that the ERRα-PGC1α mechanisms described by Torrano et al. are unlikely to occur in these models. Taken together, these data suggest that ERRα is a suppressor when PGC1α is highly expressed and as an activator when PGC1α expression is low, in turn strongly suggesting that, depending on the expression of ERRα cofactors in PCa cells, ERRα can have opposite activities. Thus, it will be important to take into account not only the expression of ERRα but also its cofactors in tumors specimens.

In conclusion, we report for the first time on the involvement of ERRα in the steps of PCa initiation and tumor progression in bone, which results from ERRα modifications of signaling molecules and the microenvironment. Our findings contribute to a new understanding of mechanisms underlying the overall negative clinical outcome associated with ERRα in prostate cancer and provide a rationale for the investigation of ERRα as a therapeutic target to treat primary prostate cancers and their associated bone metastases.

Figure 8: Schematic diagram showing ERRα pathways that can mediate tumor progression in bone. Pathway 1: Increased expression of pro-resorption factors (VEGF-A, WNT5a, TGFβ1) in PCa tumors overexpressing ERRα leads to direct stimulation of Ov formation. Pathway 2: Increased expression of pro-osteoblastic factors (VEGF-A, WNT5a, TGFβ1) in PCa tumors overexpressing ERRα that leads to direct stimulation of Ob formation. Pathway 3: Indirect stimulation of osteoclasts through osteoblasts (via the regulation of rankl in Ob for the osteolytic model). Pathway 4: Stimulation of the metastatic niche through the stimulation of postn in fibroblasts infiltrating tumors by TGFβ1 secreted by tumor cells. Ob: osteoblast; Ov: osteoclast; direct signaling (in green); indirect signaling (in orange).
MATERIALS AND METHODS

Ethics approval

SCID male mice were purchased from Charles River and handled according to the French Ministerial Decree No.87-848 of 19 October 1987. Experimental protocols were approved by the Institutional Animal Care and Use Committee at the Université Lyon-1 (France) (CEEA-55 Comité d’Ethique en Expérimentation Animale DR2014-32). Studies involving human primary prostate tumors were carried out according to the principles embodied in the Declaration of Helsinki.

PCa patient primary and bone metastases specimens for IHC

Autopsy files (dating from 1991 to 1998) from the Department of Pathology (Tumor Tissue Bank, Centre Hospitalier Universitaire de Liège, Belgium) were screened for diagnosis of disseminated prostate cancer with histologically proven bone metastases [51]. For immunohistochemistry (IHC) analysis, we selected a prostate cancer patient who died with disseminated disease, including bone metastases. We retrieved patient slides and their clinical history. Tumors were fixed and paraffin-embedded.

Meta-analysis

For correlation analysis in clinical samples, gene expression data and corresponding clinical annotations for previously published datasets on normal prostate tissue, adjacent prostate tissue, primary and bone metastases were downloaded from the Gene-Expression-Omnius (GSE6919-GSE29650-GSE32269-GSE41619-GSE21034-GSE74685) (Supplementary Table S1). To obtain z-scores, normalized data were log-transformed and calculated by subtracting the population mean from individual expression values for each gene and then dividing the difference by the population standard deviation. Correlation scores were calculated using the Pearson correlation coefficient. P-values less than 0.05 were considered statistically significant. Statistical significance was determined by GraphPad Prism v 5.02 using the two-sided student t-test.

Animal studies

SCID male mice, 6 weeks of age, were housed in barrier conditions under isolated laminar flow hoods. Mice bearing tumor xenografts were closely monitored for established signs of distress and discomfort and were humanely euthanized.

PC3 cells induce osteolytic lesions, PC3c cells induce mixed lesions with lytic and osteoblastic regions in the bone marrow cavity [28] while ACE-1 mainly induce lytic lesions in the bone marrow cavity and osteoblastic region outside the bone (spicules) [29].

