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ERRα promotes breast cancer cell dissemination to bone by increasing RANK expression in primary breast tumors

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Precis:

Our work suggests that ERRα/RANK expression in primary tumors is a predictive marker of the occurrence of bone metastases in breast cancer patients.

Abstract

Bone is the most common metastatic site for breast cancer. Estrogen-related receptor alpha (ERRα) has been implicated in cancer cell invasiveness. Here, we established that ERRα promotes spontaneous metastatic dissemination of breast cancer cells from primary mammary tumors to the skeleton. We carried out cohort studies, pharmacological inhibition, gain-of-function analyses *in vivo* and cellular and molecular studies *in vitro* to identify new biomarkers in breast cancer metastases. Meta-analysis of human primary breast tumors revealed that high ERRα expression levels were associated with bone but not lung metastases. ERRα expression was also detected in circulating tumor cells from metastatic breast cancer patients. ERRα overexpression in murine 4T1 breast cancer cells promoted spontaneous bone micro-metastases formation when tumor cells were inoculated orthotopically, whereas lung metastases occurred irrespective of ERRα expression level. RANK was identified as a target for ERRα *in vivo* and in ERRα–overexpressing breast cancer cells, RANKL stimulated tumor cell invasion and signalling pathways including mTOR phosphorylation *in vitro*. Pharmacological inhibition of ERRα reduced primary tumor growth, bone micro-metastases formation and RANK expression *in vitro* and *in vivo*. Transcriptomic study and meta-analysis confirmed a positive association between metastases and ERRα/RANK in primary breast tumors and also revealed a positive correlation between ERRα and $BRCA1^{mut}$ carriers. Taken together, our results reveal a novel ERRα/RANK axis by which ERRα in primary breast cancer promotes early dissemination of cancer cells to bone. These findings suggest that ERRα may be a useful therapeutic target to prevent bone metastases.

Abbreviations used in this paper: ANOVA: analysis of variance; BCa: Breast Cancer; BM: Bone metastases; CTC: Circulating Tumor Cells; ERR: Estrogen-Related Receptor; FP: Fat Pad; RANKL: Receptor Activator of Nuclear factor kB ligand; IHC: immunohistochemistry; LM: Lung Metastases; PBT: Primary Breast Tumors; RANK: RANKL receptor; RT: Reverse Transcription; PCR: Polymerase Chain Reaction; TCB: Tumor cell Colonization of Bone; Vegf: Vascular endothelial growth factor.

Introduction

Metastases are the primary cause of death (1) and in breast cancer (BCa), bone is the most common metastatic site (2). Bone metastases (BM) detection often occurs in the symptomatic stage and when they are diagnosed, the prognosis for the patient is generally poor and associated with significant morbidity. Unfortunately, current treatments for BM that rely on anti-resorptive agents are only palliative underlining the need of early detection of BM for early intervention (3)(4)(5).

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 natural ligands have yet been identified (6). The estrogens-related receptor alpha (ERRα) shares structural similarities with ERα/β, but it does not bind estrogens (6). Indeed, 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 oestrogens (7). Although ERRα activity is decreased by synthetic molecules such as the inverse agonists XCT790 or C29, no natural ligand has yet been found (8)(9).

ERRα is known to regulate fatty acid oxidation and the adaptive bioenergetics response (10). It is widely expressed in both healthy tissues and a range of cancerous cells (11)(12)(13). Notably, ERRα was found to be markedly increased in neoplastic *versus* normal tissues and ERRα-positive tumors were associated with more invasive disease and higher risk of recurrences (11)(13). In addition to angiogenesis, ERRα is mainly linked to tumor cells invasion (13)(14). ERRα is also highly expressed in skeletal tissues where it is known to regulate osteoblast and osteoclast differentiation and migration as well as bone remodelling *in vitro* and *in vivo* (15)(16)(17)(18). In BM, we have previously reported that overexpression

of ERRα in triple-negative BCa cells and castration-resistant prostate cancer cells results in either the inhibition or the activation of tumor cells progression in the bone microenvironment respectively (19)(20). Here, we assess whether ERRα expression in primary breast tumors (PBT) regulates the molecular mechanisms that drive BCa cell homing to bone.

Materials and Methods

Ethics approval

BALB/c female mice were purchased from Janvier 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 DR2014-44;DR2015-28). Studies involving human primary specimens were carried out according to the principles embodied in the Declaration of Helsinki.

Immunohistochemistry and Transcriptomic study on BCa patient primary tumor specimens.

Immunohistochemistry (IHC) was performed on formalin-fixed paraffin-embedded tumor tissue sections (n=100) from the Austrian Breast and Colorectal Cancer Study Group (ABCSG) Trial 6 (21) (Supplementary Methods section). For IHC, a mouse monoclonal anti-human/mouse ERRα antibody (1/50) (Santa Cruz) were used. To assess ERRα immunostaining, we used the H-score method (22). RT-PCR were performed and analyzed on specimens 446 from unilateral invasive PBT radically-resected BCa patients managed at Institut Curie-Hôpital René Huguenin (France) (Supplementary-TableS1) selected to encompass the various stages of BCa progression (Supplementary Methods section) (23)(24). This study was approved by the review board and ethics committee of the institute (23).

Detection of ERRα in circulating tumor cells (CTC)

Blood of 60 metastatic BCa patients recruited at the Cancer Institute of Montpellier in 2017 (Bioethics Committee D-RCB 2015-A00119-40) (Supplementary Methods section) was analyzed. Tumor cells were detected by using the Cellsearch® system (Silicon Biosystem, Menarini). For ERRα detection in CTCs, we used the 4^{th} channel of the CellSearch® and

anti-human ERRα/NR3B1 A488-conjugated polyclonal antibody (Novus, Littleton, USA) at a final concentration of 25 µg/mL.

Meta-analysis of ESSRA expression

Independent datasets of breast tumors for which microarray data were publicly available were analyzed: The BCa cohort "NKI" (Netherlands Cancer Institute, n=295) consisted of early-stage tumors (50% lymph node negative) with the relapse status fully documented (25) and the "METABRIC" cohort of 2051 breast cancers (26) that was used to assess the correlation of *BRCA* mutations and *ESRRA* expression. For correlation analysis, published datasets were downloaded from the Gene-Expression-Omnibus including primary Tumors, Bone, Lung and Liver metastases (GSE14020) (27) and PBT, no-metastases, Visceral and bone or only bone metastases (GSE12276-GSE2034-GSE2603)(Supplementary-TableS2)(28)(29)(30). Methods for the processing are described in the Supplementary Methods section.

