Estrogen regulates breast cancer dormancy in in vitro bone marrow microvascular niche model

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Directed by Professor Nam Hoon Cho

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ABSTRACT

Estrogen regulates breast cancer dormancy in in vitro bone marrow microvascular niche model

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Estrogen receptor-positive (ER+) breast cancer is characterized by long period of dormancy and metastatic recurrence after several years of anti-estrogen therapy, which is still not fully understood. Here we describe a noble mechanism how the dormant disseminated ER+ breast tumor cells survive and become proliferative in estrogen-deficient bone marrow (BM) niche. In general, ER+ tumor cells cannot proliferate without estrogen. In an experimental model of BM niche, however, ER+ tumor cells proliferated under estrogen deficiency, and their ERK/p38 MAP kinases activity ratio shifted from dormant to awaken phenotype. Following estrogen depletion, the BM niche cells produced angiopoietin-2, which destabilized dormancy-inducing BM niche endothelium and promoted ER+ tumor cell survival under estrogen deficiency via cell surface integrin beta1. Knock-down of angipoietin-2 completely negated estrogen deficiency-induced ER+ tumor cell awakening in the niche. In conclusion, we demonstrate that increased angiopoietin-2 signaling in BM niche can support ER+ tumor awakening from dormancy, especially after anti-estrogen therapy.

Key words: breast, bone, metastasis, dormancy, endocrine therapy resistance, cell signaling

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I. INTRODUCTION

Metastasis is a cardinal feature of human malignancy, and bone marrow (BM) is one of the most common sites that primary tumor cells drive into [1]. For breast cancer, metastatic recurrence is the main cause of patient mortality. To prevent metastatic recurrence, chemotherapies and radiation therapies are often performed along with primary tumour surgery. However, those adjuvant therapies are effective in reducing early recurrence, but have minimal effect on the development of late recurrence [2]. The lag time between primary tumor development and the rise of late metastatic tumors are reported to be as long as a decade, which is called 'tumor dormancy'. It is well established that those dormant cells are mostly found in BM as a single disseminated cell or a micrometastasis, surviving systemic chemotherapies [3], then become the origin of fatal metastatic recurrent tumors [4,5]. In particular, bone metastatic recurrence after 5 years is more common in estrogen receptor-positive (ER+) than ER- subtypes [6], comprising most of ER+ subtype patient mortalities. Overall, bone metastasis is far more frequent in ER+ subtypes than in ER- tumors [7]. This underscores the importance of understanding the biology of ER+ tumor dormancy and reactivation in BM [8], which may lead to a new therapeutic opportunity to this common cancer type [9].

Multiple mechanisms have been proposed to explain how cancer cells survive and remain in dormancy, and how they become reactivated and exit dormancy, including angiogenic

switch, immunosurveillance, and interaction with extracellular matrix and stromal cells [6,10,11]. In terms of extracellular influences, lymph node, lung or BM has a special environment, called niche, where the disseminated tumor cells usually reside [1]. Since niche exists only sparsely and dormant tumor cells are even sparser, human or animal study of tumor cell dormancy in niche is a technical challenge itself. In vitro models of tumor dormancy, therefore, have crucial importance in this field [12]. For instance, the BM niche contains many types of stromal cells, including mesenchymal stem cells (MSCs), osteoblasts, pericytes, fibroblasts, and endothelial cells (ECs) [13,14]. Among the cellular components, the role of EC is an emerging topic of importance [15]. Ghajar et al., [16] found out that endothelial components regulate breast tumor cell dormancy in lung and BM perivascular niches by utilizing both in vitro co-culture models of BM niche and in vivo models. Similarly, Marlow et al, [17] developed a three-dimensional co-culture model of BM niche by mixing MSCs, osteoblasts and ECs, which successfully reproduced dormancy of bone metastatic breast cancer in human.

In general, ER+ tumor cells need estrogen for survival and proliferation. However, many late metastatic recurrences of ER+ breast cancer come after years of adjuvant anti-estrogen therapy or after menopause when systemic estrogen levels become extremely low [6]. Furthermore, it is argued that estrogen depletion may promote, paradoxically, breast cancer metastasis. For example, Ottewell et al, [18] reported that ER- breast cancer bone metastasis in animal model did not decrease but increased after ovariectomy, especially via osteoclast activation. Similarly, McBryan et al reported that anti-estrogen treatment using tamoxifen promoted particularly metastatic recurrence of endocrine-resistant ER+ breast cancer [19]. Both results imply that estrogen deficiency can remodel the microenvironment of secondary organs, which may also support survival and metastasis of endocrine-sensitive ER+ tumor cells. The clinical relevance of this hypothesis can be found in a breast cancer patients follow-up study. Demicheli et al,. compared the hazard rate for breast cancer locoregional and distant recurrences in pre-menopausal patients and post-menopausal patients. One can imply that systemic estrogen level would be far lower in post-menopausal group than in pre-menopausal group. Interestingly, especially in lymph node positive cases, the chronological pattern of

recurrences differed in the two groups: there was a sharp two-peaks – around 1 year and 2.5 postoperative years for pre-menopausal patients, but for post-menopausal women only a broad single peak – around 2 years was seen. This suggests that systemic estrogen levels have a huge influence on dormant tumor cell behavior, particularly their metastatic reactivation.

Angiopoietin-2 (*ANGPT2*) may be the switch underling ER+ breast cancer recurrence after estrogen depletion. ANGPT1 activates the endothelial receptor tyrosine kinase Tie2 and stabilizes the vasculature. ANGPT2 is expressed by endothelium and acts as an autocrine or paracrine antagonist of ANGPT1 [20]. It has been gaining attention as a promising target in cancer therapy, and specific inhibitors are now available [21,22]. In primary organ, ANGPT2 directly stimulates tumor angiogenesis[21]. In metastatic sites, ANGPT2 loosens the endothelial cell–cell junction, which enhances extravasation of disseminated tumor cells [23,24]. Yet, its role after tumor cell extravasation, especially on tumor cell dormancy and reawakening, has not yet been explored. Since estrogen regulates angiopoietin-1 and 2 expressions in other tissues [25-28], we hypothesized that estrogen deficiency may modulate ANGPT2 signaling in the BM niche, triggering ER+ tumor cell awakening from dormancy. Herein, we demonstrate that estrogen-deficient BM niche overexpresses angiopoietin-2, which negates ER+ tumor cell dormancy and eventually promotes estrogen-independent tumor growth.

II. MATERIALS AND METHODS

1. Cell lines and culture conditions

Breast cancer cell lines MCF7, BT474, MDA-MB-361 and MDA-MB-231 were obtained from the American Tissue Culture Collection and grown in complete RPMI-1640 medium (GIBCO, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (FBS; GIBCO), 100 unit/mL penicillin, and 100 μg/mL streptomycin. Primary human umbilical vein endothelial cells at second passage were obtained commercially (PromoCell GmbH, Heidelberg, Germany) and grown in endothelial cell growth medium 2 (EGM2; PromoCell) in a humidified chamber (37℃, 5% CO2). Primary human bone marrow mesenchymal stem cells (MSCs) at second passage were obtained from Yonsei Cell Therapy Center (Seoul, Korea) and maintained in low glucose Dulbecco's Modified Eagle Medium (DMEM; GIBCO) supplemented with 10% FBS, 100 unit/mL penicillin, and 100 μg/mL streptomycin. Endothelial cells (ECs) and MSCs isolated between passages 5 and 10 were used in these experiments.

2. Generation of tumor cells expressing fluorescent tags

Tumor cell lines were tagged with red fluorescent protein (RFP) or enhanced green fluorescent protein (GFP) using a lentiviral transduction system. Briefly, pLenti CMV/TO Puro empty vector was obtained from Addgene (Addgene plasmid 17482; Cambridge, MA) 49. RFP and GFP (sequences obtained from GenBank) were cloned into pLenti CMV/TO Puro empty vector. Lentivirus were generated by co-transfection of packaging vectors pMDLg/pRRE, pMD2G, pRSV-Rev (Addgene plasmids 12251, 12253, and 12259), and pLenti CMV/TO Puro-RFP or pLenti CMV/TO Puro-GFP into 293T cells with 2.5 M calcium chloride. RFP or GFP-expressing tumor cells lines were generated by lentiviral infection and selection for 1 week in 1μg/mL puromycin.

3. *In vitro* model of bone marrow niche

MSCs and ECs were co-cultured in EGM2 for 5 to 7 days to reach confluence. For 3-dimensional (3D) culture, growth-factor reduced, phenol-red-free Matrigel® matrix (Corning Inc., NY) was used to coat the vessels before cell seeding. To discriminate ECs from MSCs, ECs were stained with carboxyfluorescein succinimidyl ester (CFSE, Life Technologies) before co-culture. Cell numbers and culture volumes were as follows: 5 x 103 MSCs, 2 x 103 ECs in microfluidic plates. 5 x 10^4 MSCs, 2 x 10^4 ECs, and 200 μL EGM2 per well in 96-well microplates; 2 x 10^5 MSCs, 5 x 10^4 ECs, and 2 mL EGM2 per well in 6-well microplates; and 2 x 10^6 MSCs, 5 x 10^5 ECs, and 10 mL EGM2 in 100 mm dishes.

4. Monitoring of cell proliferation

A microfluidic live-cell imaging platform (CellASIC™ ONIX Microfluidic Platform, EMD Millipore) was utilized to supply nutrients and oxygen and remove wastes with minimal stress to cells. Cell culture was performed according to the manufacturer's instructions. Briefly, 10 μL of MSC and EC cell suspensions (1 × 10^6 total cells/mL) was loaded onto a microfluidic culture plate (M04S, EMD Millipore), which has a compatible culture volume as that of 384-well microplate. The plate was attached to the platform controlling perfusion flow, temperature, and gas composition. An inverted fluorescence microscope was used for live-cell imaging with the 40x objective. The cells were incubated (EGM2, 37℃, 5% CO_2) for 5-7 days until reaching confluence. Then, fluorescence-expressing tumor cells were seeded sparsely onto BM niche culture plates. Tumor cell seeding numbers were as follows: 0.5 x 10^2 cells per well in microfluidic plate; 2 x 10^2 cells per well in 96-well microplates; 4 x10^3 cells per well in 6-well microplates; and 2 x 10^4 MCF7 in 100 mm dishes. For microfluidic culture, tumor cell proliferation was monitored by capturing time-lapse images of the cells using a fluorescence microscope (Nikon® Eclipse Ti, Nikon Instruments Inc.). For conventional culture, a fluorescence microplate reader (Varioskan™ Flash Multimode Reader, Thermo Scientific) was used to measure cell fluorescence every 24 hours after cell seeding.

Fluorescence intensity was measured using bottom optic readings. Excitation/emission wavelengths were 553/574 nm for RFP and 488/507 nm for GFP.

To compare proliferative capacities of tumor cells co-cultured with different stromal cell compositions (MSCs, MSCs-ECs, or no stromal cells as a control), all cells were harvested by trypsinization after co-culturing for upto 7 days. Fluorescence-positive cells were sorted and collected using fluorescence-activated cell sorting (FACS) flow cytometry (FACSAriaTM cell sorter; BD Bioscience, San Jose, CA), and seeded onto new empty plates (2 x 103 cells per well in 96-well microplates). Cell proliferative capacity was assessed again by measuring fluorescence intensity every 24 hours.

5. Flow cytometry to detect erk1/2 and p38 activities

GFP-expressing tumor cells were cultured alone or with MSCs and/or ECs in 100 mm dishes for 7 days. Cells were trypsinized, washed with phosphate-buffered saline (PBS), and fixed with 4% paraformaldehyde for 10 minutes. The fixed cells were chilled on ice and permeabilized with 90% methanol for 30 minutes. Then, 1 x 106 cells per experimental condition were aliquoted, washed, and resuspended in 100 μL of fluorochrome-conjugated primary antibodies against p38, phosphorylated p38 (Santa Cruz Biotechnology, Santa Cruz, CA), ERK1/2, and phosphorylated ERK1/2 (Cell Signaling Technology, Danvers, MA) at the manufacturer's recommended concentrations, and incubated for 1 hour. For isotype control, fluorochrome-conjugated rabbit IgG was used at the same concentration. Cells were washed, re-suspended in PBS, sorted for GFP fluorescence, and analyzed using the FACSAria™ cell sorter.

6. Estrogen depletion and supplement in culture

FBS stripped with charcoal-dextran was purchased from Gemini Bio-Products (Gemini Bio-Products, West Sacramento, CA). To evaluate the effect of estrogen depletion on BM niche, MSCs and/or ECs were cultured in phenol-red-free RPMI 1640 containing 10% charcoal-stripped FBS supplemented with or without 200 pg/mL 17β-estradiol (E2758, Sigma-Aldrich, St. Louis, MO). Because the charcoal-stripping process eliminates estrogen and other steroidal hormones essential for primary EC survival, hydrocortisone (0.2 μg/mL) was added to the final medium. To supplement estrogen, 17-β estradiol (Sigma-aldrich) was added.

7. Modulation of receptor-ligand interaction

To evaluate the effect of angiopietin-1, 2 on tumor cell proliferation, cultures were treated with 0, 50, 100 or 500 ng/mL recombinant human angiopoietin-1 and recombinant human angiopoeitin-2 (R&D Systems, Minneapolis, MN). To block angiopoietin-1 and Tie2 receptor interaction, cultures were treated with 10 μg/mL of human Tie2 affinity-purified polyclonal antibody (R&D Systems). To knock-down angiopoietin-2 in culture, short-inhibiting RNAs (siRNAs) were used. Three different SiRNAs that are known to block specifically angiopoietin-2 mRNA were generated:

#1 5′-GGAAGAGCAUGGACAGCAUAGGA-3′ [29]

#2 5’-AGAACCAGACGGCUGUGAUGAUAGAAA-3’ [30]

#3 5’-CCAGACGGCUGUGAUGAUA-3’ [31]

Negative and positive control siRNAs were obtained from GenePharma (Suzhou, China). Cells were prepared and cultured as described above and siRNAs were transfected using transfection reagent G-Fectin (Genolution Pharmaceuticals, Inc. Seoul, Korea) according to the manufacturer's protocol. Briefly, 1uL G-Fectin and 5pmol siRNA were incubated in 50 uL PBS at room temperature for 10 min, and added to cell culture plate (24-well). Culture

medium was replaced 8 h after transfection to minimize cytotoxicity. Angiopoietin-2 knockdown was assessed by PCR using total RNA extracted from cells 48 h post-transfection.

