

Supplemental Figures for:

**Interrogating Estrogen Signaling Pathways in Human ER-positive Breast Cancer Cells
Forming Bone Metastases in Mice**

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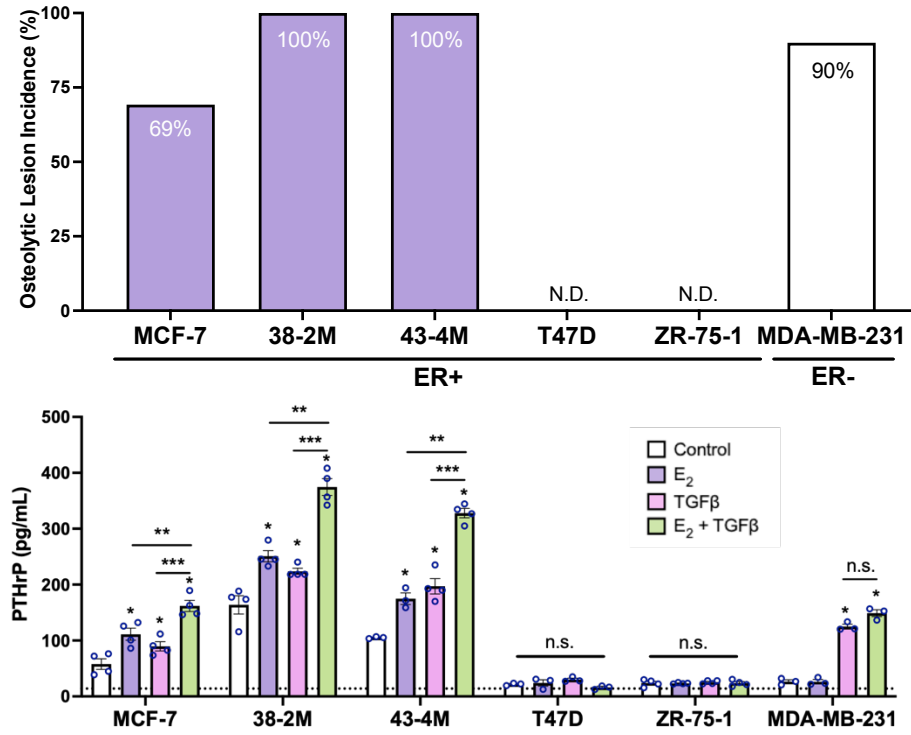
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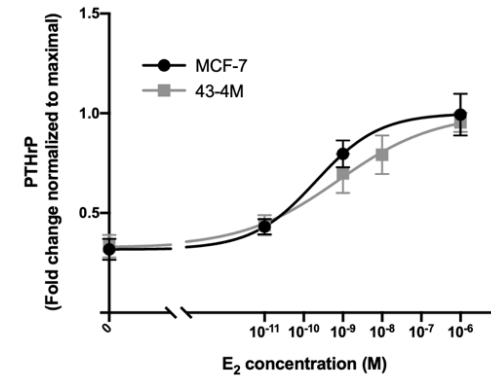
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Supplemental Figure 1

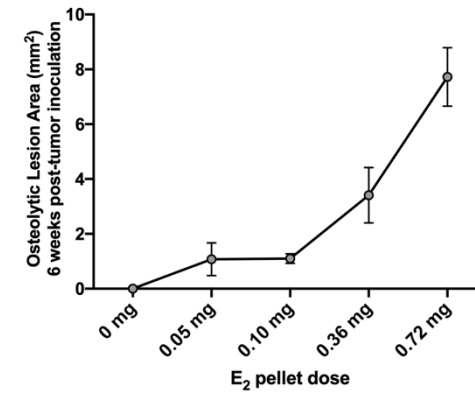
A.



B.



