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Abstract

Estrogens directly promote the growth of breast cancers that express the Estrogen Receptor α (ERα). However, the contribution of stromal expression of ERα in the tumor microenvironment to the pro-tumoral effects of estrogen has never been explored.

In this study, we evaluated the molecular and cellular mechanisms by which 17β-estradiol (E2) impacts the microenvironment and modulates tumor development of ERα-negative tumors. Using different mouse models of ER-negative cancer cells grafted subcutaneously into syngeneic ovariectomized immunocompetent mice, we found that E2 potentiates tumor growth, increases intratumoral vessel density and modifies tumor vasculature into a more regularly organized structure, thereby improving vessel stabilization to prevent tumor hypoxia and necrosis. These E2-induced effects were completely abrogated in ERα-deficient mice, demonstrating a critical role of host ERα. Notably, E2 did not accelerate tumor growth when ERα was deficient in Tie2-positive cells, but still expressed by bone marrow derived cells. These results were extended by clinical evidence of ERα-positive stromal cell labeling in the microenvironment of human breast cancers.

Together, our findings therefore suggest that E2 promotes the growth of ERα-negative cancer cells through the activation of stromal ERα (not hematopoiteic but Tie2-dependent expression of ERα), which normalizes tumor angiogenesis and allows an adaptation of blood supply to tumor demand preventing hypoxia and necrosis. These findings significantly deepen mechanistic insights into the impact of E2 on tumor development with potential consequences for cancer treatment.

Introduction

Estrogen Receptor alpha (ERα) holds a key position for diagnosis and treatment of breast cancers. Indeed, its expression by breast cancer cells dictates the use of endocrine therapies, such as tamoxifen and aromatase inhibitors, that blocks estrogen (E) activity (1). It is consistent with the fact that E could directly promote the growth of these tumors classified as ERα-positive. However, an increasing number of data sustain that tumor stromal microenvironment contributes to malignant development and progression of various cancers (2). ERα is expressed by a large number of tissues and mediates a vast range of the biological effects of E on the reproduction but also in other physiological functions (3-8). Nevertheless, the contribution of tumor microenvironment and particularly of stromal ERα to the pro-tumoral effect of E remains an open question.

Interestingly, various clinical and experimental data support this putative implication. Indeed, breast tumors are classified as ERα-positive even when only 1% of breast cancer cells express ERα, questioning the mechanisms accounting for the efficacy of targeting a so rare cell population. Additionally, ovariectomy appears to be efficient to decrease long-term recurrence risk and mortality of breast cancer classified as ERα-positive but also as ERα-negative (9) and particularly of BRCA1 tumors that generally do not express ERα (10, 11). These data point out that, beside cancer cell–associated ERα, additional E-dependent mechanisms interfere with the efficacy of endocrine therapy. This is supported by the fact that tamoxifen response rates were low but still can be found in ER-negative/progesterone receptor-negative tumors (<10%) (12). However, these ERα-negative breast tumors and particularly triple-negative breast cancer are no longer treated with endocrine therapy and exhibit poor prognosis (13). In immunodeficient animal models, pregnancy or 17β-estradiol (E2) supplementation was found to accelerate the growth of human ER-negative breast cancer cells (14, 15). E2 has been shown to impact angiogenesis (4, 14), however, there is still a paucity of information concerning the specific

molecular mechanisms by which E2 could impact tumor microenvironment and angiogenesis. Particularly, the contribution of host ERα and ERβ remains poorly documented (16, 17). In order to improve the management of cancer patients and particularly of women, it is mandatory to get mechanistic information regarding the various ways by which E2 could impact tumor development and progression. This is of particular interest knowing that E exert multiple functions in cells, depending on targeted cell-type and organ (6, 18).

The aim of the present work is to characterize which ER expressed by the tumor microenvironment and which ER-expressing cells are important for tumor development. Since the innate and adaptative immunity make crucial contributions to tumor development and to the anti-tumor effects of conventional radiation and chemotherapy, we used immunocompetent animals (19, 20). In models of ERα-negative tumor cell lines injected to syngeneic immunocompetent mice, we report here that E2 induces tumor growth through an increase and improvement of angiogenesis by triggering stromal cells *via* Tie2-dependent ERα.

Materials and Methods

Cell culture. Mouse B16K1 (MHC class I positive B16F10) melanoma cell line was used as previously described (21). Mouse Lewis lung carcinoma cell line (LL2, #CRL-2351), mouse breast tumor cells 4T1 (#CRL-2539) and mouse endothelial cell line derived by SV40 (#CRL-2181) were purchased and authenticated from ATCC, routinely cultured as recommended by manufacturer and used from passages 3 to 8. B16K1, LL2 and 4T1 were last authenticated in February 2012 by Leibniz-Institut DSMZ GmbH (Braunschweig, Germany).

Animals. Female C57BL/6J and Balb/C mice (4 weeks old) were obtained from Charles River Laboratories. ERα$^{-/-}$, ERβ$^{-/-}$ mice, Tie2-Cre^{+}/ERα-flox (Cre/$^{+}$) mice and their control wild type (WT) littermates ERα$^{+/+}$, ERβ$^{+/+}$ and Tie2-Cre^{-}/ERα-flox (lox/$^{+}$) mice were generated as described previously on C57BL/6J background (22, 23).

***In vivo* tumor models.** Four week-old female mice were ovariectomized (OVX) to prevent endogenous estrogen production. Two weeks before cancer cell injection, mice were implanted subcutaneously (s.c.) with a pellet releasing E2 (Innovative Research of America) or were sham operated (untreated control group). B16K1, LL2 or 4T1 (4×10^5 cells suspended in PBS) were injected s.c. to both flanks of WT or transgenic C57BL/6J mice (B16K1, LL2) or of Balb/C (4T1) mice.

Harvesting of tumors. Before sacrifice, mice were anesthetized and perfused by intravenous injection of FITC-conjugated lectin (Vector) that was allowed to circulate for 5 min. The tumor vasculature was then fixed by intracardiac perfusion of 4% PFA and then embedded in paraffin or OCT.