To knock-down ITGB1, we used the SureSilencing shRNA plasmid for human ITGB1 (KH00650G for the GFP) and a scrambled sequence negative control plasmid (SABiosciences, Frederick, VA), as described previously [32]. The shRNA target sequence for ITGB1 was as follows: 5′-TGT GCT CAG TCT TAC TAA TAA-3′. The cells were seeded and transfected using the Attractene Transfection Reagent (QIAGEN) according to the manufacturer's protocol.

RNA extraction and reverse transcriptionpolymerase chain reaction (RT-PCR)

As previously described[33], total RNA was isolated from cells using an RNeasy Protect Mini Kit (Qiagen) according to the manufacturer's protocol. The Super-Script III Reverse Transcriptase kit (Invitrogen) was used to synthesize cDNA. Polymerase chain reactions (PCRs) were performed with HotStarTaq DNA polymerase (Qiagen) and the following conditions: denaturation at 95℃ for 15 min, and 28 cycles of 95℃ for 40 s, 52℃ for 1 min, and 72℃ for 1 min, with a final extension for 10 min at 72℃. Expression levels of GAPDH were assessed as an internal control in all reactions. The following primers were used for PCR: forward primer for angiopoietin-1 5'-GAAGGGAACCGAGCCTATTC-3', reverse primer 5'-GGGCACATTTGCACATACAG-3'; forward primer for angiopoietin-2 5'-TGGGATTTGGTAACCCTTCA-3', reverse primer 5'-GGTTGGCTGATGCTGCTTAT-3'.

8. Western blot assay

As previously described [33], cells were trypsinized and lysed by Pro-Prep™ protein extraction kit (iNtRON biotechnology, Sungnam, Korea). Equal amounts of protein extracts (20 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Invitrogen). Blots were blocked with 5% nonfat dry milk at room temperature. The blots were incubated with antibodies specific for angiopoietin-1 (ab183701, Abcam), angiopoietin-2 (ab155106, Abcam) and β-actin (Santa Cruz Biotechnology) at specific dilution, followed by incubation with peroxidase-labeled secondary antibodies. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection kit (Santa Cruz Biotechnology).

9. Graphics and statistical analysis

For survival analysis of human patients, statistical significance was determined using Kaplan-Meier method and Log-rank test. For in vitro experiments, statistical significance was determined using Student's t-test and ANOVA. Results were considered to be significant at $P<0.05$. Statistical analyses were computed with IBM SPSS Statistics 20 software. All graphical presentations were created using GraphPad Prism 6 software. Blot images were captured by using ImageQuant™ LAS 4000 biomolecular imager (GE Heathcare Lifesciences). Blot intensities were quantified by using ImageJ 1.48v software (http://imagej.nih.gov/ij) [34].

III. RESULTS

1. ANGPT1, 2 expressions correlate with ER+ breast cancer recurrences following anti-estrogen endocrine therapy

ANGPT2 overexpression in breast cancer correlates with lymphatic metastasis and poor patient survival [35]. ANGPT1, contrastingly, has not yet been noted as a prognostic factor of breast cancer, although in vivo experiments have shown that its overexpression suppresses xenograft ER+ tumor growth [36,37]. To determine if ANGPT1, 2 expressions were associated specifically with ER+ subtype progrosis, we used a published dataset of matching gene expressions and survival [38]. Patients were first categorized into those who underwent adjuvant endocrine therapy (including ER antagonist Tamoxifen and aromatase inhibitors, n=982) or those who did not undergo such endocrine therapy (n=788). Then the patients were further stratified into two groups based on ANGPT1 and ANGPT2 mRNA levels in tumor tissue. For those with endocrine therapy, increased ANGPT1 levels were associated with a significantly increased probability of recurrence-free survival (FIGURE 1A), while increased ANGPT2 levels were associated with a significantly decreased probability of recurrence-free survival (FIGURE 1B). For those without endocrine therapy, contrastingly, neither ANGPT1 nor ANGPT2 were associated with the probability of recurrence-free survival (FIGURE 1C, D).

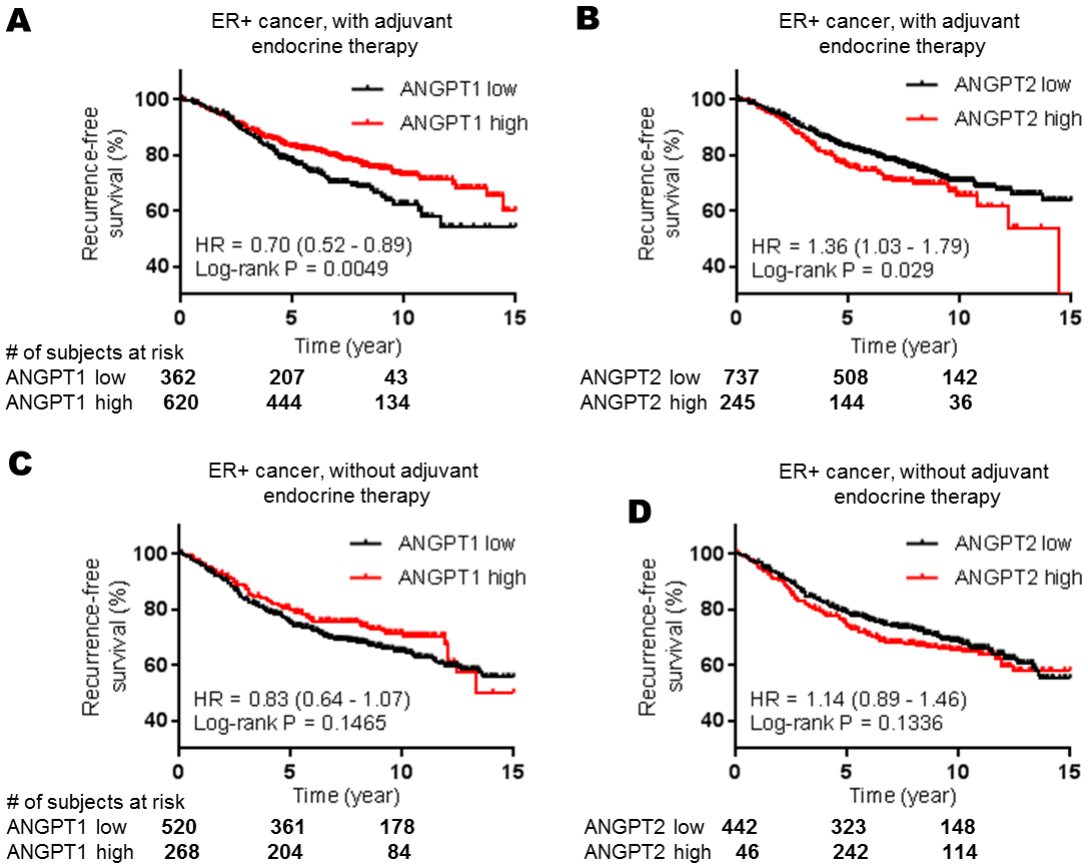

FIGURE 1. Recurrence-free survival of ER+ breast cancer patients, stratified by ANGPT1 and ANGPT2 expressions. Kaplan-Meier analysis comparing recurrence-free survival of ER+ breast cancer patients following endocrine therapy (n=982, **A-B**) and those without endocrine therapy (n=788, **C-D**), distinguished by low versus high expressions of ANGPT1 (**A, C**) or ANGPT2 (**B, D**). Gene expression data obtained from the open source KM Plotter (28). ER=Estrogen receptor; ANGPT1 = angiopoietin-1 mRNA; ANGPT2 = angiopoietin-2 mRNA.

2. Angiopoietin-1, 2 expressions correlate with ER+ breast cancer recurrences following anti-estrogen endocrine therapy

To validate the association of ANGPT1, 2 expressions and systemic estrogen activity, we analyzed the Cancer Genome Atlas (TCGA) primary breast cancer data (593 samples, data acquired from Oncomine™). Cases were divided by menopausal state – Pre / Peri / Post-menopausal or indeterminate. ANGPT2 expressions were significantly higher in peri-menopausal group than in pre-menopausal group (FIGURE 2A, B). Plus, the ratio of ANGPT2/ANGPT1 mRNAs increased step-wisely in pre / peri / post-menopausal group (FIGURE 2C). Based on these clinical data, we hypothesized that ANGPT signaling has a role in latent ER+ tumor recurrence especially after systemic estrogen depletion.

ANGPT1/Tie2 signaling is active in the BM niche, mainly participating in hematopoietic stem cell quiescence [39]. If ANGPT signaling does have a role in ER+ tumor recurrence, it is probably working also in the BM niche where dormant tumor cells are most likely to reside. Indeed, ANGPT1 and ANGPT2 is mostly expressed from stroma rather than the tumor cells (Oncomine data, FIGURE 2D).

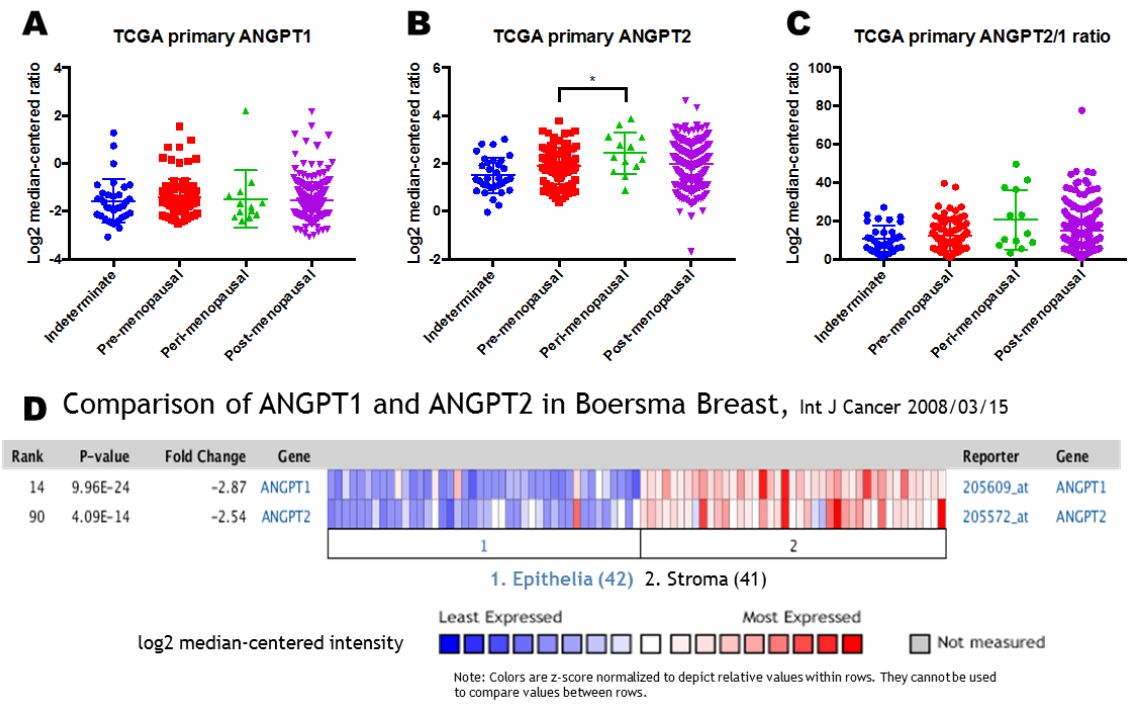

FIGURE 2. Breast cancer cohort gene expressions, categorized by menopausal status and epithelia vs stroma. (A-C) TCGA breast cancer provisional cohort (n=593, data acquired from Oncomine™). Indeterminate (Neither Pre- or Post-Menopausal); **Pre-menopausal** (<6 Months since LMP and No Prior Bilateral Ovariectomy and Not on Estrogen Replacement); **Peri-menopausal** (6-12 Months since Last Menstrual Period); **Post-menopausal** (Prior Bilateral Ovariectomy or >12 Months since LMP with No Prior Hysterectomy). **(D). Boersma breast cancer cohort (n=83, data acquired from Oncomine™).**

3. ER+ breast cancer cells become dormant in in vitro bone marrow microvascular niche model

To test the impact of ANGPT signaling on ER+ breast tumor dormancy in BM niche, we utilized existing in vitro models [12,16,17]. Briefly, to create a microenvironment similar to BM niche, Human BM-derived MSCs and/or ECs were co-seeded onto extracellular matrix-coated microplates (FIGURE 3A). These were designated as "mesenchymal niche" or "endothelial niche", based on the presence of ECs. Without ECs, BM-derived stromal cells (mesenchymal niche) generally supports breast tumor cell growth, whereas EC-contained niche (endothelial niche) inhibits tumor cell growth [17]. To optimize the system for our studies, a few modifications have been introduced: a commercial continuous fluidic cell culture system was used to supply nutrients and oxygen and remove wastes with minimal stress to cells during long-term culture; charcoal-stripping fetal bovine serum (FBS) was used to eliminate serum-derived estrogen.

The niches were incubated until their cells reaching confluence in medium containing 10% charcoal-stripped FBS supplemented with estradiol (E2) 200pg/mL. Then, fluorescence-expressing MCF7 ER+ human breast tumor cells were seeded sparsely onto the niche surface. Stromal cells and tumor cell seeding densities were determined by references and our own preliminary studies (FIGURE 4). Tumor cell proliferation was monitored by its fluorescence visualization using a microplate reader and a microscope. In results, MCF7 breast tumor cells exhibited significant growth suppressions in endothelial niche compared to those of the mesenchymal niche or cultured alone (FIGURE 3B, C). The results were repeated in other ER+ tumor cell lines BT474 and MDA-MB-361 (FIGURE 5A, B). An ER-negative cell line MDA-MB-231 did not show growth suppression in endothelial niche (compared to those cultured alone), but its proliferation rate in mesenchymal niche was significantly higher than those in endothelial niche or cultured alone (FIGURE 5C). Mitogen-activated protein (MAP) kinases activity ratio of ERK1/2 and p38 (ERK/p38 ratio) can be used as an indicator of tumor cell dormancy and awakening: proliferative tumor cells exhibit high ERK/p38 ratio,

while dormant tumor cells exhibit low ERK/p38 ratio [40]. Since both proteins are active in phosphorylated forms, we analyzed their phosphorylation status in tumor cells grown in mesenchymal niche and endothelial niche by FACS. Compared to those cultured alone or in mesenchymal niche, ER+ tumor cells in endothelial niche exhibited a shift toward low ERK/p38 ratio (FIGURE 3D). When the tumor cells cultured in endothelial niche were harvested and re-seeded in empty plates, they started replication and generated tumor, implying that their tumorigenic potentials were maintained (FIGURE 3E). Growth suppression of tumor cells in our endothelial niche model could be extended upto 3 weeks (FIGURE 3F).