Cell lines, transfection and immunoblotting

Human MCF7 (Year 2012) and mouse 4T1(Year 2012) mammary cancer cell lines were obtained from the American Type Culture Collection. The human BC-M1 cell line (generous gift from the Dr K Pantel lab) was obtained from a bone marrow aspirate of a BCa patient with no clinical signs of distant metastases at the time of the primary tumor resection (31)(32). Different conditions were used for culture (Supplementary Methods section). Murine and human ERRα cDNA (ERRα) and the dominant-negative form mutated into the co-activator domain AF2 (AF2) were prepared previously (19)(20) and transfected into 4T1 or MCF7 cells (Supplementary Methods section). Parental 4T1 and MCF7 cells were treated with the ERRα inverse-agonist C29 at 5µM and 1-5µM respectively for 48h (9)(33). DMSO was used as vehicle. For Rank studies, 4T1-CT (pool of 2 clones), 4T1-ERRα (pool of 3

clones) were plated at $5x10^5$ cells/well with complete medium. The next day, cells were starved for 24 hours and then treated with Rankl (50ng/mL) (R&D) for different times (0-5-15-25 min). Proteins extraction and immunoblotting was performed as described previously (19)(20) (Supplementary Methods section).

Animal studies

BALB/c female 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. Tumor fat pad (FP) experiments were performed using the 4T1-CT (pool of 2 clones), 4T1-ERRα (pool of 3 clones) or 4T1-AF2 (pool of 3 clones) cell lines (10^5 cells in 10μL of PBS) injected into FP of the 4th mammary gland of mice as described previously (34)(Supplementary Methods section).

FP experiments were also performed using 4T1-ERRα cells (pool of 3 clones) (10^5 cells in 10μL of PBS). When primary tumors reached approximately $30mm^3$, mice were randomized, treated every two days for 10 days by intra-peritoneal injection with vehicle (DMSO 10%, 30% PEG) or C29 (10mg/kg) (9)(35), then prepared and analyzed as above.

Immunohistochemistry on mice PBT sections

IHC analysis was performed by incubating 5μm paraffin sections overnight with mouse monoclonal anti-human/mouse RANK (1/50) (Abcam), and a mouse monoclonal anti-human/mouse ERRα antibody (1/50) (Santa Cruz) (19). Sections were then incubated with horseradish peroxidase (HRP)-conjugated anti-mouse (Dako) antibodies for 1hour and stained using 3,3'-diaminobenzidine (Dako). Counterstaining was performed using Mayer's hematoxylin (Merck).

Cell migration assay

Migration assays were carried out using the HTS FluoroBlok^{TM} Multiwell Insert System (Corning) 8 μm transwells as described previously (19). After 12 hours of starvation, 4T1-CT (2 clones) and 4T1-ERRα (3 clones) ($5x10^4$ cells) were plated in the upper chambers and the chemoattractant (0.5% FBS) with or without Rankl (50ng/mL) (R&D) in the lower chambers. After 12 hours at 37°C in 5% CO_2 incubator, cells that had migrated through the filters were fixed, stained with DAPI (Life Technologies) and counted. All experiments were run in triplicate, and migration was expressed in % of migration/wells.

Real time RT-PCR on cell lines and mice PBT specimens mRNA

Total RNA was extracted with Trizol reagent (Life Technologies) and 2μg was reverse-transcribed using SuperscriptTM II (Life-Technologies) (20). Real-time PCR was performed on a Mastercycler-ep-Realplex (Eppendorf) with primers specific to human and mouse genes (Supplementary Table S1) using Quantifast SYBR Green (Life-Technologies). PCR was carried out according to the manufacturer's instructions (20). 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 $2^{-\Delta\Delta Ct}$.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (San Diego,USA). Data were analyzed statistically by the non-parametric Mann-Whitney U test or unpaired t-test for *in vivo* studies. *In vitro* data were analyzed using ANOVA and Student t-test to assess the differences between groups. Metastases-free survival (MFS), Lung metastases-free survival (LMFS) and Bone metastases-free survival (BMFS) was determined as the interval between diagnosis and detection of the first metastases. Survival distributions was estimated by the

Kaplan-Meier method, and the significance of differences between survival rates was ascertained using the log-rank test. For all statistical tests, differences was considered significant at confidence levels greater than 95% ($p < 0.05$).

Results

High ERRα protein expression levels in primary tumors are associated with bone relapse in BCa patients

We assessed ERRα protein expression levels in primary tumors from patients of the ABCSG trial 6 cohort, which is a prospective, randomized clinical study for postmenopausal patients with hormone receptor-positive early-stage BCa, treated with tamoxifen-aminoglutethimide and followed for up to 15 years (21). Kaplan-Meier analysis of distant-recurrence-free survival stratified by ERRα protein expression level (high) (Fig.1Bb) or (low) (Fig.1Ba) showed that high levels of ERRα protein expression (Fig.1A) correlated with lower distant recurrence-free survival (%) (n=100). To further examine ERRα pathophysiologic relevance in breast tumor cells, we then asked if ERRα is expressed in circulating tumor cells (CTCs) from metastatic BCa patients. After establishing conditions for ERRα detection using cell lines (Supplementary Fig.S1), ERRα expression was detected in CTCs in three out of 60 patients with metastatic BCa (Fig.1C), further supporting the hypothesis that ERRα present in CTCs may be involved in the metastatic process. We, therefore, next analyzed the ERRα transcriptional profile in the NKI-295 cohort that includes 295 radically-resected primary BCa from patients (under 53 years) with known location of the first distant metastatic site (25). ERRα expression level was positively associated with histological grade and lymph node status (Supplementary Table S3A). Univariate Cox proportional hazard model analysis indicated that ERRα expression is associated with metastases-free survival (MFS) and bone metastases free-survival (BMFS) but not with lung metastases free-survival (LMFS) (Supplementary Table S3B). Furthermore, Kaplan Meier curves demonstrated that high *ERRα* expression is correlated with a decrease in BMFS but not LMFS (Fig.1DE). Notably, neither ERRβ nor ERRγ mRNA expression levels correlated with MFS, BMFS or LMFS (Supplementary Table S3B).