Histological analysis and quantification of angiogenesis, vessel perfusion and mural cell coverage. Morphological analysis were performed on paraffin-embedded tumor sections, stained with Hematoxylin-Eosin (H&E) or immunolabeled with CD34 (AbD-serotec) or Ki67 (Dako). For fluorescent microscopy, frozen tumor sections were immunolabeled with Cy3-conjugated

anti-αSMA (Sigma-Aldrich) and anti-CD31 (BD-Pharmingen), evidenced with a rat-IgG-specific biotinylated antibody (Vector Laboratories) and Cy3-conjugated streptavidin (Sigma-Aldrich).

Confocal microscopy analysis and quantification of tumor vascular network. Frozen tumor previously perfused with FITC-conjugated lectin were cut, then counterstained and mounted with DAPI-Mounting Medium (Vector Laboratories). The 3D images were obtained and quantified as explained in Supplementary Fig. S1. Image analysis was performed using the Matlab 7.9 software.

Hypoxia and necrosis assessment. Tumor hypoxia was evaluated with the Hypoxyprobe™-1 kit (#HP2-100, Chemicon, MA, USA) following manufacturer guidelines.

Antibody array and qRT-PCR. Proteins from size-match B16K1 tumors were extracted and analyzed on RayBio® Mouse Cytokine Antibody-Array according to manufacturer's instructions (RayBiotech). mRNA of EC were amplified on Biomark ^{TM} (Fluidigm). Data were analyzed with both Fluidigm Real Time PCR analysis and ValidPrime (24) softwares.

Statistical analysis. All quantitation experiment data are expressed as mean ± SD or mean ± SEM. Statistical analysis were conducted with GraphPad Prism^{TM} software. $P \leq 0.05$ was considered as statistically significant.

Other methods. Expanded methods are provided in Supplementary Methods online.

Results

E2 promotes ER-negative cancer cell growth in ER-positive immunocompetent microenvironment. To determine whether E2 affects the growth of ER-negative cancer cells in immunocompetent mice, we injected ER-negative tumor cell lines into syngeneic C57BL/6J (B16K1, LL2) or Balb/C (4T1) mice. Both B16K1 and LL2 cells were selected because they did not express ERα (B16K1), or very poorly (LL2), as shown by immunoblotting (Supplementary Fig. S2A). Furthermore, E2 had no effect on proliferation of B16K1 nor LL2 cells *in vitro* (Supplementary Fig. S2B and S2C). 4T1 mouse breast tumor cells did not express ERα as shown by immunocytochemistry (Supplementary Fig. S2E). Nevertheless, all B16K1 (Fig. 1A) LL2 (Fig. 1B) and 4T1 (Fig. 1C) growth were increased *in vivo* in OVX mice treated with E2. B16K1 cells were also implanted in the immunodeficient $Rag2^{-/-}$ mice to examine the role of the adaptative immune response in this E2-pro-tumoral effect (Fig. 1D). B16K1 tumor grew more rapidly in $Rag2^{-/-}$ mice as compared to control $Rag2^{+/+}$ mice, but the E2-induced acceleration was preserved in $Rag2^{-/-}$ mice. These results indicate that E2 promotes the growth of ER-negative tumors, by affecting the ER-positive stroma in immunocompetent mice, independently of the acquired immune system.

E2 increases angiogenesis in ER-negative tumors. E2 has been shown to increase physiological angiogenesis (4). As angiogenesis s a key process that sustains tumor growth in the early steps of tumor development and can also be influenced by tumor size (25), the effect of E2 on tumor angiogenesis was explored at different time points to collect tumors that time matched and size matched (Supplementary Fig. S3). Indeed, on day 11 (time-match), tumors have a mean volume of 75 and 200 mm^3 in mice untreated and treated by E2 respectively. To match the size, tumors of E2-treated mice were thus harvested earlier on day 7 (75 mm^3) and

tumors in untreated OVX mice were collected later, on day 15, when they reached a volume of 200 mm^3.

Blood vessel density was evaluated through CD31 immunostaining (Fig. 1E) and quantified by computer-assisted image analysis (Fig. 1F). E2 increased 2.1-fold the intratumoral vessel density in tumors of 75 mm^3, and this enhancement was maintained in 200 mm^3 tumors. To analyze vessel function, vessel perfusion was measured upon FITC-conjugated lectin injection (Fig. 1E, G). Computer-assisted image analysis of double staining for CD31 and lectin (Fig. 1H) revealed that the perfusion rate of tumor blood vessel reached 80% irrespective of tumor size and E2 impregnation. Altogether, the E2-induced growth of ER-negative cancer cells is associated with increased density of intratumoral perfused vessels.

E2 improves qualitatively tumor angiogenesis. Vessel morphology was evaluated with endothelial cell (EC)-marker CD34 on size-match tumors. Tumor vessels from untreated mice followed serpentine course and looked tortuous with irregular and heterogeneous structures (Fig. 2A, left panels). In contrast, vessels in E2-treated mice progressed from tumor periphery through aligned orientation (Fig. 2A, right panels). Nuclei of adjacent EC were more distant from each other in E2-treated tumors as compared to the untreated ones and EC presented an elongated morphology feature of lined EC monolayer (Fig. 2A, lower panels, pointed by arrows). Furthemore, confocal microscopy analysis of lectin-FITC staining of thick (100 µm) tumor sections followed by 2D-projection of z-slice images emphasized a major impact of E2 on tumor vessel architecture (Fig. 2B). In untreated group, vessels were uneven and dilated, whereas E2 treatment promoted a dense network of thin vessels evenly distributed with multiple branching points. Standardized computer-assisted image analysis of 3D-image constructions from z-slice images of thick tumor sections allowed vessel diameter quantification and 3D-architecture evaluation (Fig. 2C). This original method detailed in Supplementary Fig. S1 revealed a

heterogeneous distribution of vessel diameters in untreated mice ranging from 0.1 to more than 20 µm. The mode, the value that occurs most frequently, of vessel diameter distribution was 11.02±1.04 µm and 12.47±0.65 µm for tumors of 75 mm^3 (Fig. 2D) and 200 mm^3 (Fig. 2E), respectively. In contrast, E2-treated tumors were characterized by a narrow distribution of vessel diameters remaining below 15 µm. In those E2-treated mice, a highly regular vessel network was observed, in both 75 mm^3 and 200 mm^3 tumors, with a lower mode value [4.57±0.20 (N=4) and 4.55±0.23 µm (N=6) respectively]. Altogether, E2 improves the overall structure of tumor vascular network and regulates its organization.