Vascular phenotype inside tumor can be switched by angiopoeitin-1/Tie2 signaling [41]. We tested the effect angiopoietin-1/Tie2 signaling modulation on tumor cell dormancy in BM endothelial niche. We added human Tie2-receptor specific blocking antibody (10μg/mL) to culture medium of endothelial niche. Compared to untreated control, the growth of ER+ tumor cells in endothelial niche significantly increased upon Tie2 blockade (FIGURE 6A). In contrast, recombinant human angiopoietin-1 (RhAng1) 500ng/mL treatment further suppressed tumor cell proliferation in endothelial niche (FIGURE 6A). Again, Tie2 blockade significantly increased tumor cell proliferation in RhAng1-treated endothelial niche. Meanwhile, the growth of tumor cells in mesenchymal niche or those cultured alone were not affected by either Tie2 blockade or RhAng1 (FIGURE 6A). Tumor cell proliferation rates in the endothelial niches were correlated with their Tie2 receptor activities and thrombospondin-1 (TSP-1) levels (FIGURE 6B). Next, tumor cell's ERK/p38 activity ratio was examined. Upon Tie2 blockade, ERK/p38 high (awaken) MCF7 cell population in endothelial niche increased while ERK/p38 low (dormant) population decreased (FIGURE 6C). In contrast, RhAng1 further increased dormant tumor cell population in endothelial niche (FIGURE 6C). Combination of RhAng1 treatment and Tie2 blockade recovered the activity ratio of ERK/p38 MAP kinases in similar degree to those of untreated control (FIGURE 6C). We also tested the effect of recombinant human angiopoietin-2 (RhAng2), a competitive antagonist of angiopoeitin-1. Similarly, RhAng2 500ng/mL treatment promoted MCF7, BT474 and MDA-

MB-361 cell proliferations only in endothelial niche, not in mesenchymal niche or in empty plate (tumor cell alone) (FIGURE 7A-C).

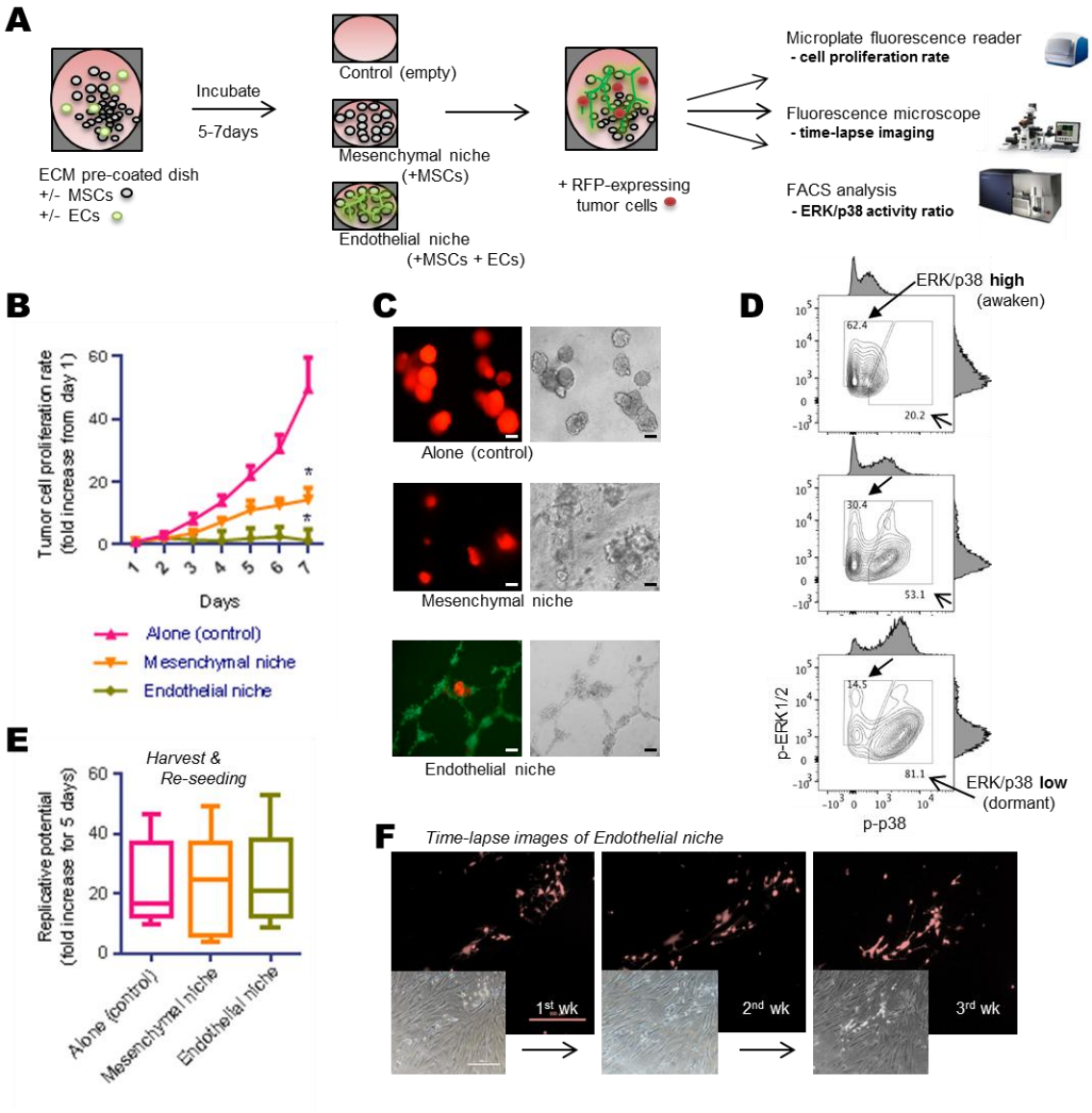

FIGURE 3. ER+ breast tumor cell dormancy in bone marrow endothelial niche models.

(**A**) Experimental scheme. BM MSCs and/or ECs were seeded on 96-well microplates and incubated until reaching confluence. For 3D cultures, Matrigel™ was coated (150μL/cm^2) onto the culture plate. Then RFP-expressing tumor cells were sparsely seeded (200 cells/well) onto the niche cells or empty surfaces (control). Tumor cell proliferations were assessed by fluorescence intensity reader and microscope, and fluorescence-activated cell sorting (FACS). (**B**) 7-day proliferation rates of ER+ tumor cells in niches (five sample sets per group; error bars: ± standard deviation (SD) *p <0.05). (**C**) Representative images of tumor cells in the niches, captured after 7-days of co-culture [Left: fluorescence (red & green); right: bright-

field. scale bar=100μm]. For visualization, ECs were pre-stained with CFSE (green). (**D**) FACS analysis of ERK1/2 and p38 MAR kinase activities of tumor cells in niche. Upper: tumor cell alone; middle: tumor cell in mesenchymal niche; lower: tumor cell in endothelial niche. Blank arrows: ERK/p38 ratio-low (dormant) population; filled arrows: ERK/p38 ratio-high (awaken) population. (**E**) Replicative potentials of dormant tumor cells. Tumor cells were harvested from niche and re-plated in 96-well microplates (500 cells/well). Box-plots represent fluorescence intensity fold changes for 7 days (five samples per condition. horizontal bar = mean). (**F**) Time-lapse images of tumor cell dormancy in niche. Tumor cells were cultured in endothelial niche for upto 21 days (fresh medium replaced every 48hr). Cells remain at almost the same number during the period. [Upper: fluoresence (red); lower: bright-field. scale bar=500μm]. ER=estrogen receptor; BM MSC=bone marrow-derived mesenchymal stem cell; EC=endothelial cell; RFP=red-fluorescence protein; MAP=Mitogen-activated protein; p-ERK1/2=phosphorylated ERK1/2; p-p38=phosphorylated p38.

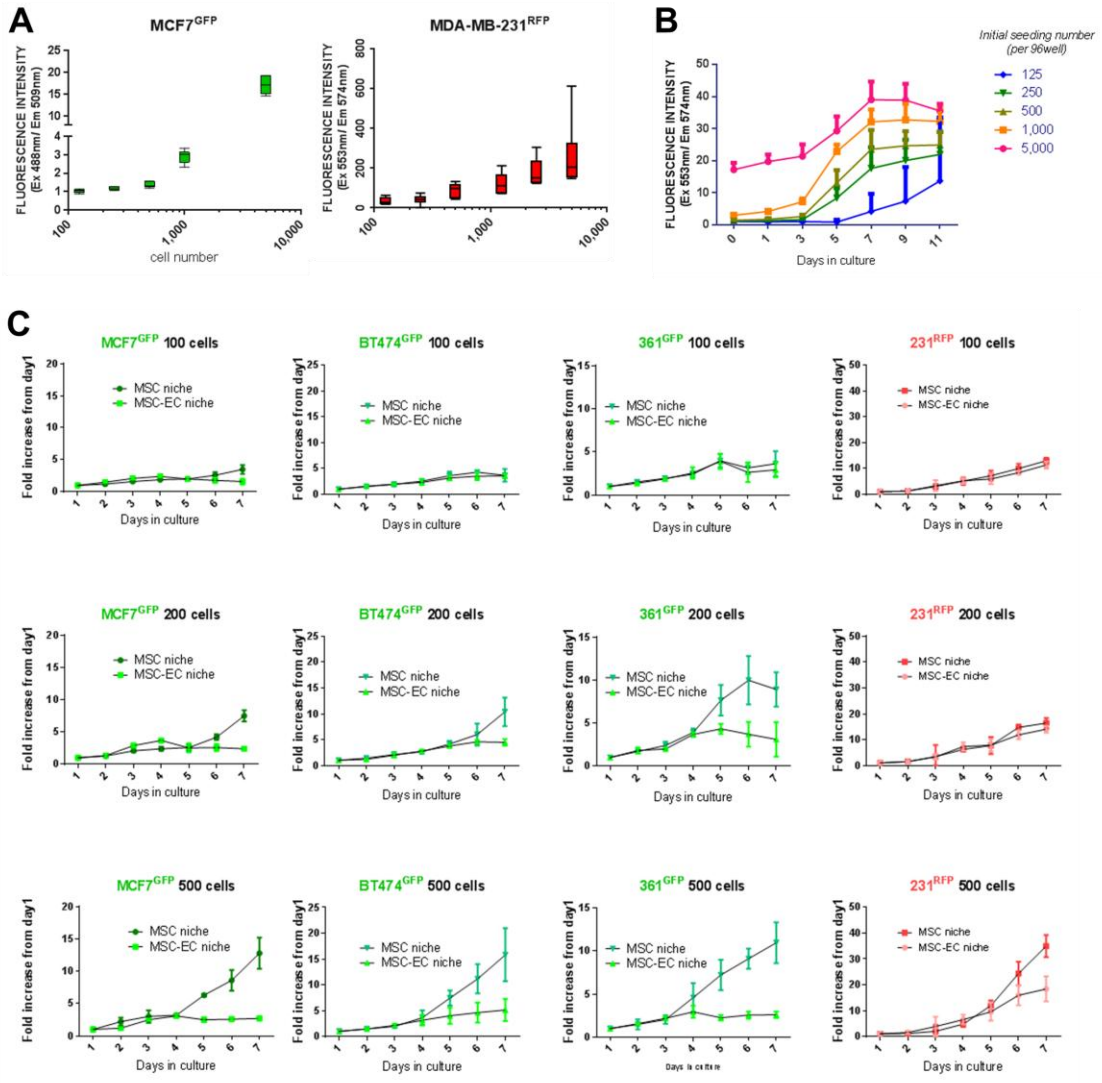

FIGURE 4. Cell number & fluorescence intensity correlation. (A)To determine the linearity in fluorescence intensity and cell number in 96-well microplate, GFP-expressing tumor cells (MCF7) and RFP-expressing tumor cells (MDA-MB-231) were plated in 100, 250, 500, 1,000, and 5,000 cells per well. Six hours later, flouresence intensities of each well were measured by microplate fluorescence reader. Fluorometric excitation wavelengths were 488nm for GFP and 553nm for RFP. Emissions were read on 509 nm (GFP) or 574nm (RFP). Band width: 5nm (GFP), 12nm (RFP). (B) To determine appropriate cell seeding density for long-term culture, different number of GFP+ MCF7 cells (125, 250, 500, 1,000, and 5,000 cell per well) were seeded and cultured for upto 11 days. As shown, initial cell seeding number between 250 to 500 per well represent the exponential growth pattern of tumor cells most promptly. (C) To determine appropriate tumor cell seeding density in niches, cell

proliferation rates in MSC niche (mesenchymal niche) vs. MSC-EC niche (endothelial niche) were compared across (MCF7, BT474, MDA-MB-361, MDA-MB-231) by varying initial seeding densities (100, 200, 500 cells per 96 well). Differences of the tumor cell proliferation rates in niches were noted most clearly after 7 days in initial cell seeding number. If fewer than 100 cells were seeded, tumor cells did not grow significantly even in (supposedly) growth-promoting condition. In contrast, if more than 1,000 cells per well were seeded in niches, growth suppressive effect of endothelial niche was not prominent. (data not shown).