ERRα in BCa cells promotes spontaneous bone micro-metastases formation in vivo

To assess the role of *ERRα* in the early steps of metastatic dissemination of BCa, we used the murine 4T1 BCa cell line, from which cells spontaneously metastasize from primary tumor to lung and bone (36). Three independent 4T1 clones overexpressing ERRα (clones 1-3), three 4T1-AF2 clones (1-3) which act as a dominant-negative form, and two empty vector controls 4T1-CT (1-2) were isolated. Western blots confirmed that the ~54kD *ERRα* protein band was increased in 4T1-ERRα (1-3) compared to 4T1 CT1-2 cells (Fig.2A) and that a band with slightly lower molecular weight, corresponding to the expected size for truncation of the AF2 domain (42aa), was present in 4T1-AF2 (1-3) cells (Supplementary Fig.S2A).

Analysis of primary mammary gland FP tumors in mice, orthotopically inoculated with 4T1-CT, 4T1-ERRα or 4T1-AF2 cells, indicated greater tumor progression in 4T1-ERRα-FP tumour-bearing animals compared to 4T1-CT-FP (Fig.2B-D) and 4T1-ERRαAF2-FP (Supplementary Fig.S2BC). In agreement with previous data (37), we found that *Vegf-a* expression was significantly increased in 4T1-ERRα-FP tumors (Supplementary Fig.S2DE).

Culturing cells collected from crushed bones of mice kept for an additional 21 days post-primary tumor resection and counting colonies (38) revealed greater tumor cell colonization of bone (TCB) by 4T1-ERRα-FP (Fig.2E), with higher incidence of dissemination to bone with 4T1-ERRα cells (77%) compared to 4T1-CT cells (12%)(Fig.2F) and a higher number of TCB (Fig.2G). In contrast, histological assessment of lung metastases (LM) revealed no difference in the number or incidence of lung colonies (Fig.2H-J) or metastases surface between both groups, in spite of maintenance of ERRα overexpression in 4T1-ERRα-LM versus 4T1-CT-LM (Supplementary Fig.S2FG). Taken together, these pre-clinical data confirm a correlation between ERRα expression level and BMFS but not LMFS (Fig.1DE;Supplementary Table S3B).

ERRα regulates RANK expression in BCa cells

To explore the underlying mechanism responsible for the ability of 4T1-ERRα cells to promote homing of cancer cells to bone *in vivo*, we quantified in PBT transcriptional expression levels of several markers involved in bone tropism. Expression of Receptor Activator of Nuclear Factor κ B (RANK) was stimulated *in vivo* in 4T1-ERRα-FP (Fig.3A) (39)(40)(41). In contrast, no effect was observed on the expression of *Cxcr4* mRNA, also involved in cancer cell bone but also in lung tropism (Fig.3A). Rank protein expression was also higher in 4T1-ERRα-FP tumors (Fig.3Bb). *RANK* regulation by ERRα was confirmed in a second human BCa cell line, MCF7, which we also engineered to overexpress full-length (wild type; MCF7-ERRα) or truncated (MCF7-ERRαAF2) human *ERRα* compared to the empty vector control (MCF7-CT) (Fig.3CD). The ERRα inverse agonist C29 (9)(33) which blocks ERRα transcriptional activity (Fig.3EF and H) also inhibited *RANK* mRNA expression in 4T1 and MCF7 parental cells (Fig.3GI). Using the human BC-M1 disseminated tumor cell (DTC) line established from the bone marrow of a breast cancer patient (31), we also demonstrated *ERRα* and *RANK* mRNA co-expression (Fig.3J). More importantly, expression of both *ERRα* and *RANK* mRNA was stimulated when BC-M1 cells were cultured under low O_2 conditions compared with normoxia.

To determine whether *Rank* stimulation has functional consequences, 4T1-ERRα cells (clones 1-3) were subjected to migration assays with and without Rankl treatment. Rankl significantly stimulates migration of 4T1-ERRα cells (4T1-ERRα versus 4T1-ERRα+Rankl) but not 4T1-CT cells (Fig.4AB). Unexpectedly, the migratory ability of untreated 4T1-ERRα cells was lower than that of untreated 4T1-CT cells but this was reversed by Rankl treatment (Fig.4B), revealing the high sensitivity of 4T1-ERRα cells to Rankl. Reinforcing the view that *ERRα* regulates Rankl signalling, we observed a more marked increase in

mTOR phosphorylation (at 5 to 25 min) and lesser increase in pSrc (25 min) and pAkt (15 min) in Rankl-treated 4T1-ERRα cells compared to 4T1-CT cells (Fig.4CD) (39).

Pharmacological inhibition of ERRα reduces mammary tumor growth, bone marrow micro-metastases formation and RANK expression in vivo

To determine whether inhibition of *ERRα* abrogates PBT progression and BCa cell dissemination to bone, BALB/c female mice were inoculated orthotopically into the mammary gland FP with 4T1-ERRα cells (clones 1-3) and treated with the ERRα inverse agonist C29 or with vehicle from the time of tumor appearance to tumor resection. Tumor weight/volume was reduced in 4T1-ERRα-FP tumor-bearing animals treated with C29 (Fig.5A-C). No toxicity from the C29 treatment was seen (Supplementary Fig.S3A-D). TCB were reduced in C29-treated mice (Fig.5DE). No difference was observed between colony numbers corresponding to tumor cells that disseminated to lung in C29 treated mice (Supplementary Fig.S3E). *ERRα* and *Rank* mRNA expression was inhibited in PBT of C29-treated (Fig.5F). Similarly, ERRα and Rank proteins level (Supplementary Fig.S4AB) was reduced in PBT in C29-treated mice.

ERRα /RANK link in BCa patients who developed bone metastases

Meta-analysis of the PBT GSE14020 dataset (27) confirmed a positive association of *ERRα* and *RANK* in patients who developed bone metastases but not in patients who developed lung and liver metastases (Table.1A). Moreover, in the Curie Institute cohort (n=446) stratification of BCa patients into subtypes showed positive correlation between *ERRα* and *RANK* in Triple Negative patients (NNN) and Luminal BCa patients (HR^+ERBB2) but not ERBB2+ patients (HR- and HR+) (Table.1B; Supplementary Table S4) (23). No correlation was observed between *ERRα* and *RANKL* (*TNFSF11*). In line with these data, we also found that expression levels of *ERRα* and *RANK* were positively correlated in primary BCa samples (HR+ERBB2-) in combined microarray datasets GSE12276, GSE2034 and GSE2603

(Table.1C; Supplementary Table S2). The strongest correlation was observed in patients who had developed only bone metastases and no correlation was observed in non-metastatic patients (No Mets).