E2 improves vessel stabilization and oxygenation of ER-negative tumors. Coverage of vessels by mural cells is a criterion of vessel stabilization and maturity (26). Double staining for lectin-FITC and the mural cell marker, α-smooth muscle actin (αSMA), was quantified by computer-assisted image analysis. Little perivascular labeling was observed at the onset of tumor growth irrespective of the treatment (size-match: 75 mm^3, Fig. 3A. upper panels). After 4 days (size-match: 200 mm^3), E2 increased the density of αSMA-positive vessels by 2.9-fold magnitude, whereas the mural cell coverage of vessel remained unchanged in untreated animals (Fig. 3A, bottom panels, B and C). Confocal microscopy (Supplementary Fig. S4) and morphological analysis of αSMA staining confirmed that the maturation of neovessels by mural cells was increased by E2 during tumor growth. Additionally, under E2 treatment, vascular cell proliferation assessed by Ki-67 immunostaining (Supplementary Fig. S5) was high at the beginning of tumor growth on day 7 and decreased on day 11. By contrast, the proliferative rate of vascular cells remained unchanged in untreated group (OVX).

Tumor hypoxia may result from an insufficient number of patent vessels or from supernumerary nonperfused vessels (27). Visualization of the hypoxic tumor area using pimonidazole, showed that E2 prevented the appearance of large hypoxic area with time and tumor size (Fig. 3D-E).

Quantification in size-match 200 mm^3 B16K1 tumors (Fig. 3F) indicated that under E2 treatment, necrotic area represented only 0 to 5% (mean score: 0.83±0.11, N=12), whereas necrosis reached 10-15% (mean score: 3.13±0.46, N=16) without E2 treatment (Fig. 3G). Later on (tumors of 400 mm^3), necrosis area increased to 30-50% without E2, but remained low (5-10%) under E2 supplementation (Supplementary Fig. S6). Similarly, necrosis in 400 mm^3 4T1 tumors remained between 5 to 10% (mean score: 1.86±0.48, N=8) under E2 treatment (Fig. 3G), but reached 15-20% (mean score: 4.86±0.99, N=7) in untreated mice. Thus, during the early steps of tumor growth, E2 promotes quantitatively angiogenesis. Then, later on, it favors vessel stabilization by mural cell recruitment associated with a decrease of vascular cell proliferation, thereby optimizing blood supply as attested by reduction of both tumor hypoxia and necrosis.

Host ERα is necessary to induce E2-dependent ER-negative tumor growth. As ERα mediates most of the vascular effects of E2 (5), the ability of E2 to stimulate the growth of B16K1 and LL2 was assessed in $ER\alpha^{-/-}$ mice and in their control $ER\alpha^{+/+}$ littermates. The pro-tumoral effect of E2 observed, to different extent, on both B16K1 and LL2 growing in $ER\alpha^{+/+}$ mice was completely abrogated when the host was ERα-deficient (Fig. 4A-B). In contrast, E2 still accelerated ER-negative cancer cell growth in ERβ deficient mice (Fig. 4C). These findings support that host ERα is absolutely required for the E2-mediated pro-tumoral effect on these ER-negative cancer cells, whereas ERβ is dispensable.

The effect of E2 on angiogenesis and oxygenation of ER-negative tumor requires host ERα. No significant difference of tumor vessel density was observed in $ER\alpha^{-/-}$ mice in response to E2 (Fig. 4D). Quantification from 3D images of lectin-FITC stained sections showed that the regular distribution of tumor vessel diameters elicited by E2 in $ER\alpha^{+/+}$ mice (Fig. 4E) was completely lost in $ER\alpha^{-/-}$ mice. Clearly, in $ER\alpha^{-/-}$ mice treated or not with E2, the tumor vessel network

remained unchanged, irregular with large vessels displaying variable diameters up to 20 µm (Fig. 4F). Moreover, staining for the pimonidazole revealed large hypoxic areas when tumors grew in ERα$^{-/-}$ mice despite E2 treatment (Fig. 4G-H). Consistently, tumor necrosis was abundant in ERα$^{-/-}$ mice and not modulated by E2 (Fig. 4I-J). Altogether, these results demonstrate that stromal ERα increases and improves tumor angiogenesis, thereby decreasing tumor hypoxia and necrosis.

Tie2-dependent ERα, but not BM-derived cells ERα, drives E2-dependent ER-negative cancer cell growth. Our results sustain that E2 acts on tumor microenvironment by modulating angiogenesis. Hence, to identify the host cells implicated in the pro-tumoral effect of E2, we used a Tie2-specific inactivation of ERα (using Tie2-Cre^{+}/ERα–flox, named Cre/$^{+}$) (28). As expected, E2 increased B16K1 tumor growth in their control littermates Tie2-Cre^{-}/ERα-flox (named lox/$^{+}$). By contrast, this effect was completely abrogated in Tie2-Cre^{+}/ERα-flox (Cre/$^{+}$) mice (Fig. 5A) demonstrating that ERα of Tie2-expressing cells is necessary to mediate the pro-tumoral effect of E2. In Tie2-Cre/$^{+}$ mice, specific inactivation of ERα appears mostly in both endothelial and hematopoietic cells (23). Since recruitment of bone marrow (BM)-derived cells contributes to vasculogenesis and tumor progression (29, 30), we evaluated the role of hematopoietic ERα. Ovariectomized C57BL/6J mice were lethally irradiated and successfully grafted with BM cells from either ERα$^{-/-}$ or ERα$^{+/+}$ mice, then treated or not with E2 (Supplementary Fig. S7). The B16K1 tumor growth was similarly accelerated by E2 in mice grafted with ERα$^{+/+}$ or ERα$^{-/-}$ BM (Fig. 5B), suggesting that BM-ERα is dispensable for the pro-tumoral effect of E2. To ascertain that BM-ERα is dispensable and that the loss of E2 effect on tumor growth in the Tie2-Cre/$^{+}$ mice is not due to the concomitant ERα inactivation in hematopoietic cells, we reconstituted Tie2-Cre/$^{+}$ mice or their control littermates with ERα$^{+/+}$

BM. In these chimeric mice with ERα-negative Tie2-expressing cells and ERα-positive hematopoietic cells, E2 do not accelerate the growth of B16K1 tumor cells anymore, although the E2 pro-tumoral effect is well visible in control littermate mice (Fig. 5C-D). These data exclude any major contribution of ERα expressed by hematopoietic cells to the pro-tumoral effect of E2. Thus, ERα of Tie2-positive cells, but not BM-derived cells ERα, is necessary to mediate the effect of E2 on the tumor growth of ER-negative cancer cells.