Intra-osseous tumor xenograft experiments were performed as previously described [28] as for technical reason, we could not obtain bone lesions after intra-articular and intra-cardiac injections. PC3, PC3c and ACE-1 cells (6 x 10⁶ in 15µL PBS) were injected in the bone marrow cavity [28]. Mice were sacrificed after 3 weeks (PC3 and ACE-1) and 6 weeks (PC3c). Radiographs (LifeRay IM PLUS, Ferrania) of animals were taken at that time after incubation using X-ray (MX-20, Faxitron X-ray Corporation). Animals were sacrificed, hind limbs were collected for histology and histomorphometric analyses. The bone lesion surface, that includes lytic and osteoblastic regions, was measured using the computerized image analysis system MorphoExpert (Exploranova). The extent of bone lesions for each animal was expressed in mm². Tibiae were scanned using microcomputed tomography (Skyscan1174, SkyScan) with an 8.1 µm voxel size and an X-ray tube (50 kV, 80 µA) with 0.5 µm aluminum filter. Three-dimensional reconstructions and rendering were performed using the manufacturer’s suite (Respectively, NRRecon TC, Vox and SkyScan). Bone Volume/Tissue Volume: (%BV/TV) includes residual trabecular and remaining cortical bone for PC3 and ACE-1 models, plus new bone formation into the bone marrow cavity for PC3c model. For the ACE-1 model, since the osteoblastic lesions is outside the cortical bone, the percent (%) of extra-new bone spicules formation (only spicules surface) with respect to total volume (trabecular, cortical and spicule) for the ACE model were quantified. %BV/TV and % of extra-new bone spicules formation were carried out with CTAn (version 1.9, SkyScan) and CTVol (version 2.0, SkyScan) software. Dissected bones were then processed for histological and histomorphometric analysis (tumor burden-to-skin tissue volume (%TB/STV)) and TRAP-positive trabecular bone surface (Oc.S) to the total bone surface (BS) as described previously for the PC3 model [22]. Results were plotted as the mean ± SD. Subcutaneous injections of PC3 cells (10⁶ in 100 µl PBS) were also performed in 6- to 8-week-old SCID mice. Mice were sacrificed after 6 weeks and tumors were fixed and embedded in paraffin.

Cell lines and transfection

The PC3 cell line was obtained from the American Type Culture Collection (ATCC) (cell line authentication was performed). The PC3c cell, a subculture cell line of PC3 was isolated in our laboratory in vitro after single cell population culture. Consequently to spontaneous derivation of the cells, we finally obtained a subculture cell line named PC3c which was chosen based on epithelial phenotype [28]. Osteoprotogerin (OPG) and endothelin-1 (ET1) are highly expressed by PC3c while...
dikkopf-1 (DKK1) expression is decreased. Finally, PC3c
highly expressed bone associated markers osteopontin,
Runx2, alkaline phosphatase, bone sialoprotein and
produced mineralized matrix in vitro in osteogenic
conditions showing a strong osteoimmunism phenotype
of PC3c cells compared to PC3 [28]. ACE-1 were obtained
from Dr T Rosol [29]. PC3 and ACE-1 stably transduced
clones were routinely cultured in F12K (Gibco/Life
Technologies) and PC3c clone were cultured in DMEM.
All cultures were supplemented with 10% (v/v) fetal
bovine serum (Perbio/Thermo Scientific) and 1% (v/v)
penicillin/streptomycin (Life Technologies) at 37°C in a
5% CO₂ incubator.

**Human ERRα cDNA (ERRα) and the dominant
negative form mutated into the co-activator domain
AF2 (AF2) was obtained previously [22]. Cells were
cultured under clonal selection for 4 weeks in the presence
of puromycin (1 μg/mL) (Life Technologies). PC3 cells
were also transfected (100 pmol) with ERRARSiRNA
sequences specific to human (ON-TARGET plus
Human ERRα SMART pool of three sequences: GGCCCCUGCGAGGAGCUUA
/GCGAGAGAGAUUGUCCUA/GCGUGGCGAGCC
UCAAAGGA) and control sequences (ON-TARGET
plus Human Non-targeting pool of three sequences:
UGGUGUAAACUGUGACUA/UGGUGUAAACUGU
UGUGUCUGAGUACUAA/UGGUGUAAACUGUU
UGUGUCUGAGUACUAA/UGGUGUAAACUGUU
UGUGUCUGAGUACUAA/UGGUGUAAACUGUU
UGUGUCUGAGUACUAA/Dharmacon) and mRNA
were extracted at 72 h after transfection. Conditioned
culture from PC3 and PC3c clones (used
on primary calvaria cells culture) and PC3 parental cells
were treated with the XCT-790 at 10⁻⁴ M (Sigma) was
obtained after 48 hours in α-MEM supplemented with 0.5% of
serum, then filter sterilized and proteins quantified to use
equal concentration of proteins for each conditions (25 μg).
Dimethyl sulfoxide (DMSO) was used as control (CT).
PC3-ERRα cells (pool of three clones) were treated with
the inverse agonist XCT-790 at 10⁻⁴ M (Sigma) for 48 h.
DMSO was used as control (CT).