ERRα is highly expressed in PBT from BRCA1-mutated carriers

Recently, *RANK/RANKL* were shown to control *BRCA1* mutation-driven mammary tumor formation and progressions (42). We therefore next asked whether *ERRα* and *BRCA1* expression are correlated. Using the combined microarray datasets GSE12276, GSE2034 and GSE2603, we found a positive correlation between *ERRα* and *BRCA1* in PBT in patients who had developed both visceral+bone and only bone metastases, and with *BRCA2* expression in patients with metastases at all sites (Table.1C). Since these datasets were not informative on the *BRCA1* and *2* mutations status, we used the METABRIC cohorts that include 2051 radically-resected primary BCa, where $BRCA1^{mut}$ or 2^{mut} were identified. *ERRα* expression level was higher in $BRCA1^{mut}$ carriers but not $BRCA2^{mut}$-carriers compared to those with no mutations (Fig.6).

Discussion

Our findings indicate that *ERRα* can be expressed in CTCs and promotes BCa cell homing to bone. The identification of *RANK* as an *ERRα*-regulated gene in our pre-clinical 4T1 cell model and *ERRα*/*RANK* positive association in BCa patient cohorts provide new mechanistic insights into ERRα-regulated pathways mediating early BCa cell invasion of bone.

Elevated ERRα expression has been observed in various tumor samples and associated with tumor aggressiveness (13). More recently, ERRα expression has been linked to resistance to several therapies including lapatinib, mTOR inhibitors and endocrine therapies such as tamoxifen (43) (44) (45). The high expression of ERRα that we documented in patient cohorts, including the ABCSG trial 6 cohort treated with tamoxifen with or without aminoglutethimide, supports the view that ERRα may be used as a prognosis factor in endocrine therapy responses at an early stage of PBT. Moreover, the fact that we have observed ERRα expression in CTCs of BCa metastatic patients and documented changes in BCa cell migration with changes in ERRα activity further strengthens the notion that ERRα may be involved into CTCs survival and metastases.

We also show that high level of ERRα was associated with bone but not with lung metastases in our mouse experimental model of spontaneous metastatic BCa and in the BCa patient cohorts. With respect to bone homing, our identification of *RANK* as a new *ERRα*–regulated gene is notable. RANK, the cognate receptor of RANKL, is highly expressed in carcinomas and is considered as a poor prognostic marker in BCa (46). RANK expression was also described as being predictive of poor prognosis in bone metastases patients but not in patients with visceral metastases (40) (41) (46). One explanation comes from evidence that RANKL is a chemoattractant: RANKL produced by osteoblasts and bone marrow stromal cells has been shown to attract RANK-expressing cancer cells and induce their migration in the bone microenvironment. This mechanism has been observed not only in

BCa but also in other cancers (41). Activation of RANK by RANKL is known to activate several signalling cascades including Src, ERK, Akt/mTOR and concomitantly to stimulate cancer cell migration, survival and protein synthesis (39). More recently, transgenic mice overexpressing RANK in mammary glands were reported to develop pre-neoplastic foci, suggesting that RANK may promote the initial stage of BCa development (47)(48). Moreover, the RANK/RANKL axis has recently been shown to be critical regulators of BRCA1-mutation-driven BCa and anti-RANKL therapy is now proposed as a preventive strategy for women carrying BRCA1 mutations (42)(49). Based on RANK functions and our new findings, ERRα appears to be a potentiel new player in the RANK/RANKL axis and BCa cancer occurrence and progression. Indeed, we demonstrated up- or downregulation of RANK expression in BCa cells after stimulation (overexpression) or pharmacologic inhibition (C29) respectively of ERRα *in vivo* and *in vitro*. Concomitant with the increase of RANK expression, we demonstrated stimulation of migratory capacity and activation of phosphorylation of mTOR, Src and Akt in BCa cells overexpressing ERRα. We also documented correlation of ERRα expression with occurrence of bone metastases but not with visceral or lung metastases in mice and in patients, suggesting that ERRα/RANK expression in primary tumors may be a predictive marker of bone metastases occurrence in BCa patients. Additionally, we found a significant correlation between high ERRα and $BRCA1^{mut}$ in $BRCA1^{mut}$ carriers, implying that ERRα may participate in the RANKL/RANK control of progenitor cell expansion and tumorigenesis in inherited breast cancer. Similarly to the suggestion that inhibition of the RANKL/RANK axis may be a therapeutic target for $BRCA1^{mut}$ carriers, inhibition of an upstream RANK regulator, ERRα, may also constitute a useful new therapeutic approach for these patients (50). In conclusion, we report for the first time a RANK/ERRα axis in BCa that contributes new mechanistic understanding of the overall negative outcome associated with ERRα in breast cancer.

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Legends

Fig.1: **ERRα is a bone metastases-associated gene in BCa. (A)** Kaplan-Meier curves show correlation between high expression of ERRα, categorized with median value, and distant recurrence-free survival in patients treated with tamoxifen alone or aminoglutethimide with tamoxifen (n=100). **(B)** Visualization of ERRα protein expression at low (a) and high level (b) by IHC on sections of PBT. **(C)** Representative $EpCAM^{(+)}$, pan-$keratin^{(+)}$ (K-PE), $CD45^{(-)}$ CTCs and ERRα-$AF488^{(+)/(-)}$ from metastatic BCa patients detected by the CellSearch® System. DAPI was used for nuclear counter staining. **(D)** Kaplan-Meier curves constructed after segmentation into two groups on the basis of the median value for ERRα expression show correlation between high expression of ERRα and lower bone metastases free-survival (BMFS) in NKI cohort (n=295, P=0.01); Low ≤ 50% quartile; high ≥ 50%. **(E)** No correlation was found between high level of ERRα and lung metastases free-survival (LMFS).

Fig.2: **ERRα promotes BCa cell homing to bone *in vivo*. (A)** Three independent clones (4T1-ERRα 1-3) overexpressing full-length ERRα and two control clones with empty vector (4T1-CT 1-2) were obtained after stable transfection of 4T1 cells. ERRα (~54kD) expression was assessed by Western blotting and compared to α-tubulin as a protein loading control. **(B)** 4T1-ERRα (pool of 3 clones) or 4T1-CT (pool of 2 clones) cells were inoculated into the mammary gland fat pad of BALB/c mice. Greater tumor expansion was observed in mice with 4T1-ERRα-FP **(C-D)** Weight and volume of tumors dissected at endpoint (n=10, Mann-Whitney, weight: P=0.0156; volume: P=0.0074). **(E)** After resection, animals were kept for 21 days, bones were then crushed and released cells were cultured for 3 weeks. **(F, G)** Counting of tumor cells that colonized the bone (TCB) revealed a high incidence with 4T1-ERRα cells which was associated with an increase in TCB number (n=9, 262±175 versus

32±55, Mann-Whitney, P=0.0083). **(H-J)** Histological assessment of lung metastases (LM) **(H)** shows no difference between LM incidence **(I)** and LM number/lungs **(J)** (n=9). Asterisk represents lung metastases.