E2 modulates angiogenic factor expression in ER-negative tumors. To further characterize whether intratumoral angiogenic factors can be influenced by E2 treatment, expression of a set of proteins known to regulate angiogenesis was analyzed using an antibody array on whole protein extracts of size-match tumors. Of all candidates analyzed, levels of basic fibroblast growth factor (bFGF) and of vascular endothelial growth factor (VEGF)-D remain unchanged whereas both VEGF-A and platelet platelet factor-4 (PF4) were upregulated by E2 (Fig. 6A). Additionally, among the VEGF receptors, VEGFR-3, whose expression was very low in untreated tumors as compared to VEGFR-1 and VEGFR-2, was strongly upregulated in E2-treated tumors. As we demonstrated that angiogenesis contributes to E2-mediated ER-negative cancer cell growth, we evaluated *in vitro* if E2 could modulate the angiogenic expression profile of an endothelial ER-positive cell line, CRL-2181 (Fig. 6B). Among the set of genes analyzed, expression of VEGF-A, VEGF-D, VEGFR-1, sVEGFR-1, thrombospondin-1 (TSP-1) and PECAM was significantly upregulated by E2 treatment. Transcripts of VEGF-B, VEGFR-3, TGFβ1 and VCAM-1 tended to increase under E2 treatment while those of PlGF, Notch1 and Dll4 decreased.

Altogether, these data indicate that E2 could modulate intratumoral angiogenic factor levels and that endothelial expression of some angiogenic factors could be impacted by E2 treatment.

ERα is expressed by peritumoral microenvironment of human breast cancers. To assess whether the expression of ERα occurs *in vivo* in the stroma of human tumors, immunohistochemistry on human ERα-positive and ERα-negative breast cancer tissues was performed. Close to the strong staining of malignant ERα-positive tumors cells (Fig. 7A), a relevant ERα staining was observed in some stromal cells. Stromal expression, detected in some fusiform-shape fibroblast-like cells (thin arrows), was also detected in human breast tumors classified as ERα-negative tumors (Fig. 7B-C). Additionally, double immunostaining for ERα combined either with CD45 (Fig. 7D) or SMA antibody (Fig. 7E) revealed that ERα-positive stromal cells were not leucocytes and were SMA-negative. However, some rare co-stainings for ERα and CD31 (Fig. 7F) were observed in human breast cancer stroma. These data indicate that ERα immunostaining can be found in stromal cells in the microenvironment of human breast cancers irrespectively of the ERα expression by cancer cells.

Discussion

Until now, several evidences have suggested that E2 affects tumor microenvironment independently of its direct effect on tumor cell growth. However, the cellular and molecular mechanisms driving these interactions remained undetermined in immunocompetent mice. The present study describes novel insights showing that E2 acts through ERα expressed by tumor microenvironment to promote tumor growth *via* an increased and normalized angiogenesis.

The cellular effectors of inflammation are key constituents of the microenvironment. Moreover, E2 is known to increase the production of pro-inflammatory cytokines, in various cell populations such as CD4+ lymphocytes (31), Natural Killer cells (32) and macrophages (33). First, we evidence here that the pro-tumoral impact of E2 on ERα-negative tumor cell growth was also present in $Rag2^{-/-}$ immunodeficient mice that lack T and B lymphocytes, indicating that

these immune cells do not play a major role in the E2 effect. Secondly, using chimeric mice reconstituted with BM from ERα$^{-/-}$ or ERα$^{+/+}$ mice, we show that ERα of BM-derived cells is not required for acceleration of B16K1 tumor growth in C57BL/6J mice by E2. This result diverges from Gupta et al. (14) who described that BM cell recruitment was sufficient to mediate the E2-induced growth of human ER-negative tumorigenic breast epithelial cells implanted to immunodeficient mice. Major differences in the experimental protocols, i.e. selective ERα ablation in BM of immunocompetent animals (present work) *versus* co-injection of a mix of immunodeficient BM cells, epithelial tumor cells and Matrigel into immunodeficient mice (14) probably accounts for the apparent discrepancy.

The present data clearly show that E2 impregnation not only increases vessel density, but also improves qualitatively tumor angiogenesis, by improving vessel structure, organization, and stabilization by mural cell recruitment. This impact of E2 completely relies on host ERα, as tumor growth and vascular network in ERα$^{-/-}$ mice were unresponsive to E2 treatment. In tumors that grew in E2-treated host, the vessel network was dense with multiple branching points and clearly oriented. Vessels were composed of lined EC presenting flattened and elongated morphology with distant nuclei, their diameter were thin and even resulting in a more regular shape. E2 also improved their maturation by increasing neovessel coverage by mural cells, and drastically reducing both tumor hypoxia and necrosis. Modulation of intratumoral angiogenic factors is a likely mechanism underlying this improvement of angiogenesis. Upregulation of VEGF-A by E2 was already documented (4), whereas upregulation of VEGFR-3 is of particular interest since it was recently reported to control the rapid conversion of tip cells to stalk cells during angiogenesis (34). At the EC level, transcription of various genes reported to be related to angiogenesis and vessel maturation was modulated by E2. Indeed, the increase of VEGFR-1 and sVEGFR-1 has been implicated in vessel maturation (26, 35). The anti-angiogenic factor TSP-1, was also upregulated in EC by E2, a modulation already reported in T47-D and MCF-7 cells

(36). Knowing the high degree of complexity by which E2 impacts cell physiology, all these data indicate that the balance between pro- and anti-angiogenic factors can be modulated by E2 and contributes through paracrine interactions between EC, cancer cells and other stromal cells to increase and improve tumor angiogenesis and thus tumor growth.