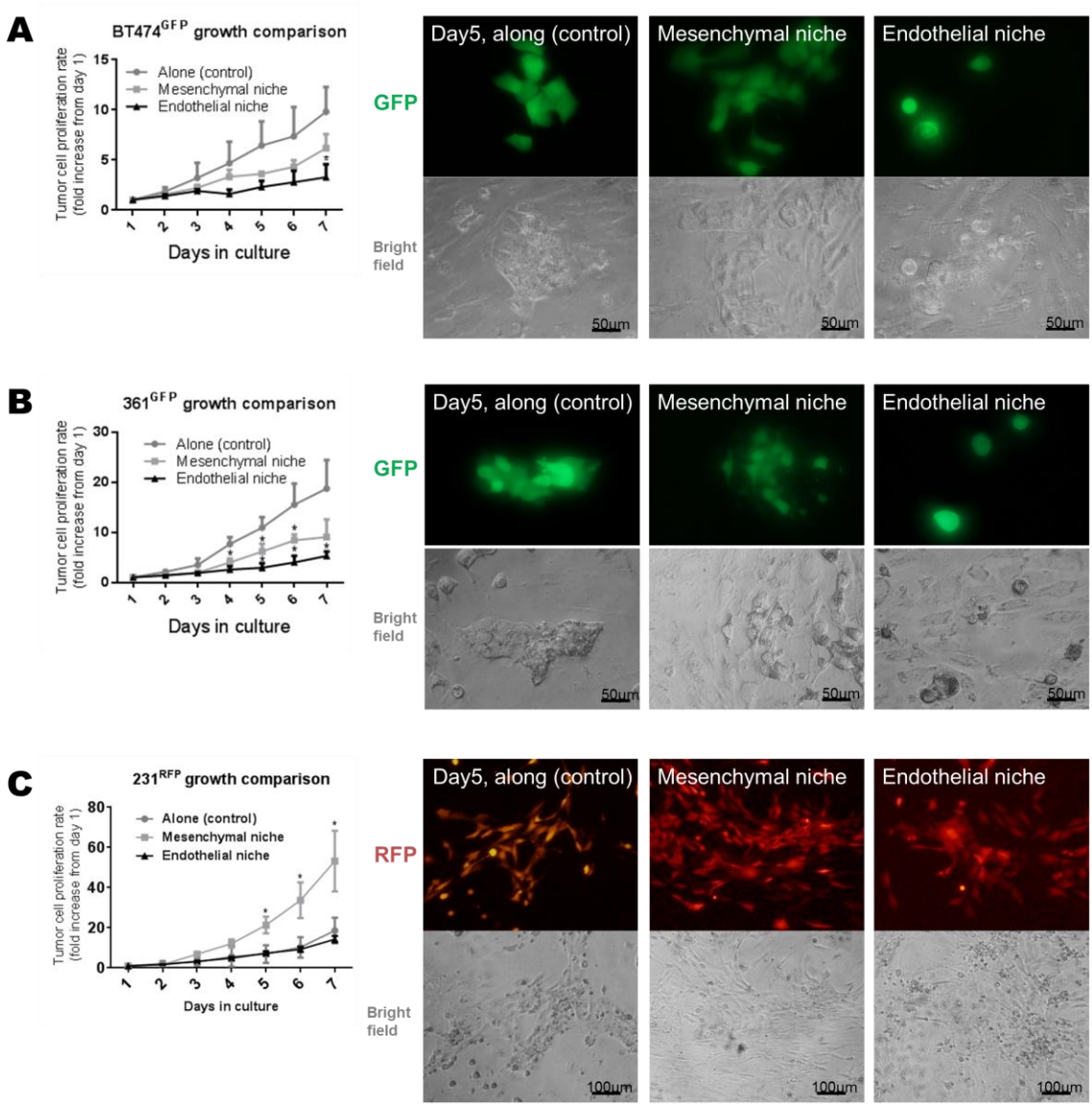

FIGURE 5. Breast tumor cell dormancy in bone marrow endothelial niche models. (A-C) BM MSCs and/or ECs were seeded on 96-well microplates and incubated until reaching confluence. For 3D cultures, Matrigel™ was coated (150μL/cm2) onto the culture plate. Then GFP-expressing BT474 or MDA-MB-361 cells, or RFP-expressing MDA-MB-231 cells were sparsely seeded (200 cells/well) onto the niche cells or empty surfaces (control). Tumor cell proliferations were assessed by fluorescence intensity reader and microscope. Graphs represent 7-day proliferation rates of tumor cells in niches (five sample sets per group; error bars: ± standard deviation (SD) *p <0.05). (C) Representative images of tumor cells in the niches, captured after 5-days of co-culture [Upper: fluorescence (RFP or GFP); lower: bright-field].

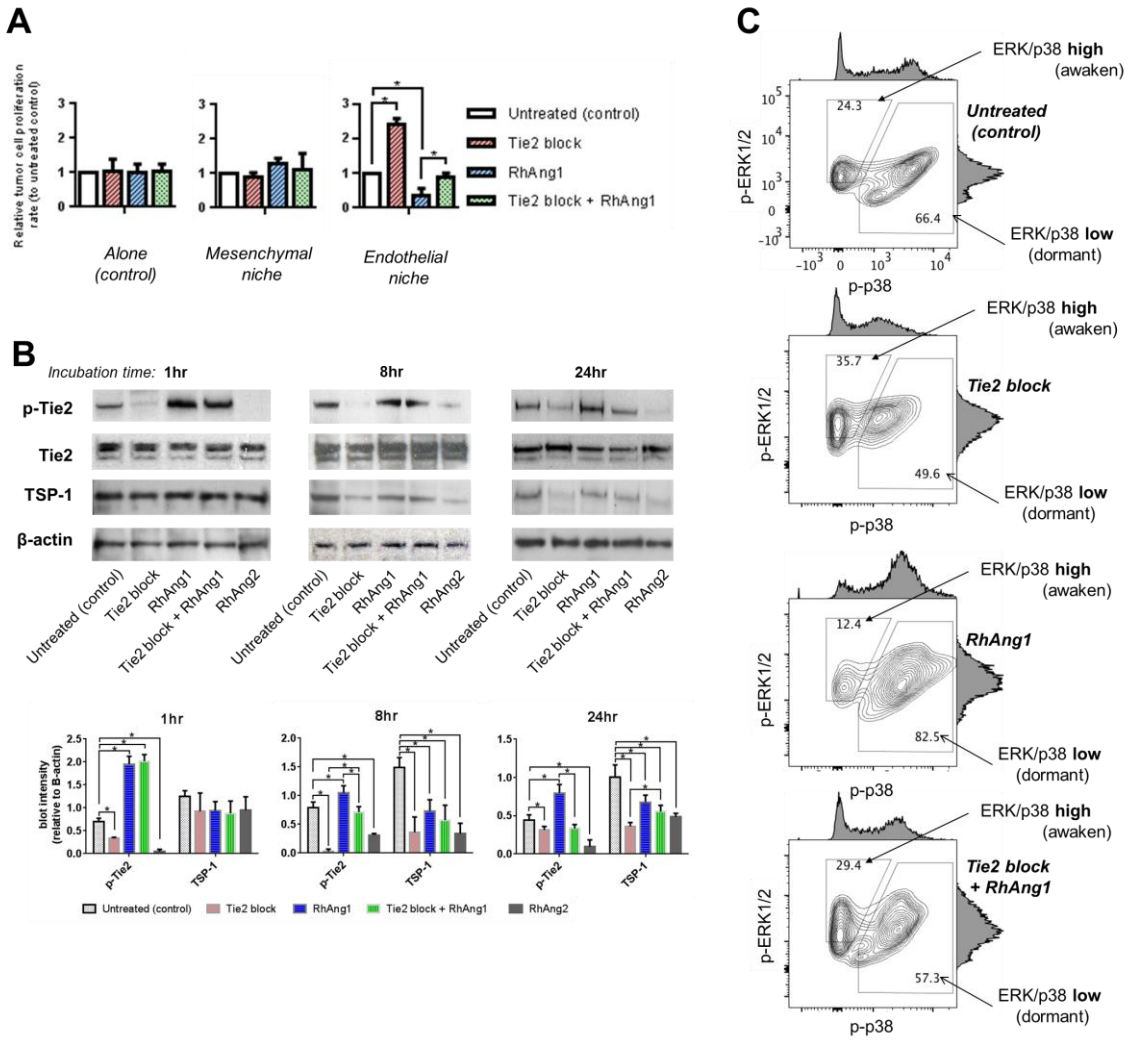

FIGURE 6. Disruption of endothelial Angiopoietin-1/Tie2 signaling elicits awakening of dormant tumor cells in niche. (**A-C**) To modulate endothelial angiopoietin-1/Tie2 signaling in culture, recombinant human angiopoietin-1 (RhAng1) 500ng/mL or human Tie2 receptor specific blocking antibody 10 μg/mL were treated on the niche models. ER+ tumor cell dormancy/awakening states in the niches were assessed by fluorescence intensity reader and FACS. (**A**) Relative tumor cell proliferation rates in niches. Cell proliferation rates for 3 days of RhAng1 and/or Tie2 blockade were analyzed. Data are presented as mean signaling intensity changes, relative to untreated control (n=3 per condition. Error bar: ±SD. * $p < 0.05$). (**B**) Endothelial niche Tie2 receptor phosphorylation and thrombospondin-1 (TSP-1) level changes upon Tie2 block, RhAng1, Tie2 block+RhAng1, or RhAng2 500ng/mL. *Above:* representative blot images. *Below:* Blot quantification. Normalized to B-actin. Error bar: SD. Cells were harvested after 1hr, 8hr or 24hr of incubation. Experiments were done triplicated.

(**C**) ERK1/2 and p38 MAR kinase activities of tumor cells in RhAng1 and/or Tie2 blocking antibody treated niches. Filled arrows: ERK/p38 ratio-high population. Blank arrows: ERK/p38 ratio-low population. Black=untreated; red=Tie2 blocked; blue=RhAng1-treated; green=Tie2 blocked & RhAng1-treated endothelial niches.

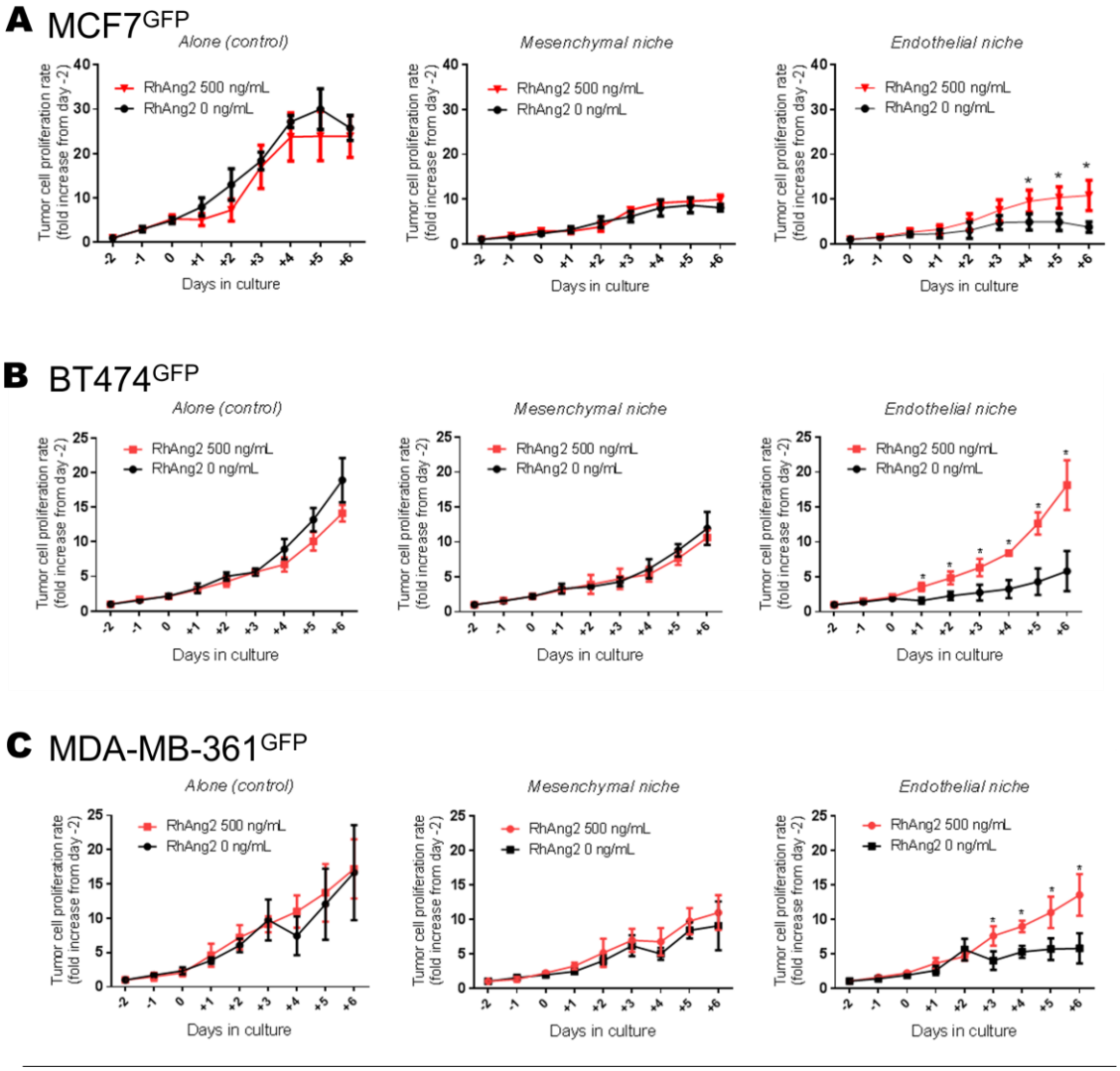

FIGURE 7. Recombinant angiopoietin-2 promotes tumor cells growth in endothelial niche. (**A-C**) To antagonize angiopoieint-1/Tie2 signaling, recombinant human angiopoietin-2 (RhAng2) 500ng/mL was added two days after tumor cell seeding (Day 0). Following 6-day proliferation rates of ER+ tumor cells in niches were measured by fluorescence intensity reader (five sample sets per group; error bars: ± standard deviation (SD) *p <0.05).

4. Estrogen depletion negates ER+ tumor cell dormancy in BM endothelial niche via increased angiopoietin-2 signaling

To test the effect of estrogen deficiency on ER+ breast tumor dormancy in BM niche, the growth rates and ERK/p38 activity ratio of breast tumor cells in E2-supplemented and unsupplemented niches were compared (FIGURE 8A). Three ER+ breast cancer cell lines (MCF7, BT474 and MDA-MB-361) and one ER- cell line (MDA-MB-231) were used. MCF7 represents luminal A (ER+, PR+, HER2-), which is totally dependent on estrogen to grow in culture. BT474 represents luminal B (mild ER+, PR+, strong HER2+), MDA-MB-361 also represents luminal B (ER+, PR+, strong HER2+), both of which are amenable to anti-estrogen hormone therapy. MDA-MB-231 represents basal-like subtype (ER-, PR-, HER2-) and is independent of estrogen for growth. When cultured alone, ER+ tumor cells grew significantly slower in absence of E2 than in presence of E2 (FIGURE 8B), implying that estrogen promotes their growths. Similar growth trends were noted in mesenchymal niche, too (FIGURE 8B). In endothelial niche, contrastingly, ER+ tumor cells grew significantly faster in absence of E2 than in presence of E2 (FIGURE 8B). Such reverse growth trends were not shown in experiments with ER- tumor cells (FIGURE 8B). We also tested the effect of 4-hydroxytamoxifen (4-OHT), an active metabolite of tamoxifen [42], which can effectively suppress ER signaling and ER+ cell growth (FIGURE 9A, B). Treatment of 4-OHT stimulated MCF7, BT474 and MDA-MB-361 cell growth in endothelial niches, contrary to those cultured alone (FIGURE 9C, D).