Fig.3: Correlation between ERRα and Rank expression in BCa cells. (A) Real-time PCR performed on mRNA extracted from 4T1 primary tumors confirmed increased expression of ERRα in 4T1-ERRα-FP and show increased expression of Rank by ERRα (ANOVA: P<0.0001 and unpaired t-test P<0.0001 for both *ERRα* and *Rank*). Cxcr4 mRNA expression was not altered. **(B)** Increased expression of Rank was visualized by IHC in 4T1-ERRα-FP (b) compared to 4T1-CT-FP (a). **(C)** MCF7 clones overexpressing ERRα or ERRαAF2 were obtained after retroviral transfection. ERRα overexpression was assessed by Western blotting and comparison to α-tubulin. **(D)** Real-time RT-PCR was performed on triplicate samples and normalized against the ribosomal protein gene *L32* to evaluate ERRα and RANK expression (ANOVA: p<0.0001, unpaired t-test p<0.0001 for *ERRα* and ANOVA: p=0.0135, unpaired t-test p=0.0041 for *RANK*). **(E-I)** C29 treatment induced a decrease in Rank expression in both parental 4T1 and MCF7 cells lines. Inhibition of ERRα protein and mRNA induced by C29 at 5μM after 48h is shown by Western blotting and by real-time PCR (unpaired t-test, p<0.0001) respectively **(E,F)** concomitant with a decrease in Rank expression (unpaired t-test P=0.0419) **(G)** Inhibition of ERRα protein induced by C29 at 1 and 5μM after 48h in MCF7 cells **(H)** is associated with a decrease of RANK mRNA expression (ANOVA: P=0.0016, unpaired t-test P=0.0127 and P=0.0062)**(I)**. **(J)** Real-time PCR was performed on triplicate samples to evaluate *ERRα* and *RANK* expression in the BC-M1 cells under low O_2 conditions compared with normoxia. ERRα and RANK expression was higher under low O2 conditions (unpaired t-test P=0.0143 and P=0.0016 for *ERRα* and *RANK* respectively).

Fig.4: Increased migration capacity of BCa cells overexpressing ERRα after Rankl treatment. (A, B) Migration was increased in 4T1-ERRα cells (clones 1-3) after Rankl treatment (50ng/ml) for 12h (4T1-ERRα versus 4T1-ERRα+Rankl: ANOVA: P=0.0005, unpaired t-test P=0.0001). The percentage of migration of 4T1-CT cells (clones1-2) was unchanged after Rankl treatment (4T1-CT versus 4T1-CT+Rankl). In the absence of added RankL, migration of 4T1-ERRα cells was lower than that of 4T1-CT cells (P=0.0019), but the opposite was observed after Rankl treatment (P=0.002: 4T1-CT+Rankl versus 4T1-ERRα+Rankl). **(C)** Rankl signalling was assessed by treating 4T1-ERRα cells (clones1-3) and 4T1-CT cells (clones1-2) cells with RankL for different times (0, 5, 15, 25 min). Activation of Src, mTor and Akt (pSrc, pmTor and pAkt) was assessed with α-tubulin as loading control. **(D)** mTor was markedly activated in 4T1-ERRα cells at 5 min to 25min; Src and Akt were also stimulated in 4T1-ERRα cells but to a lesser extent than mTor at 25 min and 5 min respectively.

Fig.5: Pharmacological inhibition of ERRα. (A) 4T1-ERRα (clones1-3) cells were inoculated into the FP of the mammary gland of BALB/c mice and mice were treated with C29 (10mg/kg) for 10 days from the time of tumor appearance to tumor resection (day 14). C29 (C29-Tumor-FP)(d-f) decreased tumor expansion compared to tumor growth in vehicle treated mice (a-c) **(B, C)** Weight and volume of tumors dissected at endpoint (n=9, unpaired t-test, weight: P=0.014; Mann and Whitney: volume: P=0.019). **(D, E)** After tumor resection, animals were kept for an additional 21 days, bones were then crushed and released cells were cultured for 3 weeks. C29 treatment (D-d-f; E) decreased TCB number compared Veh (D-a-c; E) (n=5, 142 ±138 versus 416 ±138, unpaired t-test, P=0.035). **(F)** Real-time PCR performed on RNA extracted from C29 (C29-Tumour-FP) versus vehicle treated (Veh-

Tumor-FP) mice (n=3, unpaired t-test P=0.004 for *ERRα* and P=0.0032 for *Rank*) confirmed a decrease in the expression of ERRα and Rank in treated animals.

Fig.6: Meta-analysis of the METABRIC cohorts that include 2051 radically-resected primary BCa, where BRCA1 and 2 mutations were identified, revealed correlation between high *ERRα* and BRCA1 mutation (Mann-Whitney P=0.02). No link was found between ERRα expression and BRCA2 mutation.

Table.1: Correlation between ERRα and Rank expression in BCa patients. (A) Meta-analysis of the public dataset GSE14020 revealed correlation between *ERRα* and *RANK* in all tumors and in patients with bone but not lung or liver metastases. **(B)** Meta-analysis of the Curie BCa cohorts (n=446) revealed correlation between *ERRα* and *RANK (TNFRSF11A)* mRNA expression in the total tumor population, in the triple negative group and in the luminal group (HR+ERBB2-). No correlation was found in ERBB2+ patients (HR- and HR+) or with *RANKL* (*TNFSF11*). **(C)** Meta-analysis of public datasets GSE12276, GSE2034 and GSE2603 revealed correlation between *ERRα* and *RANK, BRCA1* and *BRCA2* expression levels in all tumors, in patients with visceral and bone metastases or only bone metastases.