Altogether, these data demonstrate that E2 optimizes blood supply to ER-negative cancer cells through an important modification of tumor vasculature that appears normalized as described by Carmeliet and Jain (26, 37). This concept of normalization was documented by Mazzone et al. (26) who observed that tumor vessel normalization improves tumor perfusion and oxygenation and decreases metastasis. Importantly, hypoxia is a negative prognostic factor associated to chemo- and radio-resistance and is described to promote tumor invasion and metastatic dissemination (37-40). As vessel normalization emerges actually as a potential therapeutic option (25, 37), the stromal ERα-mediated impact of E2 on ER-negative cancer cell growth and angiogenesis is of peculiar interest. Indeed, even if E2 indirectly contributes to promote ER-negative cancer cell growth, the decreased tumor hypoxia and necrosis conferred by E2 could present a therapeutic advantage in terms of metastasis and efficacy of chemo- and radiotherapies. This idea is supported by the Southwest Oncology Group run trials which reported that E2 increases response to chemotherapy in young pre-menopausal women treated for lung cancer, turning the negative pro-tumoral effect of E2 into a positive factor in response to chemotherapy (41).

Using the Tie2 promoter-driven ERα expression, we further demonstrated that ERα expressed by Tie2-positive cells plays a crucial role in the pro-tumoral effect of E2. Tie2 is largely reported to be an EC marker as it is expressed mostly by EC-lineage (28). However, more recently, it has also been reported in hematopoietic lineage, in pericytes, fibroblasts/myofibroblasts (42-44). Tie2-expressing monocytes (TEM) have been shown to promote tumor angiogenesis in various mouse tumor models (45). Additionally, BM-derived

endothelial progenitor cells (EPC) could also have contributed to the formation of tumor endothelium, but it remains highly controversial (29). In the present work, E2 elicited similar pro-tumoral effects in chimeric mice irrespective of the presence or absence of ERα in BM-derived cells. Moreover, the pro-tumoral effect of E2 was abrogated in chimeric mice harboring ERα-negative Tie2-expressing cells, but with ERα-positive hematopoietic cells, demonstrating a minor role of E2 on BM-derived EPC in tumor vasculature development. This result corroborates studies showing that tumor endothelium does not predominantly originate from BM cells, particularly in C57BL/6 mice (46, 47). As we demonstrated that the pro-tumoral effect of E2 relies on ERα-dependent promotion and improvement of angiogenesis, this is in line with the crucial role played by endothelial ERα in the various effects exerted by E2 on endothelium i.e. EC migration and proliferation *in vitro*, re-endothelialization acceleration *in vivo* (5, 23, 48-50) and with the major contribution of pericytes to vessel stabilization (26, 37, 40). Thus, Tie2-positive cells expressing ERα, but not BM ERα, are necessary to promote the E2-induced growth of ER-negative tumors.

Finally, we clearly detected ERα in human stromal cells surrounding human ER-positive but also ER-negative breast cancers. These stromal ERα-positive cells were negative for CD45 and SMA, and were rarely positive for CD31. However, the lack of selective fibroblast markers led the phenotype of the stromal ERα-positive cells difficult to be clearly identified. Until now, this stromal expression of ERα is not routinely evaluated during diagnosis of human breast tumors. Nevertheless, it could be relevant if a correlation between stromal ERα expression, prognosis and/or treatment response could be addressed in women.

In summary, we demonstrate here that ERα of microenvironment plays a crucial role in the *in vivo* growth of ERα-negative cancer cells under E2 treatment. Indeed, stromal ERα is necessary to induce tumor growth, mediating adaptation of tumor angiogenesis and vessel stabilization that subsequently improves oxygen and nutrients delivery, thereby preventing

hypoxia and necrosis. This could have implications in the management of patients, particularly in the diagnosis and the schedule of hormono-, radio- and chemotherapies.

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Figure Legends

Figure 1. E2 promotes ER-negative tumor growth *in vivo* and increases angiogenesis. *In vivo* growth curve of B16K1 **(A)**, LL2 **(B)** and 4T1 **(C)**, untreated (OVX, N=6) or treated with E2 (+E2, N=6). **(D)** *In vivo* growth curve of B16K1 injected to $Rag2^{-/-}$ or WT mice, untreated (OVX, N=7) or treated with E2 (+E2, N=8). **(E)** Double staining for CD31 EC-marker (red) and lectin-FITC (green) on size-match B16K1 tumors, scale bars = 135 µm. **(F)** Quantifications of CD31-positive density, **(G)** Perfused vessel density as measured by lectin-FITC density **(H)** Percentage of perfused vessels. N=5 to 8 tumors; optical fields: 8 to 12 per tumor. All results are mean ± SEM. For all statistical analysis: *p<0.05, **p<0.01, ***p<0.001 E2 *versus* OVX.

Figure 2. E2 modifies the structure of tumor vasculature in B16K1 tumors. B16K1 tumors untreated (OVX) or treated with E2, harvested after 7, 11 or 15 days (D7-D15) for time- and size-match analysis. **(A)** Staining for CD34 EC-marker, scale bars = 50 µm. **(B)** 2D-projections and **(C)** 3D-images from computer-assisted construction of z-sections recorded by confocal microscopy of lectin-FITC positive vascular network; scale bars = 50 µm. **(D-E)** Vessel diameter distributions of lectin-FITC positive vascular network; N = 3 to 6. Statistical analysis: **p<0.01 E2 *versus* OVX.

Figure 3. E2 improves vessel maturation and oxygenation of B16K1 tumors. B16K1 tumors untreated (OVX) or treated with E2 harvested after 7, 11 or 15 days (D7-D15) for time- and size-match analysis. **(A)** Double staining for lectin-FITC (green) and αSMA (red); scale bars = 67 µm, **(B)** quantification of αSMA-positive vessel, **(C)** variation of vessel coverage by mural cells between tumors of 75mm^3 and 200 mm^3. For (B) and (C): N=5 to 8 tumors, optical fields: 8 to 12 per tumor. **(D)** Double staining for PIMO (green) and dapi (blue); scale bars = 1 mm. **(E)** Quantification of hypoxic (PIMO-positive) tumor area N=5 to 7. **(F)** H&E

coloration revealing necrosis area; scale bars = 500 µm. **(G)** Scored quantification of tumor necrosis area of size-match (200 mm^3) B16K1 and of size-match (400 mm^3) 4T1 tumors. B16K1: OVX, N=16; +E2, N=12; 4T1: OVX, N=7; +E2, N=8. All results are mean ± SEM. For all statistical analysis: *p<0.05, **p<0.01, ***p<0.001 E2 *versus* OVX.