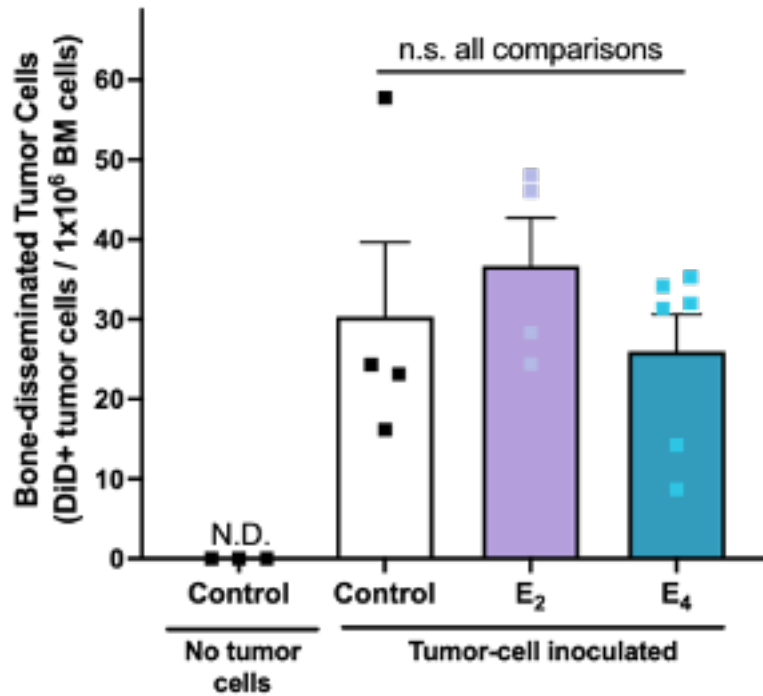
C.



Supplemental Figure 1. Correlation between in vivo osteolytic bone metastases (BMET) formation and regulated PTHrP

secretion in ER+ or ER- human breast cancer cell lines. A) In vivo osteolytic lesion incidence (%; top panel) and in vitro constitutive and inducible (E₂ and/or TGFβ) parathyroid hormone-related protein (PTHrP) secretion (bottom panel) for human breast cancer cell lines. Osteolytic lesion incidence (%) in hind limbs, as measured by radiographs with histologic confirmation of BMETs: 1) 6-weeks post tumor inoculation with ER+ MCF-7 (n=13), 38-2M (n=8), 43-4M (n=7), T47D (n=6), or ZR-75-1 cells (n=9) in mice pelleted with 0.72 mg 17β-estradiol (E₂); or 2) 3-weeks post-inoculation of non-pelleted mice with ER-negative MDA-MB-231 cells (n=10). Not detected radiographically or histologically (N.D.). PTHrP secretion from ER+ MCF-7, MCF-7 BMET-derived = (38-2M and 43-4M), T47D, or ZR-75-1 human breast cancer cells maintained in E₂-deplete media for 4 days prior to treatment with E₂ (10⁻⁷ M) and/or TGFβ (5 ng/ml) vs media control for 48 (72 hours for T47D) (n=3-4/group). ER-negative MDA-MB-231 cells (n=3/group) were maintained in E₂-deplete media for 2 days prior to 24 hours of treatment with E₂ (10⁻⁷ M) and/or TGFβ (5 ng/ml), or media control. *p≤0.05 vs control, **p≤0.05 E₂ vs E₂+TGFβ, ***p≤0.05 TGFβ vs E₂+TGFβ, not significant (n.s.) by two-way ANOVA with Holm-Sidak's post-test. **B)** E₂ dose-dependency of radiographic osteolytic bone lesion area (6 weeks post-tumor cell inoculation, with histologic confirmation of tumors) in hind limbs of MCF-7 inoculated mice supplemented with varying E₂ doses (0.05, 0.10, 0.36, and 0.72 mg; n=3-10/group). No osteolytic lesions were detected in animals lacking E₂ supplementation ("0 mg"). **C)** E₂ dose-dependency of osteolytic E₂-inducible PTHrP secretion, normalized to maximum secretion per cell line, in MCF-7 and MCF-7 BMET-derived 43-4M cells maintained in E₂-deplete media for 4 days prior to E₂ treatment, as indicated for 52 hrs (43-4M) or 72 hrs (MCF-7, due to lower levels of secretion) (n=3-4/group).