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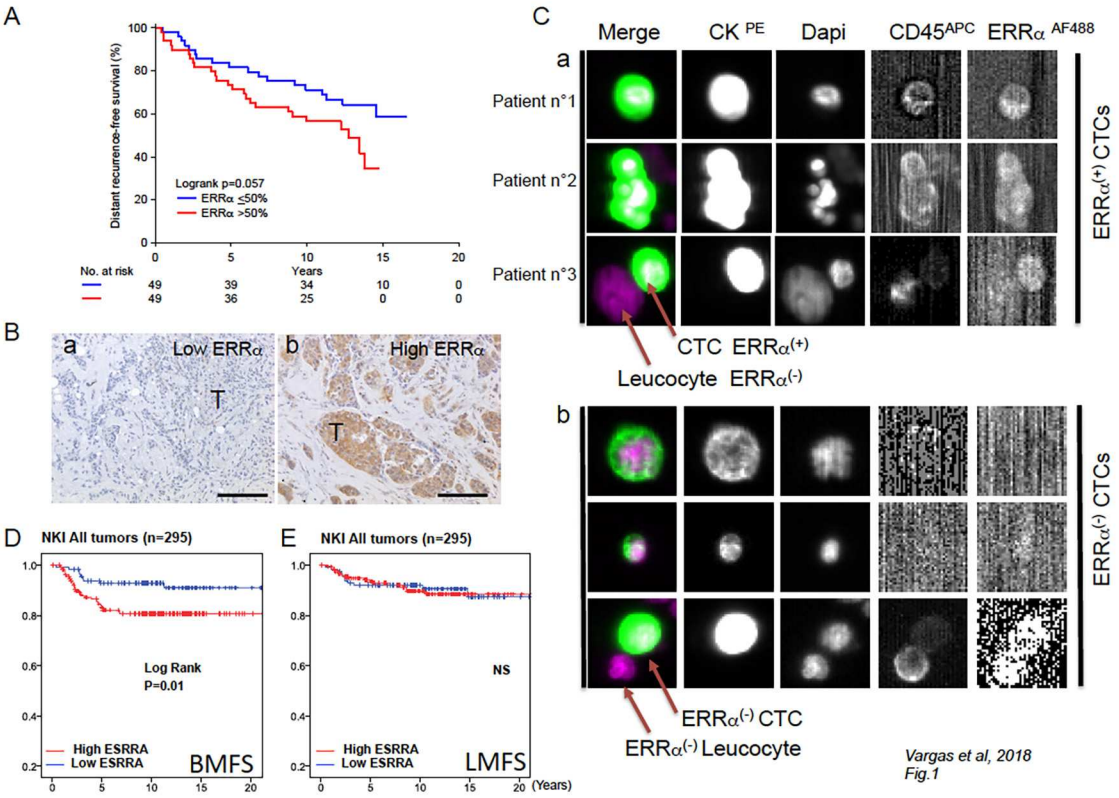