Figure 4. ERα is necessary to mediate the E2-dependent increase of tumor angiogenesis, oxygenation and growth. *In vivo* growth curve of B16K1 **(A)** or LL2 **(B)** in ER$\alpha^{-/-}$ mice; **(C)** of B16K1 in ER$\beta^{-/-}$ mice, untreated (OVX) or treated with E2 . For (D) to (J) B16K1 cells were implanted in ER$\alpha^{-/-}$ mice and collected at size-match (200 mm^3)**. (D)** Tumor vessel density quantified by CD31-positive staining, N=8. Vessel diameter distributions of lectin-FITC positive vascular network in B16K1 tumors from E2-treated ER$\alpha^{+/+}$ *versus* ER$\alpha^{-/-}$ mice **(E)** or from untreated (OVX) *versus* E2-treated ER$\alpha^{-/-}$ mice **(F)**; N=4 to 6. **(G)** Double staining for PIMO (green) and dapi (blue); scale bars = 600 µm. **(H)** Quantification of hypoxic (PIMO-positive) tumor area, N=5 to 7. **(I)** Hematoxylin coloration revealing necrosis area; scale bars = 500 µm. **(J)** Scored quantification of tumor necrosis area; N=10 to 16. All results are mean ± SEM. For all statistical analysis: NS= no statistically different (p>0.05), *p<0.05, **p<0.01, ***p<0.001 E2 *versus* OVX.

Figure 5. Tie2-dependent ERα is implicated in E2-dependent ER-negative cancer cell growth. (A) *In vivo* growth curve of B16K1 injected in Tie2-Cre^{+}/ERα-flox (named $Cre/^{+}$), or in Tie2-Cre^{-}/ERα-flox (named $lox/^{+}$), untreated (OVX) or treated with E2, N=10. **(B-D)** Mice were lethally irradiated, then grafted with bone marrow cells (BM) from ER$\alpha^{-/-}$ or ER$\alpha^{+/+}$ mice and after complete BM recovery, untreated (OVX) or treated with E2 before B16K1 implantation. **(B)** *In vivo* growth curve of B16K1 in chimeric C57BL/6J mice, N=16. **(C)** *In vivo* growth curve of B16K1 in chimeric Tie2-Cre^{+}/ERα-flox (named $Cre/^{+}$) and **(D)** in Tie2-Cre^{-}/ERα-flox (named

lox/$^{+}$), N=18. All results are mean ± SEM. For all statistical analysis: NS= no statistically different (p>0.05), *p<0.05, **p<0.01, ***p<0.001 E2 *versus* OVX.

Figure 6. E2 modulates angiogenic factor expression in ER-negative tumors.

(A) Intratumoral protein expression of bFGF, VEGF-A, VEGF-D, PF4, VEGFR-1, VEGFR-2 and VEGFR-3 in size-match B16K1 tumors, untreated (OVX) or treated with E2, N=5. **(B)** qRT-PCR analysis of angiogenic genes from EC-CRL-2181, results are expressed as change in mRNA expression in E2-treated EC (percent of OVX level), N=4. All results are mean ± SEM. For all statistical analysis: NS= no statistically different (p>0.05), *p<0.05, **p<0.01, ***p<0.001 E2 *versus* OVX.

Figure 7. Detection of ERα by immunohistochemistry in the peritumoral microenvironment of human breast cancers. ERα–staining of stromal cells (large arrows), fibroblast-like cells (thin arrows) and cancer cells (surrounded by the line) in **(A)** ERα-positive breast tumors and in **(B-C)** ERα-negative breast tumors. Double staining for ERα (red) and **(D)** CD45 (green), **(E)** SMA (green) or **(F)** CD31 (green). Scale bars = 50 μm excepted for panel D where scale bar = 10 μm.