We analyzed ERK/p38 activity ratio in ER+ MCF7 cells cultured alone, in mesenchymal niche or in endothelial niche (FIGURE 8C). Tumor cells cultured alone or in mesenchymal niche showed decreases in awaken population upon estrogen depletion (ERK/p38 activity ratio-high, 89.3% to 20.2% and 43.3% to 17.8%, respectively, FIGURE 8C). Meanwhile, tumor cells cultured in endothelial niche exhibited increase in awaken subpopulation upon estrogen depletion (ERK/p38 activity ratio-high; 16.7% to 33.6%, FIGURE 8C), suggesting disruption of niche-induced tumor cell dormancy.

The results described above suggest that upon estrogen depletion, some tumor dormancy-related factors in endothelial niche changes. We focused on angiopoietin-1 and 2 specifically. In ER+ breast tumor cells, estrogen down –regulates angiopoeitin-1 mRNA in an ER-dependent manner [27]. Such regulations may also be active in stromal cells, too. Since the paradoxical growth promoting effect of estrogen depletion was seen only in endothelial niche, we analyzed the expressions of angiopoietin-1, 2 mRNAs and proteins in endothelial niche cells, in E2- supplemented and un-supplemented (depleted) conditions. Angiopoietin-1 levels, both as mRNA and protein forms, were not significantly different in between E2-supplemented and depleted endothelial niches (FIGURE 10A, B). In contrast, angiopoietin-2 mRNA was expressed significantly higher in estrogen-depleted niche than in supplemented niche (FIGURE 10A). Protein expression levels exhibited similar trends as well (FIGURE 10B). To note, the levels of angiopoietin-1 and angiopoietin-2 mRNA and proteins in each experimental set were inversely proportional (FIGURE 10A, B).

Increased angiopoietin-2 in estrogen-depleted endothelial niche may have shifted tumor cell behavior from dormancy to awakening. This hypothesis was examined by specific knock-down of angiopoietin-2 on niches. Three short-inhibiting RNAs (siRNAs) binding to different angiopoietin-2 mRNA (ANGPT2) sequence (siRNA ANGPT2 #1, #2, #3) significantly attenuated its expressions in endothelial niche (FIGURE 10A). Then, tumor cell proliferation rates upon ANGPT2 knock-down in niche were examined. All three ER+ tumor cells in estrogen-deficient endothelial niche did not proliferate when ANGPT2 was knocked-down (FIGURE 11A, B). In contrast, ANGPT2 knock-down had no significant effect on ER+ tumor cell proliferations in estrogen-deficient mesenchymal niche (FIGURE 11A, B).

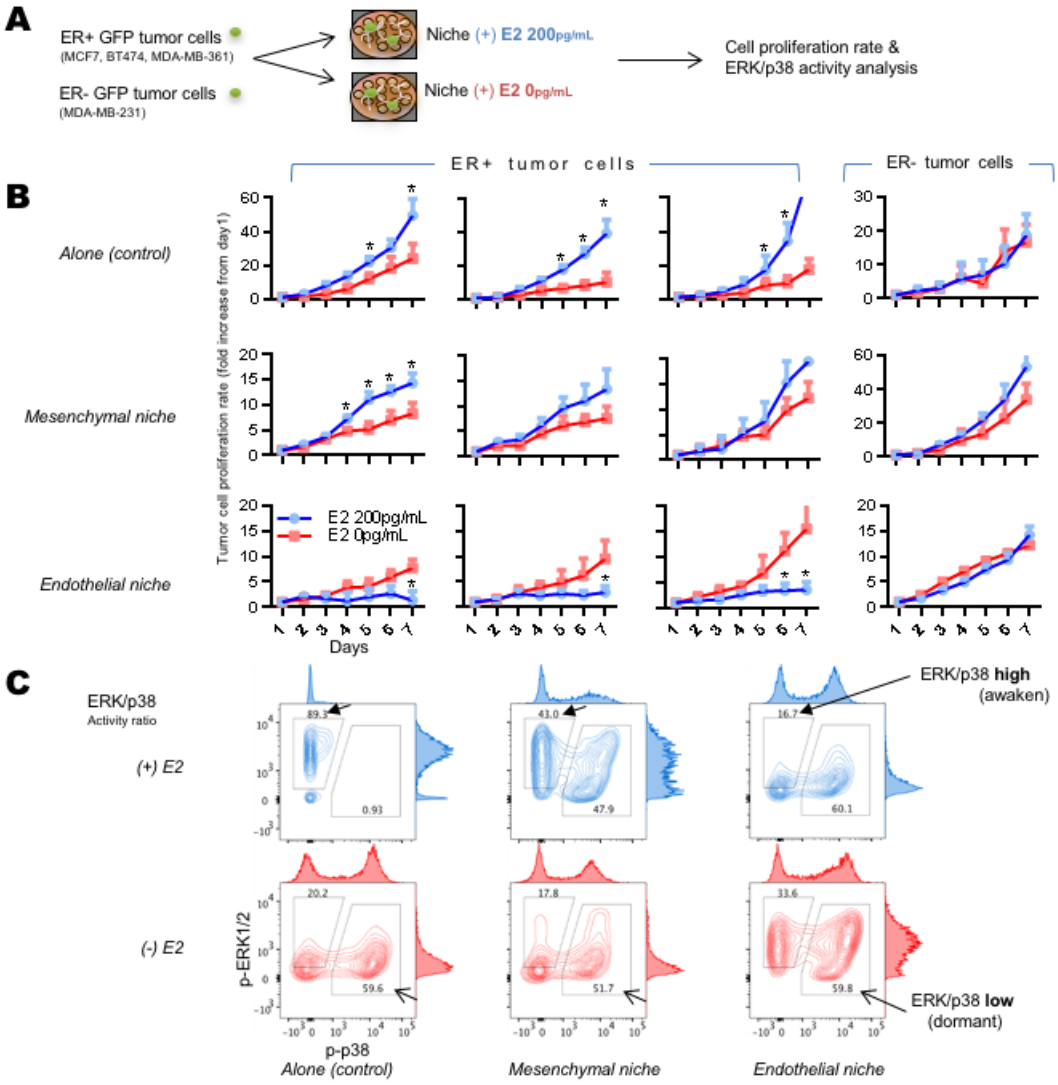

FIGURE 8. Increased ER+ tumor cell proliferation after estrogen depletion in bone marrow endothelial niche. (**A**) Experimental scheme. BM niche models were generated by co-culturing BM MSCs and/or ECs on ECM-coated microplates. GFP-expressing tumor cells were sparsely seeded (200 cells/well) onto the niche cells or empty surfaces (control). Tumor cell proliferations were compared in niches cultured by using estrogen-supplemented (200pg/mL) or un-supplemented (depleted) media. (**B**) Seven-day proliferation rates of tumor cells in niches. From left to right: ER+ MCF7, ER+ BT474, ER+ MDA-MB-361 and ER- MDA-MB-231. (five sample sets per group; error bars: ±SD * $p < 0.05$). (**C**) FACS analysis of ERK1/2 and p38 MAR kinase activities of tumor cells in estrogen-supplemented (blue) and

estrogen-depleted (red) niches. Filled arrows: ERK/p38 ratio-high population. Blank arrows: ERK/p38 ratio-low population.

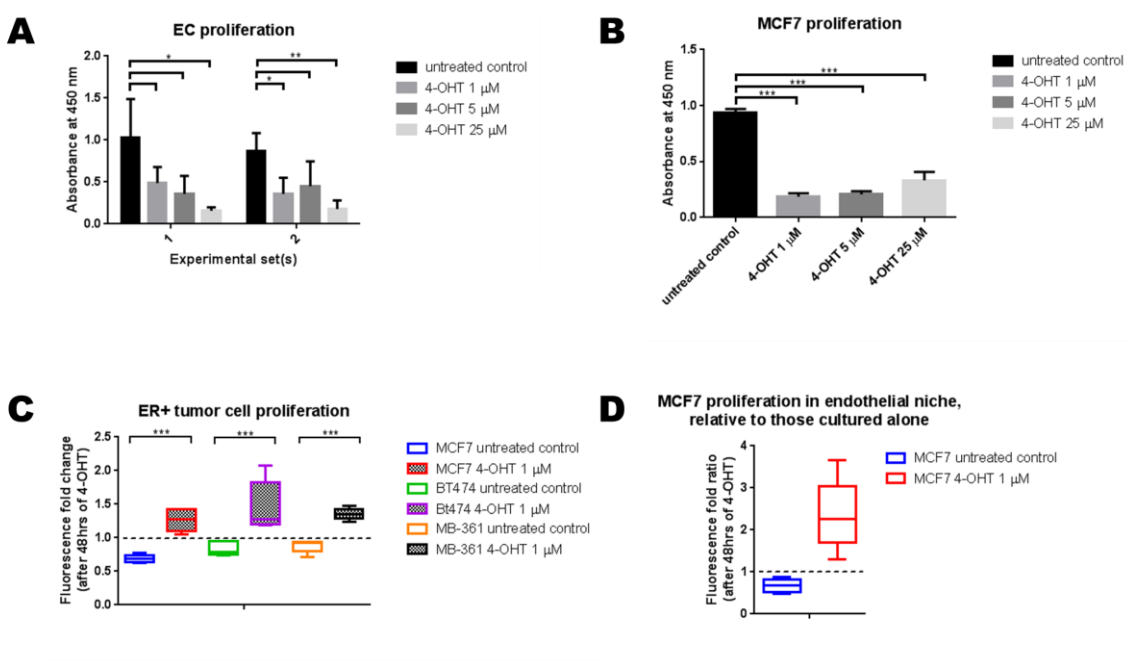

FIGURE 9. 4-hydroxytamoxifen impact on ER+ tumor cell growth in endothelial niche.

(**A-B**) 4-hydroxytamoxifen (4-OHT) impact on EC and MCF7 cell proliferation. 4-OHT 1uM, 5uM or 25uM were treated 12hrs after tumor cell seeding. After 48hrs, tumor cell proliferation/viability of each condition was assessed by using cell counting kit-8 (Dojindo Molecular Technoligies). Three sample sets per condition, replicated more than twice. Error bar: ±SD. * $p < 0.05$.(**C-D**) ER+ MCF7, BT474 and MDA-MB-361 cell proliferation in endothelial niches with or without 4-OHT. Tumor cell proliferations were compared by measuring GFP fluorescence intensities before and 48hr after 4-OHT or mock (same volume of 99% ethanol) treatment on endothelial niches. MCF7 proliferation in endothelial niche was also compared to those cultured alone before and after 4-OHT treatment (**D**). Five sample sets per condition. Box and whiskers (min to max) plots were used.

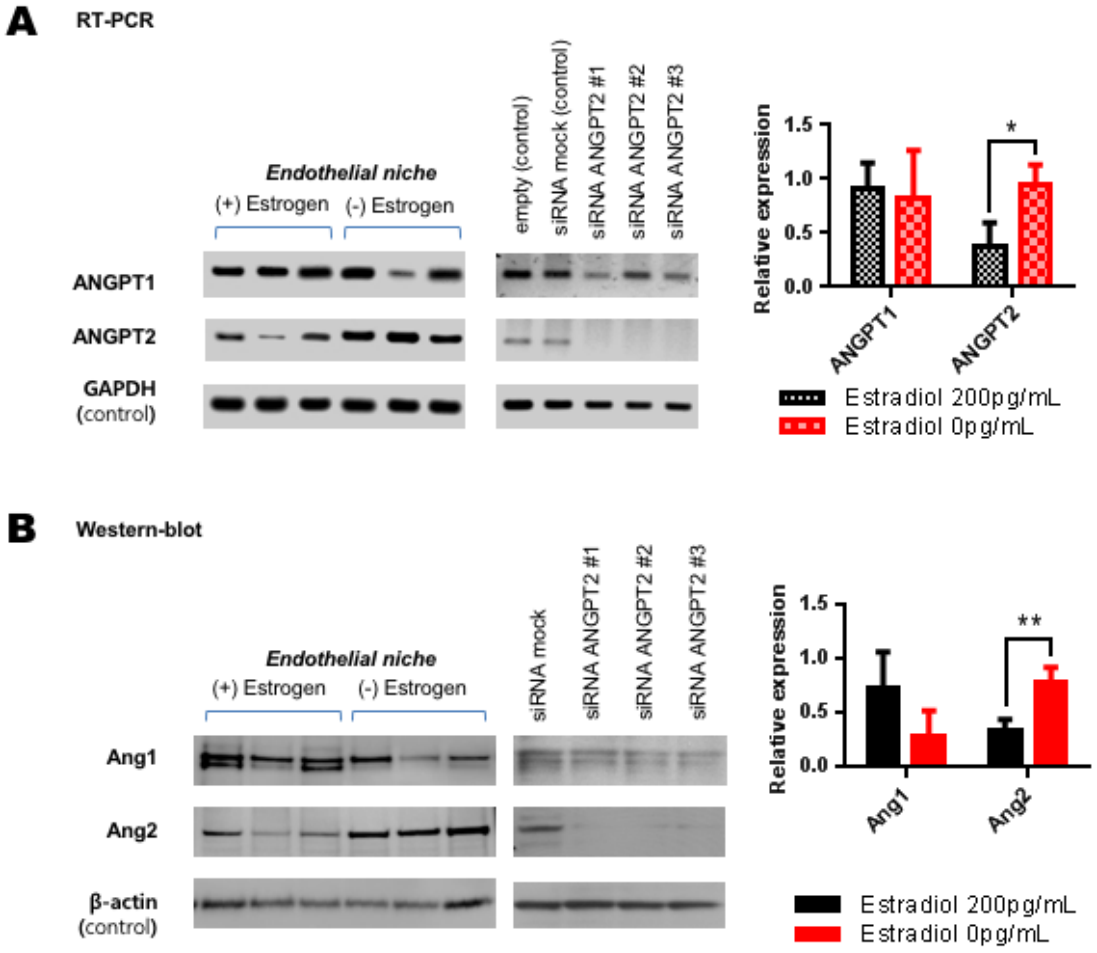

Figure 10. Effect of estrogen depletion on niche endothelial cell angiopoietin-1 and -2 expressions. (**A-B**) BM MSC and EC cells were co-cultured with or without estradiol supplementation (200pg/mL) for 3 days (RT-PCR) or 5 days (Western blot). Cells were harvested and their expressions of angiopoietin-1, 2 mRNAs (ANGPT1, 2) and proteins (Ang1, 2) were compared by RT PCR (**A**) and Western blot (**B**). Specific knock-down of ANGPT2 was performed by transfecting siRNA ANGPT2 #1, #2, #3 in 6hrs after tumor cell seeding. See "Materials and Methods" sections for siRNA information. Data in graph are presented as mean blot intensity relative to GAPDH (RT-PCR) or β-actin (Western blot). Blots were quantified by using ImageJ software (IJ 1.46r). (Three sample sets per group. Error bar: SD. * $p < 0.05$.)