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Fig.1

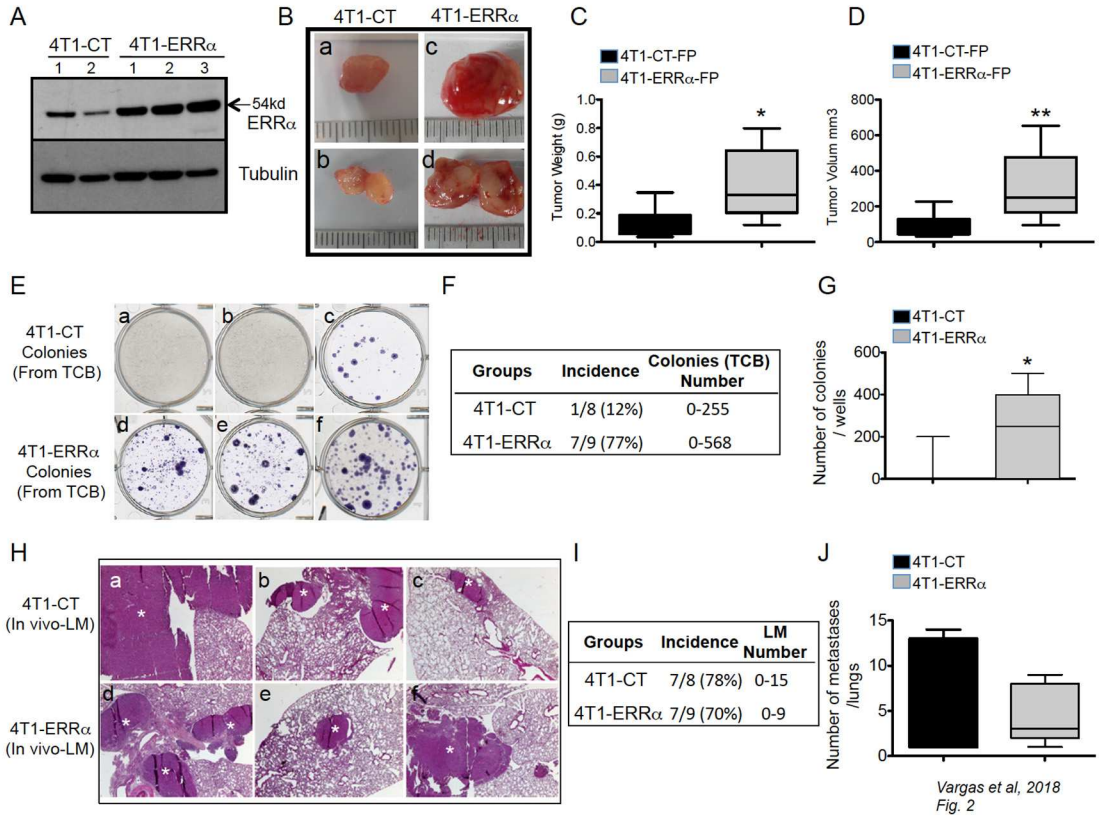

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Fig. 2

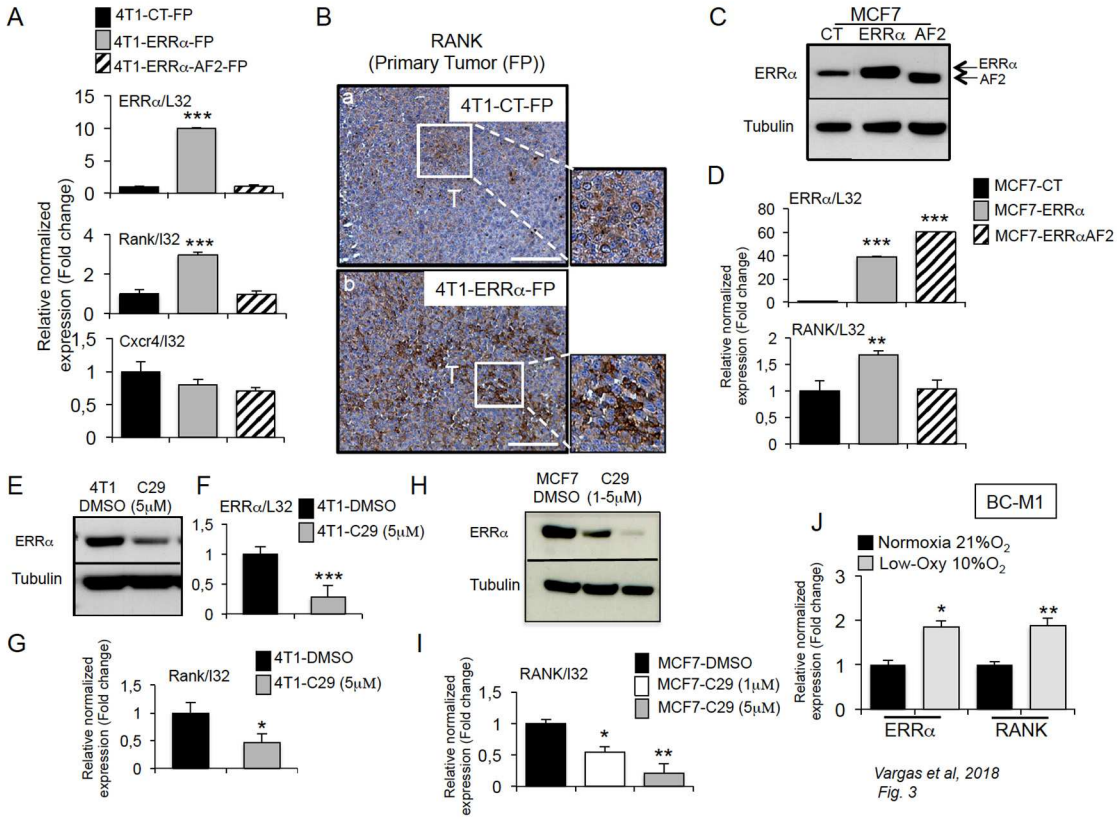

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Fig. 3

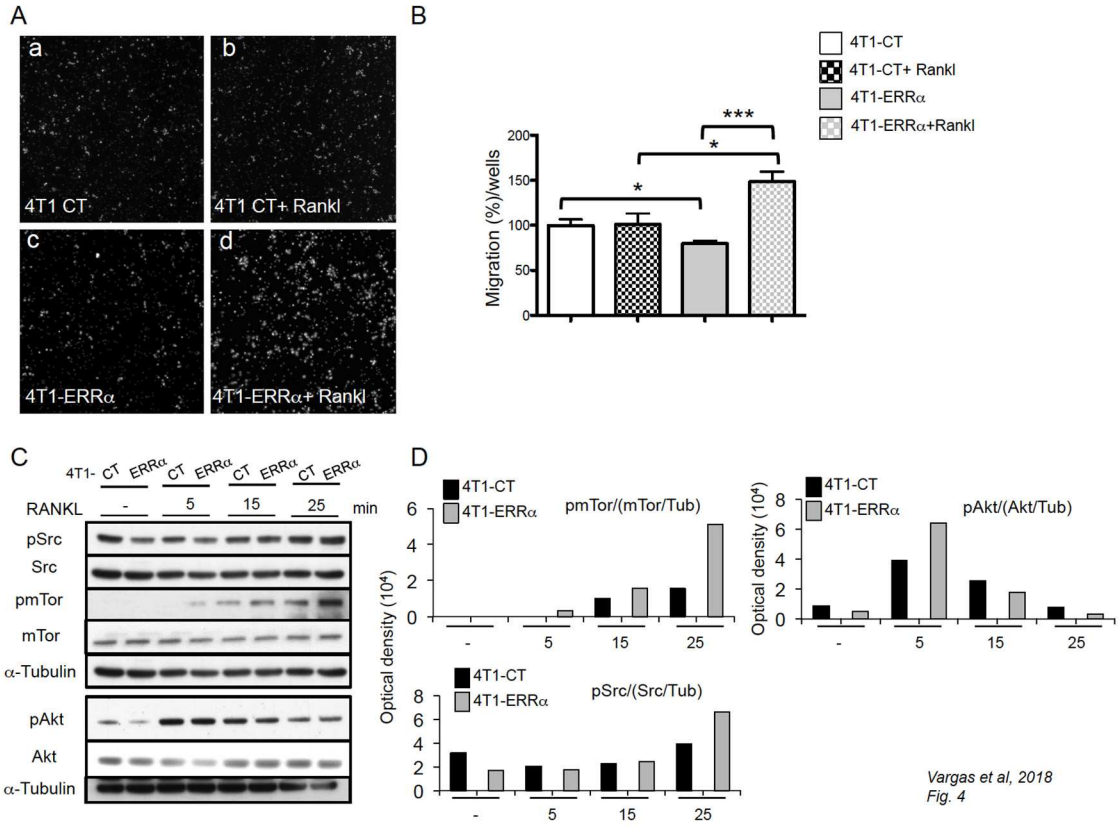
A
a
4T1 CT-
b
4T1 CT+ Rankl
c
4T1-ERRα
d
4T1-ERRα+ Rankl
B
4T1-CT
4T1-CT+ Rankl
4T1-ERRα
4T1-ERRα+Rankl
Migration (%)/wells
200
150
100
50
0
*
*
***
C
4T1- CT ERRα CT ERRα CT ERRα CT ERRα
RANKL - 5 15 25 min
pSrc
Src
pmTor
mTor
α-Tubulin
pAkt
Akt
α-Tubulin
D
4T1-CT
4T1-ERRα
pmTor/(mTor/Tub)
Optical density (10⁴)
6
4
2
0
- 5 15 25
4T1-CT
4T1-ERRα
pAkt/(Akt/Tub)
Optical density (10⁴)
8
6
4
2
0
- 5 15 25
4T1-CT
4T1-ERRα
pSrc/(Src/Tub)
8
6
4
2
0
- 5 15 25
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Fig. 4