Figure 1

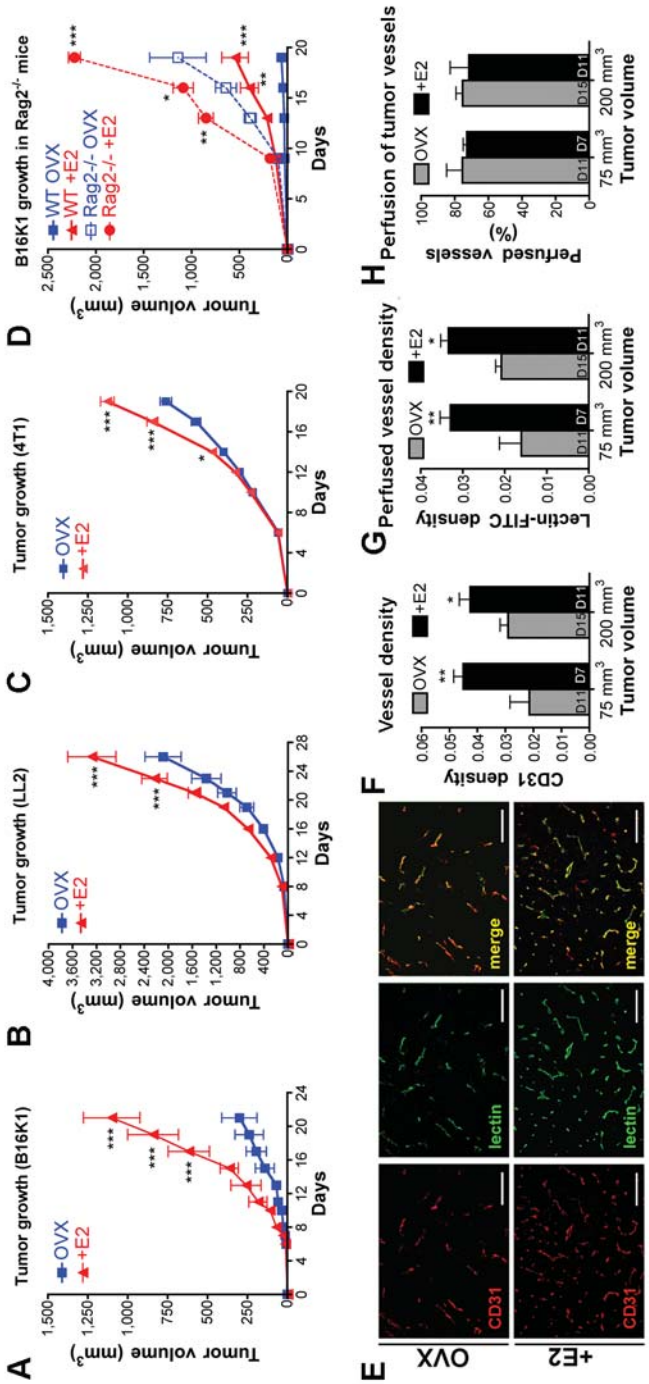
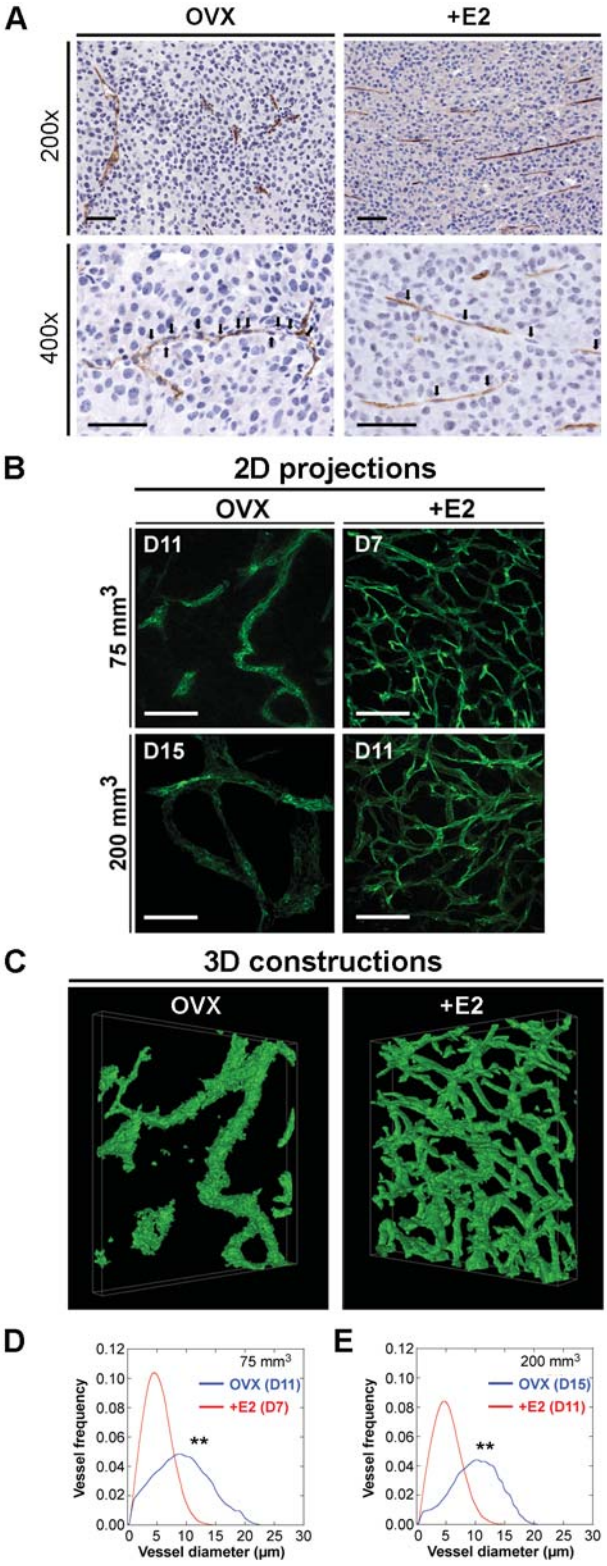


Figure 2



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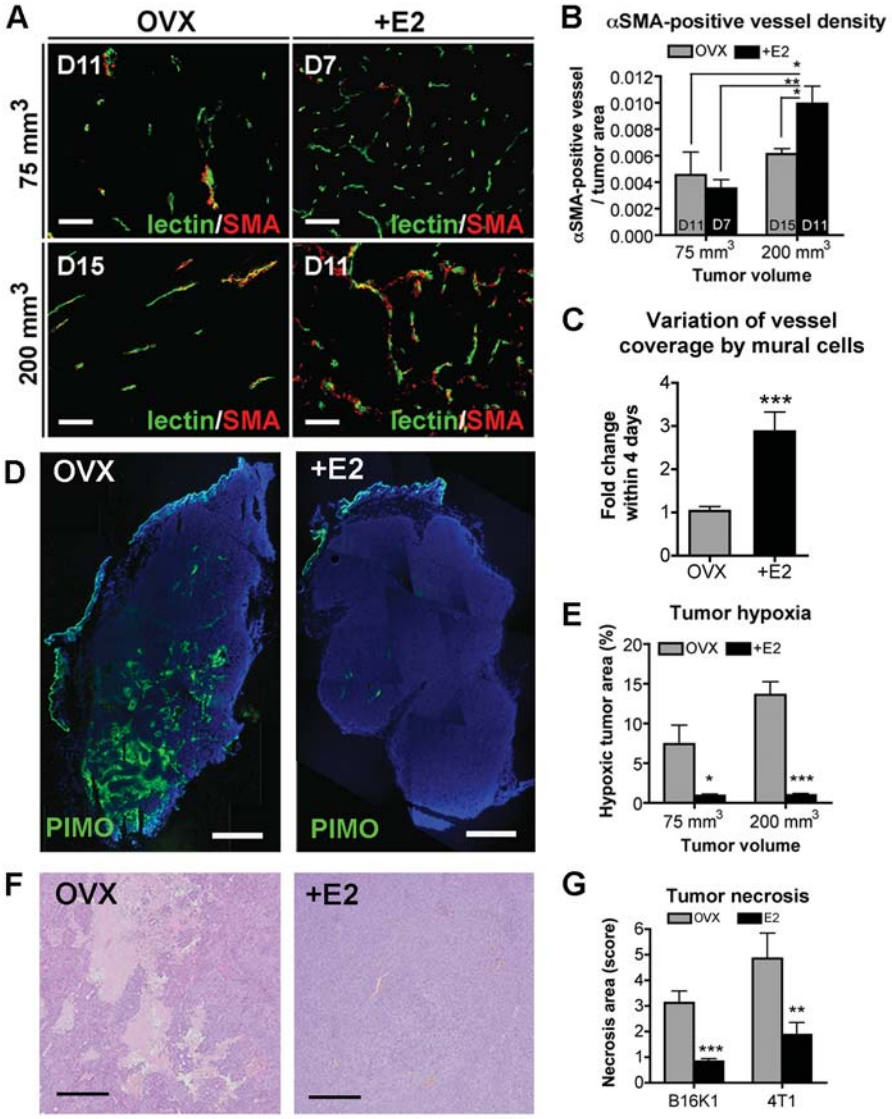