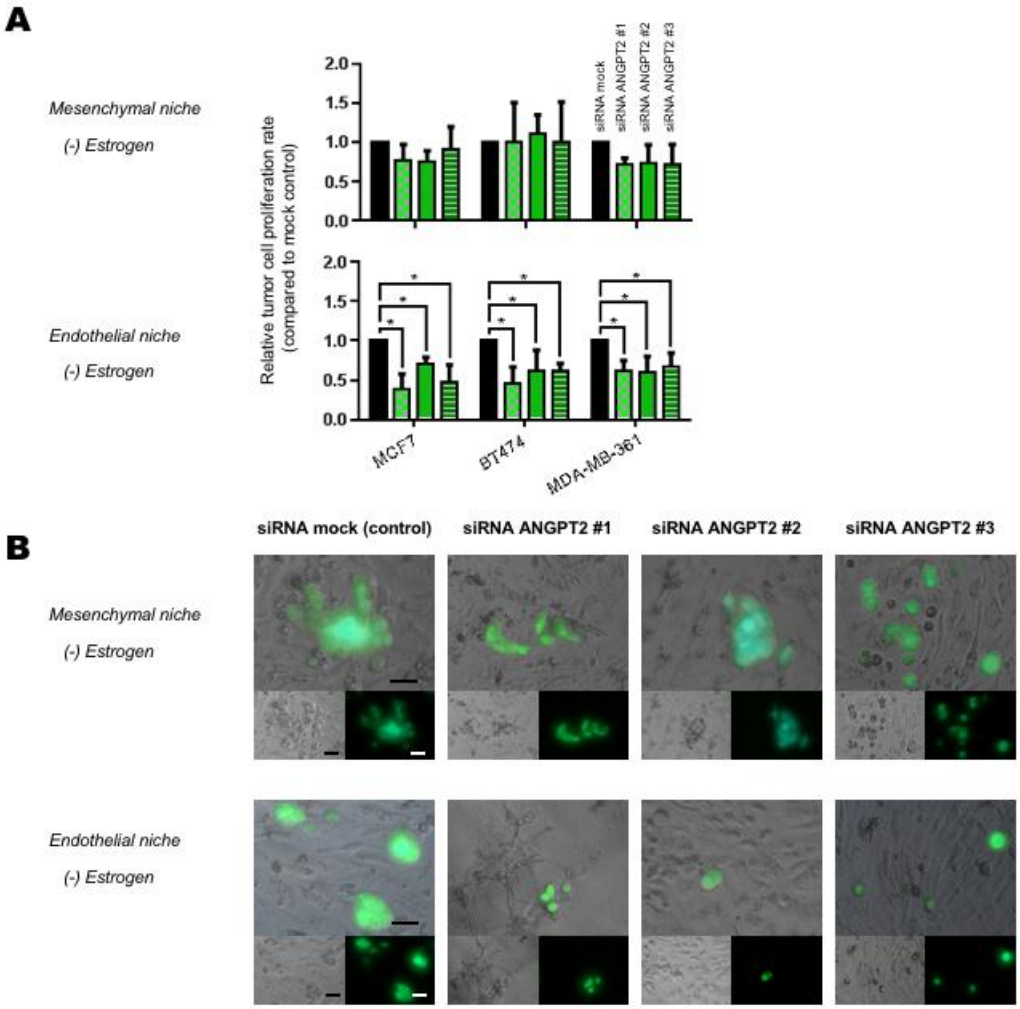

Figure 11. SiRNA knock-down of ANGPT2 suppresses tumor cell proliferation in estrogen-deficient bone marrow endothelial niche. (**A-B**) GFP-expressing ER+ tumor cell lines were cultured in niches for 3 days with or without estradiol supplementation (200ng/mL). Specific knock-down of ANGPT2 mRNA in niche was performed by transfecting siRNA #1, #2 or #3 in 6 hrs after tumor cell seeding. (**A**) Relative tumor cell proliferation rates in niches. Cell proliferation rates for 3 days following siRNA transfection were analyzed by using fluoresence intensity reader. Data are presented as mean signaling intensity change from day0 to day3 relative to siRNA mock control (three sample sets per group; error bar: ±SD. * $p < 0.05$, by student's t-test.) (**B**) Representative images of mesenchymal niches and endothelial niches treated with siRNAs. Images were captured by

fluorescence microscope after 3-days of co-culture [Upper: merged; left lower: bright-field; right lower: fluorescence (green). Scale bar=50μm].

5. Angiopoietin-2 promotes ER+ tumor cell survival under estrogen depletion via integrin β1

As described above, RhAng2 treatment (500ng/mL) increased ER+ tumor cell proliferations in otherwise growth-suppressing E2-supplemented endothelial niches (FIGURE 7A-C). RhAng2 had no effect on their proliferations in E2-supplemented mesenchymal niche (FIGURE 7A-C). However, in estrogen-depleted mesenchymal niches, RhAng2 increased tumor cell proliferations, although not always in significant degree and in dose-dependent manner (FIGURE 12A-C). Since there is no Tie2-expressing ECs in mesenchymal niche, we hypothesized angiopoietin-2 may act on tumor cells directly. Interestingly, Imanishi et al. [43,44] reported that angiopoeitin-2 acted directly on ER+ tumor cells via ITGB1 and promoted their initial survival and metastatic growth at lung. Encouraged by the previous reports, we evaluated the effect of RhAng2 on ER+ tumor cell proliferation under estrogen deficiency. Tumor cells were cultured for 3 days in E2-supplemented or un-supplemented growth media containing varying concentrations of RhAng2 (0, 50, 100, 500ng/mL, FIGURE 13A). In presence of E2, RhAng2 had no effect on ER+ and ER- tumor cell proliferations (FIGURE 13B). In absence of E2, however, RhAng2 promoted ER+ tumor cell proliferations in dose-dependent manner (FIGURE 13C). ER- tumor cell proliferation was not affected by RhAng2 (FIGURE 13C). To find-out whether this was dependent on tumor cell ITGB1, we performed ITGB1 knock-down by using shRNAs (FIGURE 14). ITGB1 knock-down itself had no effect on tumor cell growth under estrogen deficiency (FIGURE 13D). In contrast, ITGB1 knock-down completely negated RhAng2's proliferative effect under estrogen depletion (FIGURE 13E).

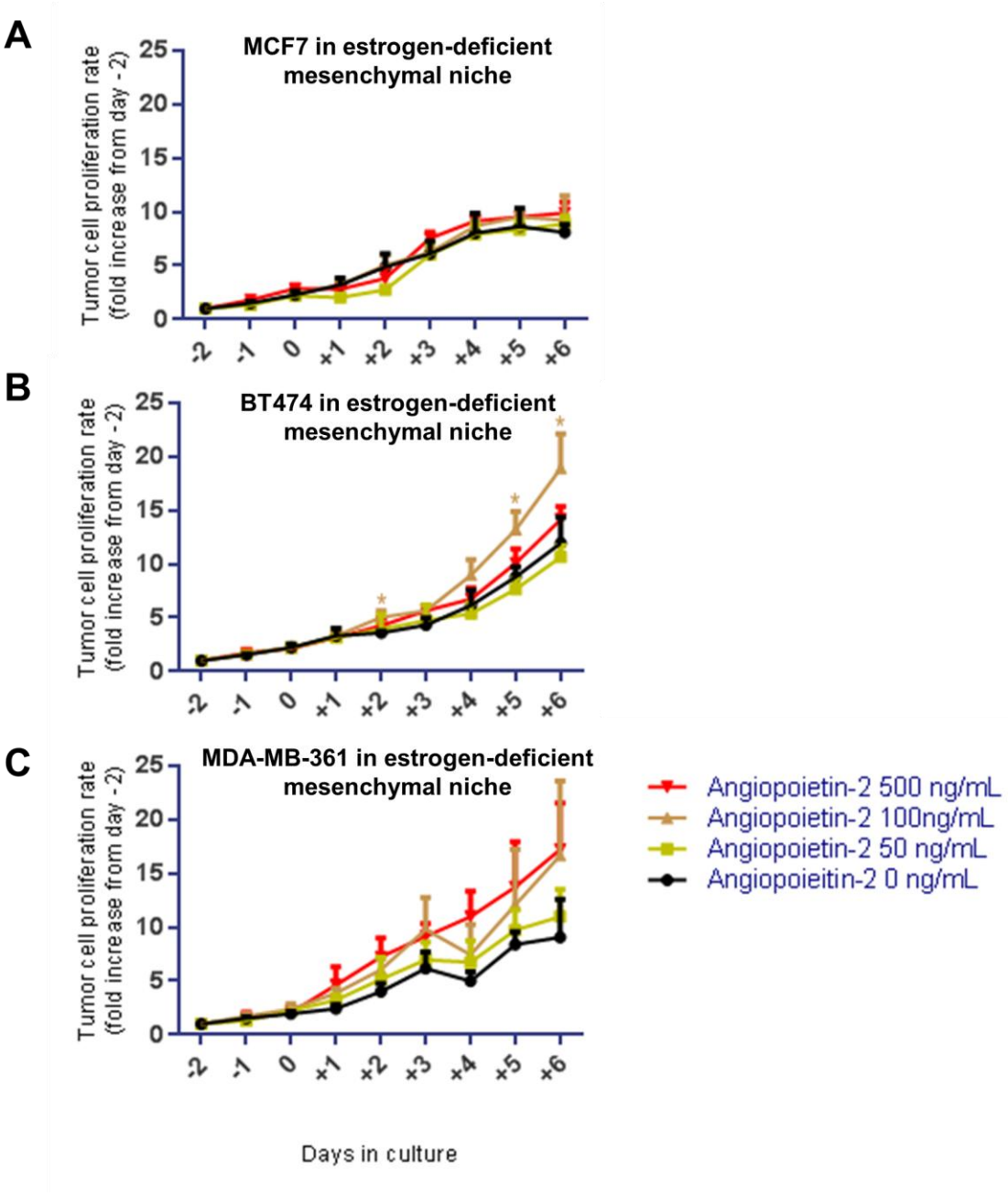

FIGURE 12. Effect of recombinant human angiopoietin-2 treatment on estrogen-depleted ER+ tumor cell proliferation in mesenchymal niche (A-C) Mesenchymal niches were generated by culturing BM MSCs on Matrigel™ precoated 96-well microplates. Then GFP+ ER+ tumor cells were sparsely seeded (200 cells/well) onto the niches (Day -2). Recombinant human angiopoietin-2 0, 50, 100 and 500ng/mL were added two days after tumor cell seeding (Day 0). The niches were cultured by using 10% charcoal-stripped FBS containing media. Following 6-day proliferation

rates of ER+ tumor cells in niches were measured by fluorescence intensity reader. (A) MCF7; (B) BT474; (C) MDA-MB-361. (five sample sets per group; error bars: ± standard deviation. *p <0.05).

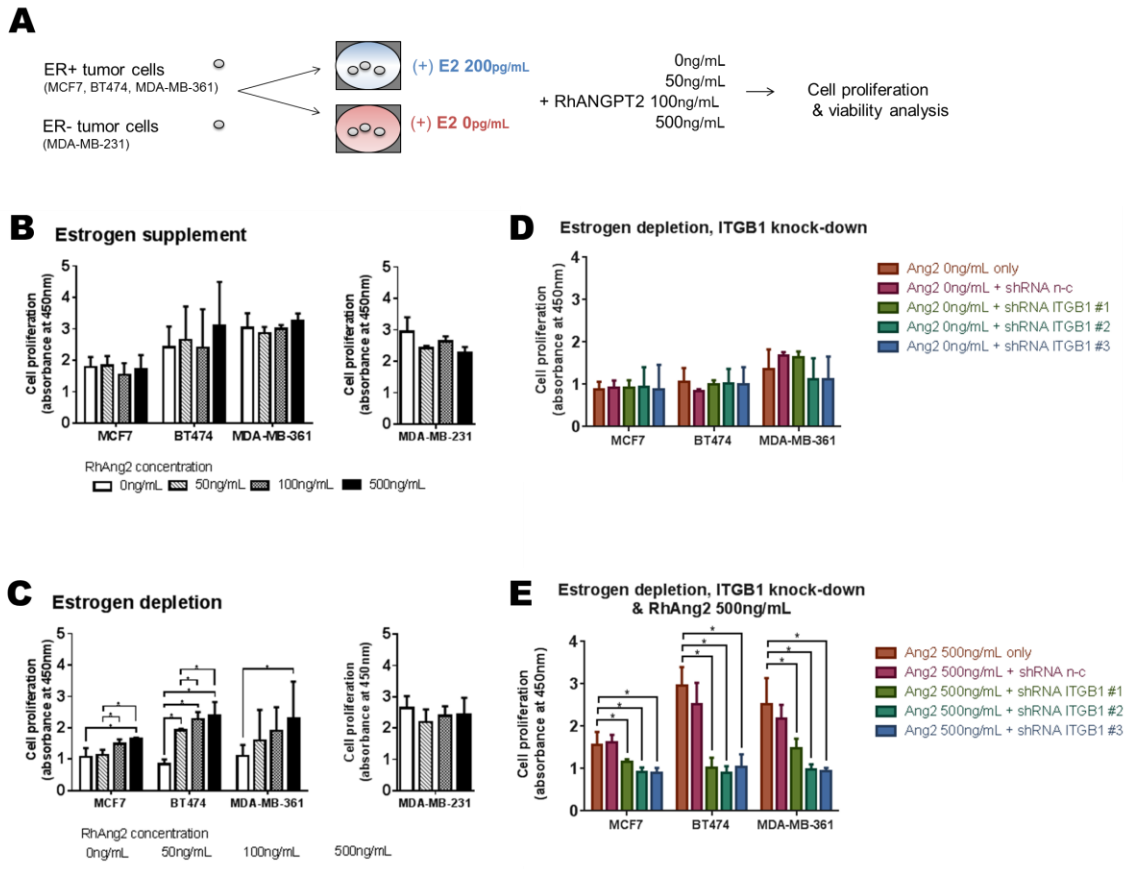

FIGURE 13. Exogenous angiopoietin-2 promotes ER+ tumor cell survival under estrogen deficiency via cell surface integrin β1. (**A**) Experimental scheme. ER+ breast tumor cell lines MCF7, BT474, MDA-MB-361 and ER- cell line MDA-MB-231 were cultured in 96-well microplates by using estradiol-supplemented (200pg/mL) or un-supplemented (depleted) media. Recombinant human angiopoietin-2 (RhAng2, 0ng/mL, 50ng/mL, 100ng/mL, 500ng/mL) were treated 1hr after tumor cell seeding. After 48hrs, tumor cell proliferation/viability of each condition was assessed by using cell counting kit-8 (Dojindo Molecular Technoligies). (**B-E**) Tumor cell proliferation / viability in presence of RhAng2. (**B**) Tumor cells cultured in estrogen-supplemented media. (**C**) Tumor cells cultured in estrogen-depleted media. (**D, E**) Integrin β1 (ITGB1) knock-down tumor cells and untreated cells cultured in estrogen-depleted media, in presence and absence of RhAng2 500ng/mL. Integrin β1 knock-down was performed by using short-hairpin RNAs (shRNAs),