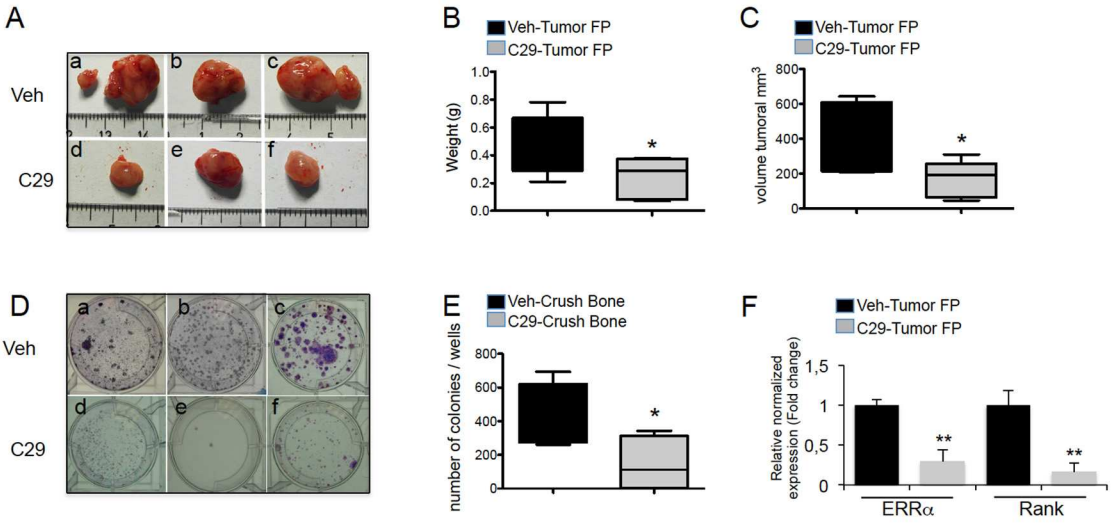

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Fig.5

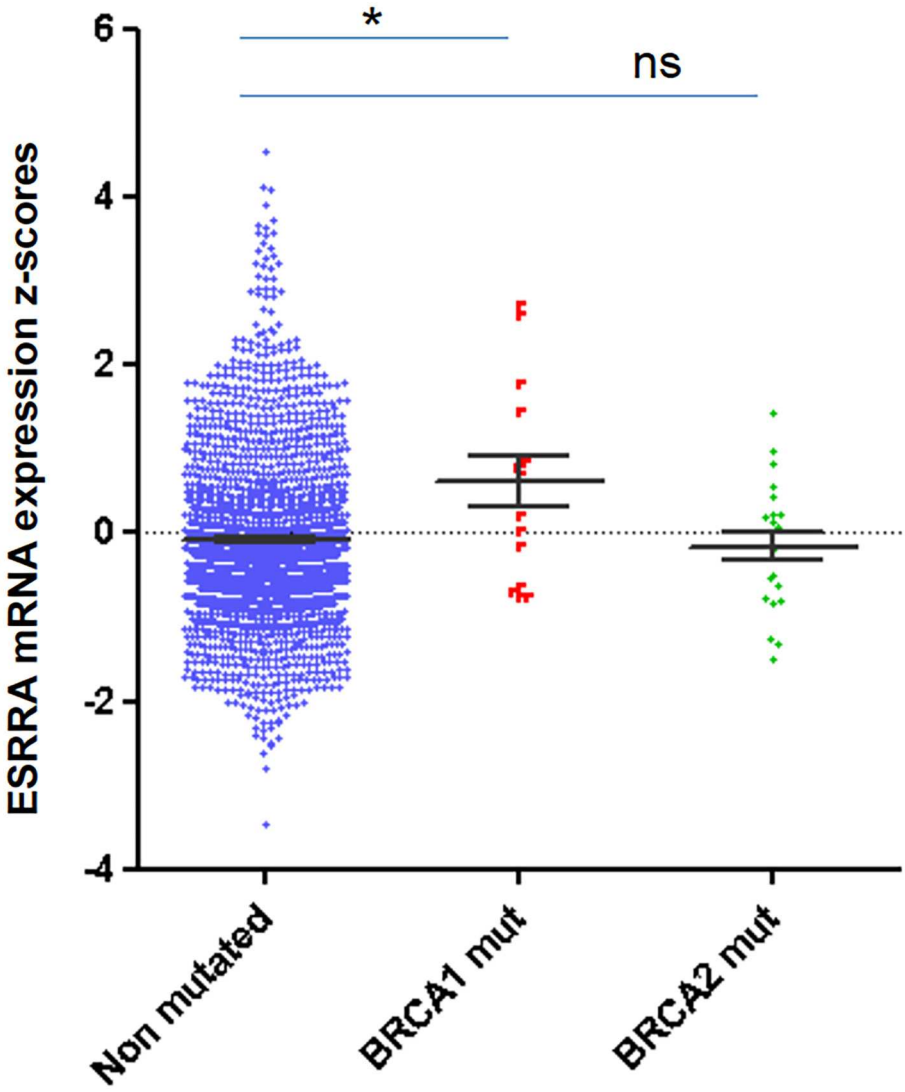

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Fig.6

A

GSE14020 n=	All ESRRA 65	Bone Mets ESRRA 18	Lung Mets ESRRA 20	Liver Mets ESRRA 5	
TNFRSF11A (RANK)	0,510 **<0,0001**	0,515 **0,0287**	0,457 0.0504 (NS)	0,009 0,884 (NS)	

B

Curie n=	All ESRRA 446	NNN ESRRA 68	HR-ERBB2+ ESRRA 42	HR+ERBB2- ESRRA 285	HR+ERBB2+ ESRRA 51
TNFRSF11A (RANK)	0,318 **<0,0000001**	0,299 **0,013**	0,25 0,11 (NS)	0,303 **0,00000099**	0,203 0,15 (NS)
TNFSF11 (RANKL)	0,054 0,25 (NS)	0,146 0,23 (NS)	0,058 0,71 (NS)	-0,001 0,99 (NS)	-0,017 0,90 (NS)

C

GSE12276 2034-2603 n=	All ESRRA 248	No Mets ESRRA 121	Visceral+Bone ESRRA 53	Bone only ESRRA 74
TNFRSF11A (RANK)	0.4063 **<0,0001**	0.1050 0.2517(NS)	0.4667 **0.0005**	0.5145 **<0,0001**
BRCA1	0.2949 **<0,0001**	0.1470 0.1075 (NS)	0.3318 **0.0152**	0.2846 **0.0140**
BRCA2	0.5113 **<0,0001**	0.3559 **<0,0001**	0.4254 **0.0015**	0.5026 **<0,0001**

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Table.1