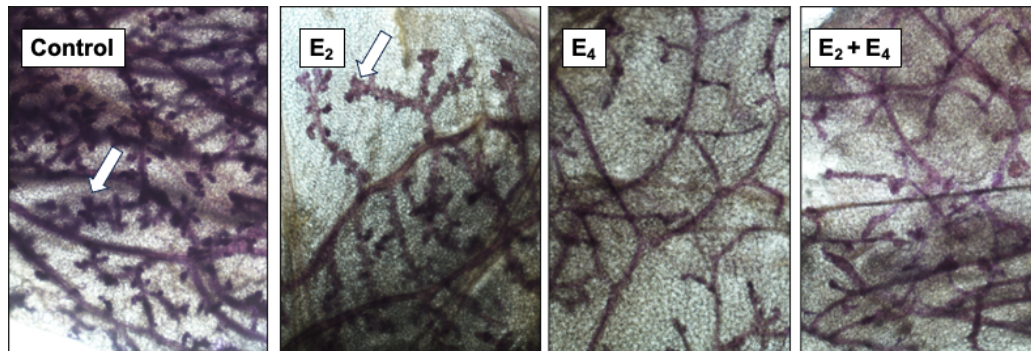
Supplemental Figure 2.



Supplemental Figure 2. Effect of E₄ vs E₂ on dissemination of ER+ 43-4M cells to bone.

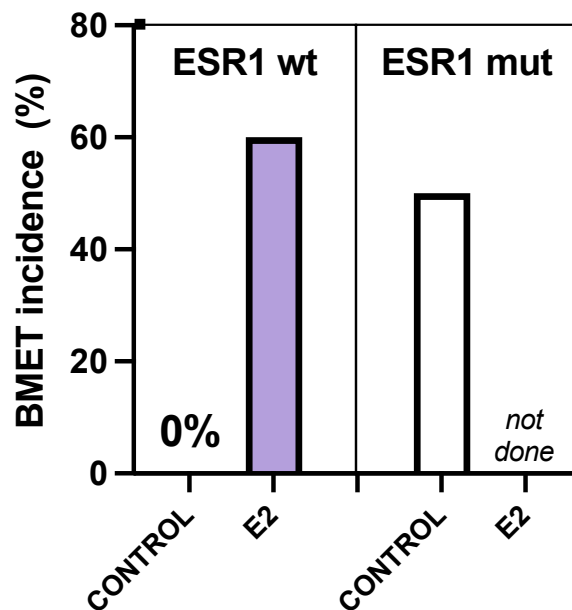
DiD-labeled ER+ 43-4M cell dissemination to bone was quantified in 4-week-old mice treated (beginning 3 days prior to tumor cell inoculation) with E₂ (0.72 mg 60-day-pellet) or daily estetrol (E₄) (4 mg/kg/d via gavage) vs untreated mice, with cells were harvested from hind limbs 3 days post-inoculation (n=6/group). There were no significant differences (n.s.) in DiD+ tumor cell number between E₂ vs E₄ treatment or between estrogen-treatments and untreated controls, as tested by one-way ANOVA with Sidak's post-test.

Supplemental Figure 3.



Supplemental Figure 3. Effect of E₄ vs. E₂ alone or in combination on mammary gland morphology. Representative images of carmine-stained, fixed and defatted glands 42 days post-tumor-inoculation of 5-week-old mice treated with 0.72 mg E₂, 4 mg/kg/d E₄, or a combination of E₂ and E₄, as compared to naïve controls, highlighting representative effects on mammary duct terminal end buds (white arrows) .

Supplemental Figure 4.



Supplemental Figure 4. E₂-dependence of ER+ BMET progression in mice inoculated with ER+ MCF7 cells with ESR1 wild type vs ESR1 activating mutations. Osteolytic BMET lesion incidence 10-weeks post-inoculation of 4 to 5-week-old mice with either (right panel) a 1:1 mixture of MCF-7luc derived clonal cell lines expressing one of two activating ESR1 mutations (MCF-7-Y537S CL3, MCF-7-D538G CL4) *without* E₂ supplementation (control), or (left panel) “parental” MCF-7luc cells (Cambridge Bioscience, Cambridge, UK), without (control) or with E₂ (0.72 mg pellet) supplementation, beginning 4 days prior to inoculation (n=4-5/group).