as described in figure 14. Data presented are mean values of light absorbance rate at 450nm, representing proliferative activity of each well; Error bar: ±SD. * $p < 0.05$.

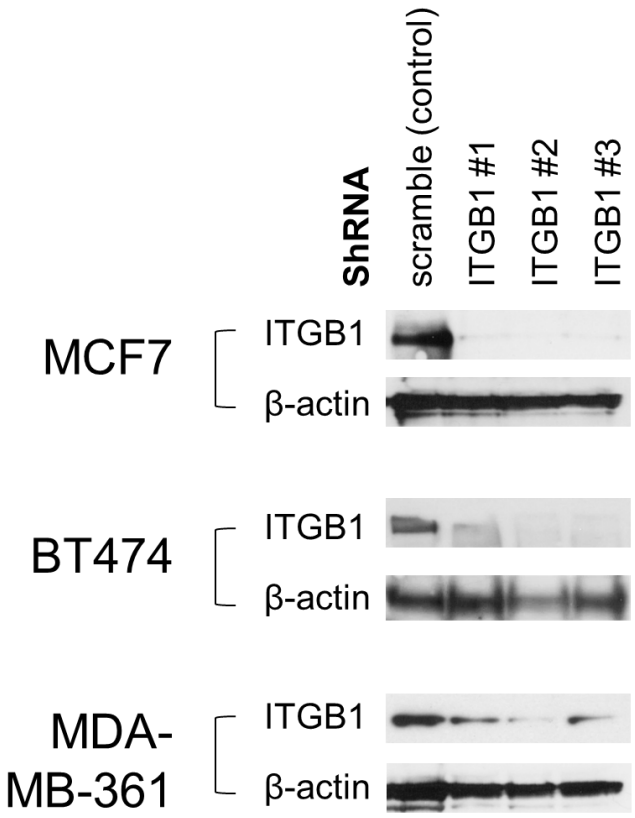

FIGURE 14. Silencing of β1 integrin by shRNAs. After shRNA transfection of MCF7, BT474, MDA-MB-361 cells, protein expression was down-regulated by more than 90%. Reduction of target protein by shRNA #1, #2 and #3 in three different cell lines at 96 h after each shRNA transfection. Scramble shRNA was used for control.

6. Angiopoietin-1, 2 expressions correlate with ER+ breast cancer metastatic recurrences following anti-estrogen endocrine therapy

Angiopoietin-2 overexpression in breast cancer is known to correlate with poor patient survival [35]. According to our experimental data, its expression would be particularly associated with metastatic recurrence of ER+ subtype after adjuvant anti-estrogen therapy. To test the hypothesis, we used a published dataset and a web-based program "KM plotter (http://kmplot.com) that contain both gene expression and human patient survival data [45]. Patients were first categorized into those who underwent adjuvant endocrine therapy (including estrogen receptor blocker tamoxifen and aromatase inhibitor, n=561) or those who did not undergo endocrine therapy (n=435). Then the patients were further stratified into two groups based on angiopoietin-2 mRNA (ANGPT2) levels in tumor tissue. For those who underwent anti-estrogen endocrine therapy, increased *ANGPT2* level was associated with a significantly decreased probability of distant metastasis-free survival (DMFS) (FIGURE 15A). For those without endocrine therapy, contrastingly, *ANGPT2* level was not associated with the probability of DMFS (FIGURE 15B). We did not perform a multivariate analysis incorporating known prognostic indicators such as tumor size, grade, and number of axillary lymph nodes involved.

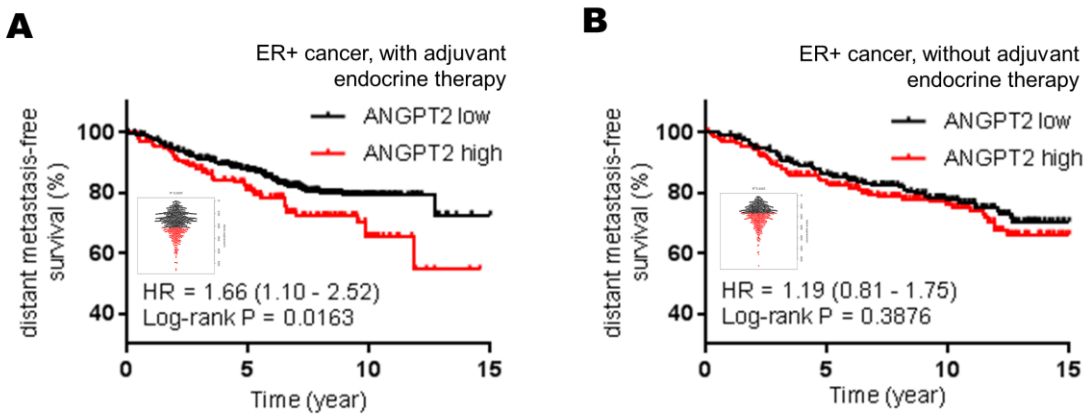

FIGURE 15. Distant metastasis-free survival of ER+ breast cancer patients, stratified by *ANGPT2* expression. Kaplan-Meier analysis comparing distant metastasis-free survival of ER+ breast cancer patients following endocrine therapy (n=561, A) and those without endocrine therapy (n=435, B), distinguished by low versus high expressions of *ANGPT2*. Gene expression data obtained from the open source KM Plotter. Beeswarm graph plots each RNA prove distribution. ER=Estrogen receptor; *ANGPT2* = angiopoietin-2 mRNA.

IV. DISCUSSION

The objective of this study was to investigate the effects of estrogen-deficiency on BM microenvironment and dormancy of residing ER+ breast tumor cells. We found that prolonged estrogen depletion up-regulated angiopoietin-2 in BM endothelial niche, which ameliorated its dormancy-inducing effect on ER+ tumor cells and promoted tumor cell survival under estrogen-deficiency. Angiopoietin-1 and 2 have two important physiologic functions: (1) regulation of vascular stability [20]; (2) regulation of hematopoietic stem cell (HSC) quiescence in BM niche [39]. Indeed, quiescent HSCs reside specifically in arteriolar niches of BM [14]. Angiopoietin-1 and 2 act on cells not only via Tie2 receptor, but also integrin β1 (ITGB1) [46]. Unlike Tie2 receptor which is expressed only in ECs and HSCs, ITGB1 is expressed in most epithelial tumor cells [47]. Indeed, activation of ITGB1 along with uPAR can turn dormant tumor cells into awakening state [48].

While our finding that angiopoietin-2 promotes ER+ tumor cell survival under estrogen deficiency via ITGB1 add significance to the previous results, it needs verification whether the effect of angiopoietin-2 was due to increased tumor cell division or decreased apoptosis, or both.

Angiopoietin-2 signaling can affect tumor progression by disrupting the microenvironment, too. In ovarian cancer, angiopoietins promotes intraperitoneal tumor growth by accumulating cancer-promoting fibroblasts and enhancing tumor angiogenesis [49]. In pancreatic cancer, angiopoietin-2 drives lymphatic metastasis [50]. In breast cancer, it impairs the blood-brain barrier and support tumor cell colonization in brain [23]. In addition we've found that angiopoietin-2 also triggers breast tumor cell awakening from dormancy, probably via destabilizing the BM endothelial niches [16].

There appears to be a complex relationship between estrogen level and angiopoietin-1, 2 expressions. Glinskii et al. [51] reported that cessation of ovarian hormone significantly reduced angiopoietin-1 expression in meningeal microvasculature. Similarly, Ardelt et al.[25] reported that estradiol (E2) increased angiopoietin-1 expression in cerebral vascular beds, and

Bonagura et al.[28] reported that E2 increased angiopoietin-1 expression in endometrial glandular epithelial cells. In contrast, other reports showed that estrogen administration reduced angiopoietin-1 levels in placenta [52], in ER+ breast tumor cells [27], and in some non-reproductive organs [53]. Furthermore, E2 treatment transiently increased angiopoietin-2 expression in ER+ T47D and ZR75.1 cells, while estrogen-depletion also increased angiopoeitin-2 expression in these cells slowly but gradually [35]. These discrepancies may reflect differences in experimental conditions and tissue-specific regulatory mechanisms. Nevertheless, all studies suggest that sudden changes in estrogen levels do affect angiopoietin-2 expressions. Therefore, it will be valuable to compare systemic and local angiopoietin-2 levels in pre and post-menopausal women of breast cancer.

Estrogen receptor blockers such as tamoxifen and aromatase inhibitors (AI) are two mainstays of adjuvant therapy to prevent metastatic recurrences of breast cancer [54]. However, both AIs or ER blockers should be used in long-term, over 5 to 10 years to effectively prevent metastatic recurrences. Our results imply that eliminating locally available estrogen by AIs can increase angiopoietin-2, triggering the awakening of dormant ER+ tumor cells and even supporting their survival. If such, adding ANGPT2 inhibitors onto current endocrine therapy regimen may further decrease metastatic recurrence of ER+ breast cancer.

The *in vitro* culture system described here potentially limits the results of our study. However, we believe the experimental design and results are consistent with existing in vivo models and results from studies of human patients. In vivo and human studies using existing drugs such as angiopoietin-2 inhibitors could add significance on our findings.

Treatment strategies for primary breast cancer have been successful. However, for metastatic disease currently available therapeutic tools are still considered inadequate. ER+ breast cancer is of particular concern because its metastasis often precedes a period of dormancy. Dormant ER+ tumor cells in BM niche are thought to be the seed of metastatic recurrence, indicating an opportunity to control metastatic disease even before becoming apparent. Further studies are mandatory to verify the effect of ang-2 signaling manipulation

on late recurrence prevention in women with breast cancer, particularly those under risk of estrogen deficiency.

V. CONCLUSION

This study is about estrogen deficiency-induced awakening of breast tumor cells from dormancy. Human breast cancer has two special characteristics: First is ‘dormancy’, and second is ‘sex hormone estrogen dependency’. Unlike other types of malignancy, breast cancer often halts its growth for significantly long periods – months, years, or even decades until finally develop metastatic recurrences. During this pause, tumor cells rarely undergo cell division and consume relatively small amount of energy, so they are resistant to conventional anti-cancer therapeutics (targeting rapidly dividing cells). Still, it is not fully understood how the tumor cells become reactivated from dormancy and begin metastatic tumor formation. Understanding the mechanism of tumor dormancy and awakening is of great importance to provide a new therapeutic insight on prevention of metastatic recurrence. Hopefully, there had been significant advances in this field and we now know that bone marrow (BM) microenvironment plays an active role in regulating tumor dormancy. Especially, vascular endothelial cells (ECs) of BM seems to provide growth suppression signals onto those disseminated tumor cells.

Bases on these researches, we sought to find the key signal that reactivates dormant breast tumor cells in BM niche, paying attention to another characteristic of breast cancer – estrogen dependency. Estrogen is a steroid hormone that promotes growth and expansion of mammary glands. Tumor cells expressing its receptor (ER) generally shows estrogen-dependent proliferation pattern. ER+ breast cancers are more likely to become dormant in secondary organ such as the BM than ER-negatives. Interesting point is that ER+ cancer recurs years after primary tumor treatment, especially in post-menopausal women in whom systemic estrogen level is fairly low. We analyzed two important vascular homeostasis regulators, angiopoietin-1 and angiopoietin-2 in published ER+ breast cancer dataset, and found out that after therapeutic estrogen depletion, increased angiopoeitin-2 expression was prognostic in ER+ breast cancer metastatic recurrence. Based on the clinical data, we tested the effect of

estrogen-deficiency on ER+ tumor cell dormancy and adjacent BM niche stromal cells - mesenchymal stem cell (MSCs) and endothelial cells (ECs). We utilized part of the previous experimental tumor dormancy models and modified it for our experiment. In results, surprisingly, ER+ tumor cells proliferated in otherwise growth-suppressing BM niche model under estrogen deficiency. We found that angiopoietin-2 was neo-expressed by estrogen-deficient BM MSCs and ECs, and siRNA knock-down of angiopoietin-2 mRNA have restored the growth-suppressing effect of the niche. Further studies using recombinant human angiopoietin-2 protein show that angiopoietin-2 promoted ER+ tumor cell proliferation under estrogen deficiency via tumor cell surface integrin β1.