**Osteoclastogenesis assay**

Bone marrow cells from 6-week-old OF1 male mice
were cultured for 7 days as previously described [22].
Pools of PC3-CT/PC3-ERRα clones or PC3c-CTX/PC3c-
ERRα or ACE-1-CTX/ERRα/AF2 were plated (10⁶ cells
on day 1 on bone marrow cells culture in presence of rankl
and m-csf). Mature osteoclasts were stained for TRAP
activity (Sigma-Aldrich). Multinucleated TRAP-positive
cells containing three or more nuclei were counted as
osteoclasts. Results were plotted as the mean ± SD of three
wells for each condition and were representative of two
independent experiments.

**Osteoblastogenesis assay**

Cells were enzymatically isolated from the calvaria
as described previously [31]. Mouse calvaria cells were
continuously exposed (21 days) to conditioned medium
isolated from cultures of PC3 pooled clones or PC3c
clones. Wells were stained with von Kossa stain for ALP
and bone nodules were counted on a grid [31]. Results
were plotted as the mean number of nodules ± SD of
three wells and were representative of two independent
experiments. The murine osteoblastic MC3T3-E1 cell
line was cultured for 2 weeks with pooled PC3-CTX versus
PC3-ERRα clones or ACE-1-CTX/ERRα/AF2 that were
plated (10⁶ cells) on day 1 for 2 weeks. mRNA were then
extracted and osteoblastic markers expression addressed
by real-time PCR.

**Immunohistochemistry**

Tibia bearing metastases were fixed and embedded
in paraffin. IHC analysis was performed by incubating
sections overnight with rabbit monoclonal against
human and mouse anti-VEGF-A (1/50) (Abcam), rabbit
polyclonal antibodies against human and mouse WNT5a
(1/700)(Abcam), goat anti-mouse postn (1/200)(R&DE)
mouse monoclonal anti-human/mouse TGFβ1 (1/40)
(R&D), rabbit polyclonal anti-mouse vimentin/1:50
(Biovision) and mouse monoclonal anti-human/mouse
ERRα antibody (1/50)(Santa Cruz) [22]. Sections were
then incubated with HRP-conjugated anti-mouse (Dako),
anti-rabbit (Dako) or the anti-goat (Jackson) antibodies
for 1 hour and stained using 3,3-diaminobenzidine
(Dako). Counterstaining was performed using Mayer’s
hematoxylin (Merck).

**Immunoblotting**

Protein extraction and immunoblotting were
performed as described previously [22]. As the mouse
monoclonal anti-human/mouse ERRα antibody (Santa Cruz)
did not work in Western-blots, we used a rabbit monoclonal
antibody against ERRα (Epitomics) (1:400) [22] and a rabbit
polyclonal antibody against GAPDH (Abcam) (1:10,000)
(Amersham) as a protein loading control.

**Real time RT-PCR**

Total RNA was extracted with Trizol reagent
(Life Technologies) and 2 μg was reverse-transcribed
using Superscript™II (Life Technologies) [22]. Real-
time PCR was performed on a Mastercycler ep-Realplex
(Eppendorf) with primers specific to human and mouse
genes (Supplementary Table S2) using Quantifast SYBR
Green (Life Technologies). PCR was carried out according
to the manufacturer’s instructions. Melting curve analysis
was used to verify that a single peak was obtained for each
product with a 95–100% PCR efficiency (Roche software).
Relative gene expression levels were normalized according
to the Ct value of the housekeeping gene encoding the
ribosomal protein L32 and results were expressed as fold
differences equal to 2⁻ΔΔCt.
Statistical analysis

Statistical analysis was performed using GraphPad Prism software (San Diego, USA). Pairwise comparisons were tested using a non-parametric Mann-Whitney U test for bone metastases. In vitro data were analyzed using ANOVA and Student’s t-tests to assess the differences between groups. Results of $P < 0.05$ were considered significant.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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