As described above, previous studies on tumor dormancy point out that dormant tumor cells reside in BM, especially near perivascular niche. In addition, our results add that estrogen depletion can promote ER+ tumor cell awakening by modulating the niche angiopoietin-2 signaling. We believe that this paper will be of interest to oncologists. Postmenopausal women undergo over 5 to 10 years of endocrine therapy such as aromatase inhibitors or tamoxifen to prevent metastatic recurrence. Our results propose a new way of enchancing the efficacy of current treatment regimen by adding angiopoietin-2 inhibitors. To cell biologists, the identification of estrogen's shadow role as an inducer of dormant cell awakening could provide an evidence of previously unknown endocrine control of cell behavior via tissue-level modification.

REFERENCES

1. Aguirre-Ghiso JA. Models, mechanisms and clinical evidence for cancer dormancy. Nat Rev Cancer 2007;7:834-46.

2. Early Breast Cancer Trialists' Collaborative G. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 2005;365:1687-717.

3. Braun S, Kentenich C, Janni W, Hepp F, de Waal J, Willgeroth F, et al. Lack of effect of adjuvant chemotherapy on the elimination of single dormant tumor cells in bone marrow of high-risk breast cancer patients. J Clin Oncol 2000;18:80-6.

4. Janni W, Vogl FD, Wiedswang G, Synnestvedt M, Fehm T, Juckstock J, et al. Persistence of disseminated tumor cells in the bone marrow of breast cancer patients predicts increased risk for relapse--a European pooled analysis. Clin Cancer Res 2011;17:2967-76.

5. Janni W, Rack B, Schindlbeck C, Strobl B, Rjosk D, Braun S, et al. The persistence of isolated tumor cells in bone marrow from patients with breast carcinoma predicts an increased risk for recurrence. Cancer 2005;103:884-91.

6. Zhang XH, Giuliano M, Trivedi MV, Schiff R, Osborne CK. Metastasis dormancy in estrogen receptor-positive breast cancer. Clin Cancer Res 2013;19:6389-97.

7. Coleman RE, Rubens RD. The clinical course of bone metastases from breast cancer. British Journal of Cancer 1987;55:61-6.

8. Giancotti FG. Mechanisms governing metastatic dormancy and reactivation. Cell 2013;155:750-64.

9. Goss PE, Chambers AF. Does tumour dormancy offer a therapeutic target? Nat Rev Cancer 2010;10:871-7.

10. Kim RS, Avivar-Valderas A, Estrada Y, Bragado P, Sosa MS, Aguirre-Ghiso JA, et al. Dormancy signatures and metastasis in estrogen receptor positive and negative breast cancer. PLoS One 2012;7:e35569.

11. Brackstone M, Townson JL, Chambers AF. Tumour dormancy in breast cancer: an update. Breast Cancer Res 2007;9:208.

12. Barkan D, Green JE. An in vitro system to study tumor dormancy and the switch to metastatic growth. J Vis Exp 2011; doi:10.3791/2914.

13. Mendez-Ferrer S, Michurina TV, Ferraro F, Mazloom AR, Macarthur BD, Lira SA, et al. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. Nature 2010;466:829-34.

14. Kunisaki Y, Bruns I, Scheiermann C, Ahmed J, Pinho S, Zhang D, et al. Arteriolar niches maintain haematopoietic stem cell quiescence. Nature 2013;502:637-43.

15. Ghajar CM. Metastasis prevention by targeting the dormant niche. Nat Rev Cancer 2015;15:238-47.

16. Ghajar CM, Peinado H, Mori H, Matei IR, Evason KJ, Brazier H, et al. The perivascular niche regulates breast tumour dormancy. Nat Cell Biol 2013;15:807-17.

17. Marlow R, Honeth G, Lombardi S, Cariati M, Hessey S, Pipili A, et al. A novel model of dormancy for bone metastatic breast cancer cells. Cancer Res 2013;73:6886-99.

18. Ottewell PD, Wang N, Brown HK, Reeves KJ, Fowles CA, Croucher PI, et al. Zoledronic acid has differential antitumor activity in the pre- and postmenopausal bone microenvironment in vivo. Clin Cancer Res 2014;20:2922-32.

19. McBryan J, Fagan A, McCartan D, Bane FT, Vareslija D, Cocchiglia S, et al. Transcriptomic Profiling of Sequential Tumors from Breast Cancer Patients Provides a Global View of Metastatic Expression Changes Following Endocrine Therapy. Clin Cancer Res 2015;21:5371-9.

20. Augustin HG, Koh GY, Thurston G, Alitalo K. Control of vascular morphogenesis and homeostasis through the angiopoietin-Tie system. Nat Rev Mol Cell Biol 2009;10:165-77.

21. Eroglu Z, Stein CA, Pal SK. Targeting angiopoietin-2 signaling in cancer therapy. Expert Opin Investig Drugs 2013;22:813-25.

22. Saharinen P, Eklund L, Pulkki K, Bono P, Alitalo K. VEGF and angiopoietin signaling in tumor angiogenesis and metastasis. Trends Mol Med 2011;17:347-62.

23. Avraham HK, Jiang S, Fu Y, Nakshatri H, Ovadia H, Avraham S. Angiopoietin-2 mediates blood-brain barrier impairment and colonization of triple-negative breast cancer cells in brain. J Pathol 2014;232:369-81.

24. Holopainen T, Saharinen P, D'Amico G, Lampinen A, Eklund L, Sormunen R, et al. Effects of angiopoietin-2-blocking antibody on endothelial cell-cell junctions and lung metastasis. J Natl Cancer Inst 2012;104:461-75.

25. Ardelt AA, McCullough LD, Korach KS, Wang MM, Munzenmaier DH, Hurn PD. Estradiol regulates angiopoietin-1 mRNA expression through estrogen receptor-alpha in a rodent experimental stroke model. Stroke 2005;36:337-41.

26. Matsuoka-Sakata A, Tamura H, Asada H, Miwa I, Taketani T, Yamagata Y, et al. Changes in vascular leakage and expression of angiopoietins in the corpus luteum during pregnancy in rats. Reproduction 2006;131:351-60.

27. Harfouche R, Echavarria R, Rabbani SA, Arakelian A, Hussein MA, Hussain SN. Estradiol-dependent regulation of angiopoietin expression in breast cancer cells. J Steroid Biochem Mol Biol 2011;123:17-24.

28. Bonagura TW, Aberdeen GW, Babischkin JS, Koos RD, Pepe GJ, Albrecht ED. Divergent regulation of angiopoietin-1 and -2, Tie-2, and thrombospondin-1 expression by estrogen in the baboon endometrium. Mol Reprod Dev 2010;77:430-8.

29. D'Souza SS, Scherzinger-Laude K, Simon M, Salimath BP, Rossler J. Angiopoietin-2 inhibition using siRNA or the peptide antagonist L1-10 results in antitumor activity in human neuroblastoma. J Cancer Res Clin Oncol 2012;138:2017-26.

30. Daly C, Pasnikowski E, Burova E, Wong V, Aldrich TH, Griffiths J, et al. Angiopoietin-2 functions as an autocrine protective factor in stressed endothelial cells. Proc Natl Acad Sci U S A 2006;103:15491-6.

31. Mangan SH, Van Campenhout A, Rush C, Golledge J. Osteoprotegerin upregulates endothelial cell adhesion molecule response to tumor necrosis factor-alpha associated with induction of angiopoietin-2. Cardiovasc Res 2007;76:494-505.

32. Choi YP, Kim BG, Gao MQ, Kang S, Cho NH. Targeting ILK and beta4 integrin abrogates the invasive potential of ovarian cancer. Biochem Biophys Res Commun 2012;427:642-8.

33. Kang KS, Choi YP, Gao MQ, Kang S, Kim BG, Lee JH, et al. CD24(+) ovary cancer cells exhibit an invasive mesenchymal phenotype. Biochem Biophys Res Commun 2013;432:333-8.

34. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods 2012;9:671-5.

35. Sfiligoi C, de Luca A, Cascone I, Sorbello V, Fuso L, Ponzone R, et al. Angiopoietin-2 expression in breast cancer correlates with lymph node invasion and short survival. Int J Cancer 2003;103:466-74.

36. Hayes AJ, Huang WQ, Yu J, Maisonpierre PC, Liu A, Kern FG, et al. Expression and function of angiopoietin-1 in breast cancer. Br J Cancer 2000;83:1154-60.

37. Tian S, Hayes AJ, Metheny-Barlow LJ, Li LY. Stabilization of breast cancer xenograft tumour neovasculature by angiopoietin-1. Br J Cancer 2002;86:645-51.

38. Gyorffy B, Lanczky A, Eklund AC, Denkert C, Budczies J, Li Q, et al. An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1,809 patients. Breast Cancer Res Treat 2010;123:725-31.

39. Arai F, Hirao A, Ohmura M, Sato H, Matsuoka S, Takubo K, et al. Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell 2004;118:149-61.

40. Sosa MS, Avivar-Valderas A, Bragado P, Wen HC, Aguirre-Ghiso JA. ERK1/2 and p38alpha/beta signaling in tumor cell quiescence: opportunities to control dormant residual disease. Clin Cancer Res 2011;17:5850-7.

41. Reiss Y, Knedla A, Tal AO, Schmidt MH, Jugold M, Kiessling F, et al. Switching of vascular phenotypes within a murine breast cancer model induced by angiopoietin-2. J Pathol 2009;217:571-80.

42. Mandlekar S, Kong AN. Mechanisms of tamoxifen-induced apoptosis. Apoptosis 2001;6:469-77.

43. Imanishi Y, Hu B, Jarzynka MJ, Guo P, Elishaev E, Bar-Joseph I, et al. Angiopoietin-2 stimulates breast cancer metastasis through the alpha(5)beta(1) integrin-mediated pathway. Cancer Res 2007;67:4254-63.

44. Imanishi Y, Hu B, Xiao G, Yao X, Cheng SY. Angiopoietin-2, an angiogenic regulator, promotes initial growth and survival of breast cancer metastases to the lung through the integrin-linked kinase (ILK)-AKT-B cell lymphoma 2 (Bcl-2) pathway. J Biol Chem 2011;286:29249-60.

45. Potter SR, Epstein JI, Partin AW. Seminal vesicle invasion by prostate cancer: prognostic significance and therapeutic implications. Rev Urol 2000;2:190-5.

46. Hakanpaa L, Sipila T, Leppanen V-M, Gautam P, Nurmi H, Jacquemet G, et al. Endothelial destabilization by angiopoietin-2 via integrin β1 activation. Nat Commun 2015;6.

47. Guo W, Giancotti FG. Integrin signalling during tumour progression. Nat Rev Mol Cell Biol 2004;5:816-26.

48. White DE, Kurpios NA, Zuo D, Hassell JA, Blaess S, Mueller U, et al. Targeted disruption of beta1-integrin in a transgenic mouse model of human breast cancer reveals an essential role in mammary tumor induction. Cancer Cell 2004;6:159-70.

49. Brunckhorst MK, Xu Y, Lu R, Yu Q. Angiopoietins promote ovarian cancer progression by establishing a procancer microenvironment. Am J Pathol 2014;184:2285-96.

50. Schulz P, Fischer C, Detjen KM, Rieke S, Hilfenhaus G, von Marschall Z, et al. Angiopoietin-2 drives lymphatic metastasis of pancreatic cancer. FASEB J 2011;25:3325-35.

51. Glinskii OV, Abraha TW, Turk JR, Rubin LJ, Huxley VH, Glinsky VV. Microvascular network remodeling in dura mater of ovariectomized pigs: role for angiopoietin-1 in estrogen-dependent control of vascular stability. Am J Physiol Heart Circ Physiol 2007;293:H1131-7.

52. Albrecht ED, Babischkin JS, Pepe GJ. Regulation of placental villous angiopoietin-1 and -2 expression by estrogen during baboon pregnancy. Mol Reprod Dev 2008;75:504-11.

53. Ye F, Florian M, Magder SA, Hussain SN. Regulation of angiopoietin and Tie-2 receptor expression in non-reproductive tissues by estrogen. Steroids 2002;67:305-10.

54. Howell A, Dowsett M. Endocrinology and hormone therapy in breast cancer: aromatase inhibitors versus antioestrogens. Breast Cancer Res 2004;6:269-74.

ABSTRACT (IN KOREAN)

In vitro 골수 혈관주변 보금자리 모델을 이용한 에스트로겐의 유방암 휴면기 조절 기전 연구

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한현호

에스트로겐 호르몬 수용체 양성 유방암은 원발 종양의 성공적인 제거 후 원격전이까지 장기간 휴면기를 가지는 것을 특징으로 한다. 휴면 상태의 수용체 양성 유방암 세포는 주로 골수 조혈모세포 보금자리에서 발견된다. 본 연구에서는 유방암 세포가 골수 보금자리에서 휴면 상태로 전환되는 과정 및 이후 에스트로겐호르몬 억제요법 하에 재활성화되는 기전을 밝힌다. 호르몬 수용체 양성 유방암은 호르몬 공급이 없는 상태에서 장기간 생존할 수 없다. 그러니 골수 보금자리 실험모델에서 생장한 유방암 세포는 호르몬 공급이 없는 상태에서도 장기간 생존할 뿐 아니라, 오히려 휴면상태에서 활성상태로 전환되는 경향을 보였다. 호르몬 결핍상태에서 골수보금자리 실험모델의 중간엽줄기세포가 angipoietin-2 를 과발현하는데, angipoietin-2 는 유방암세포 표면의 integrin beta1 과의 상호작용에 의해 세포생존을 촉진하였다. 중간엽줄기세포에서 angipoietin-2 를 발현억제하자 에스트로겐 호르몬 결핍에 의한 휴면유방암세포주 재활성화 현상이 발생하지 않았다. 결론적으로, 골수보금자리의 중간엽줄기세포에서 발현되는 angipoietin-2 가 호르몬수용체양성 유방암의 호르몬결핍 상태에서의 재활성화를 유도한다고 할 수 있다.

핵심되는 말: 유방, 골, 전이, 휴면기, 내분비 치료 저항성, 세포 신호

PUBLICATION LIST

1. **Han HH,** Kim BG, Lee JH, Kang S, Kim JE, Cho NH. Angiopoietin-2 promotes ER+ breast cancer cell survival in bone marrow niche. Endocrine Related Cancer. 2016 Aug; 23(8): 609–623.

2. **Han HH**, Lee SH, Kim BG, Lee JH, Kang S, Cho NH. Estrogen Receptor Status Predicts Late-Onset Skeletal Recurrence in Breast Cancer Patients. Medicine (Baltimore). 2016 Feb;95(8):e2909.