Figure 4

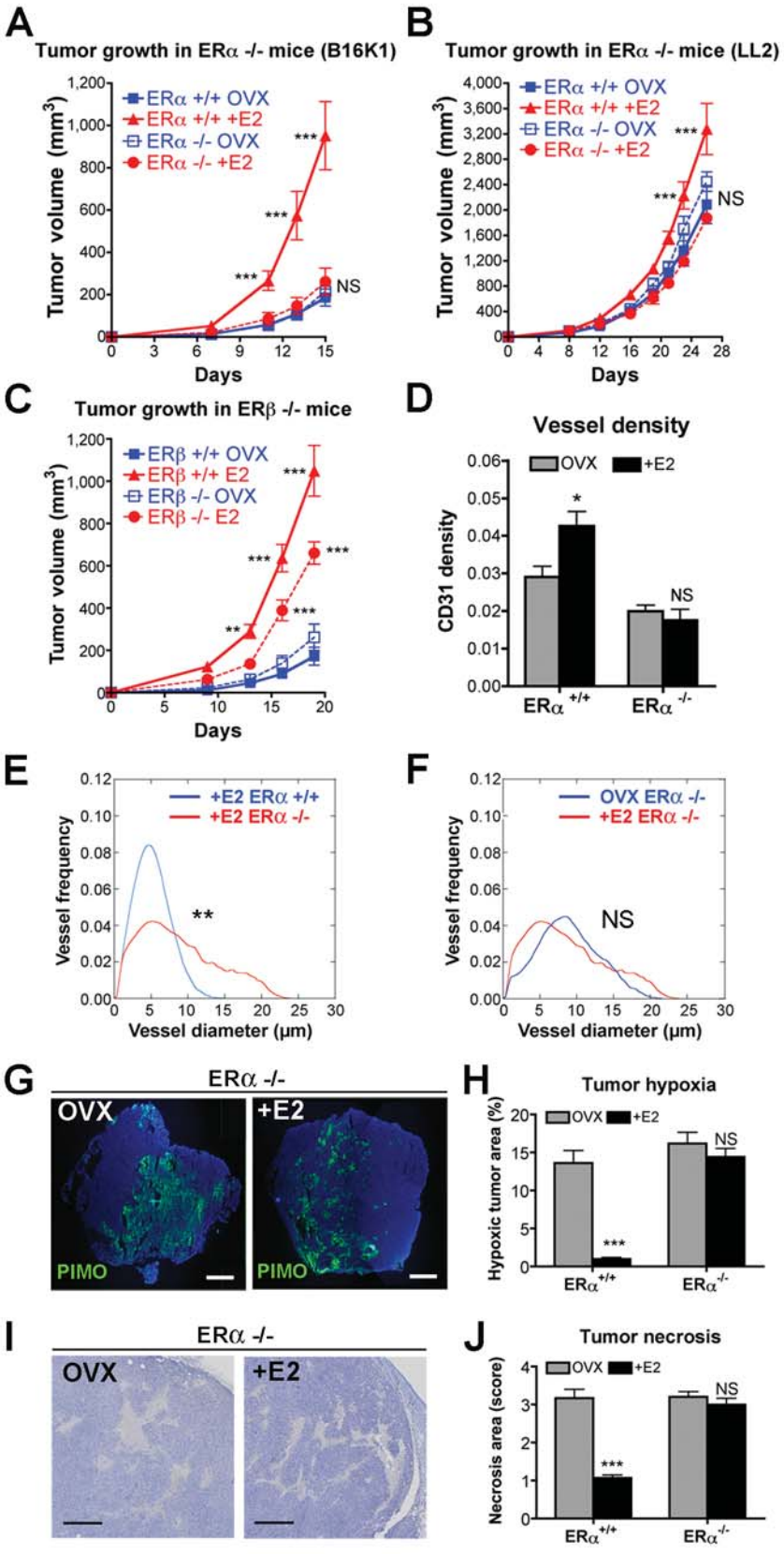

Figure 5

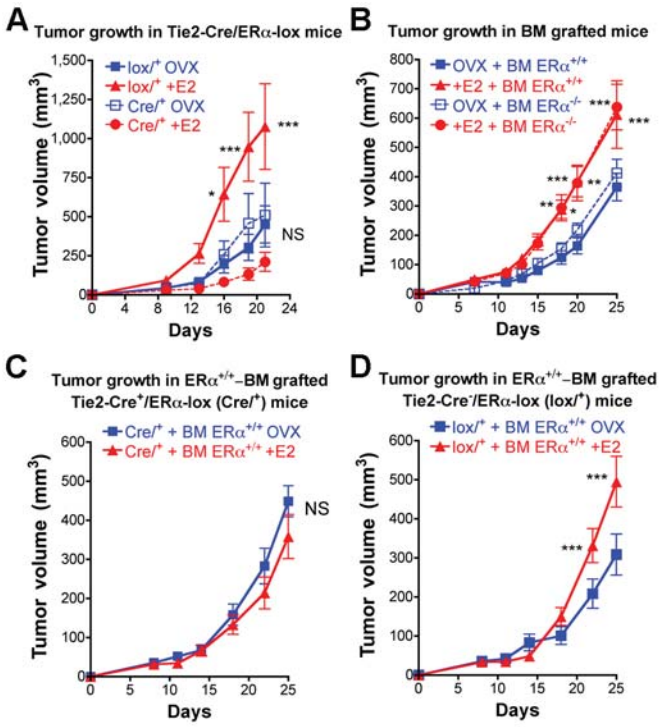

Figure 6

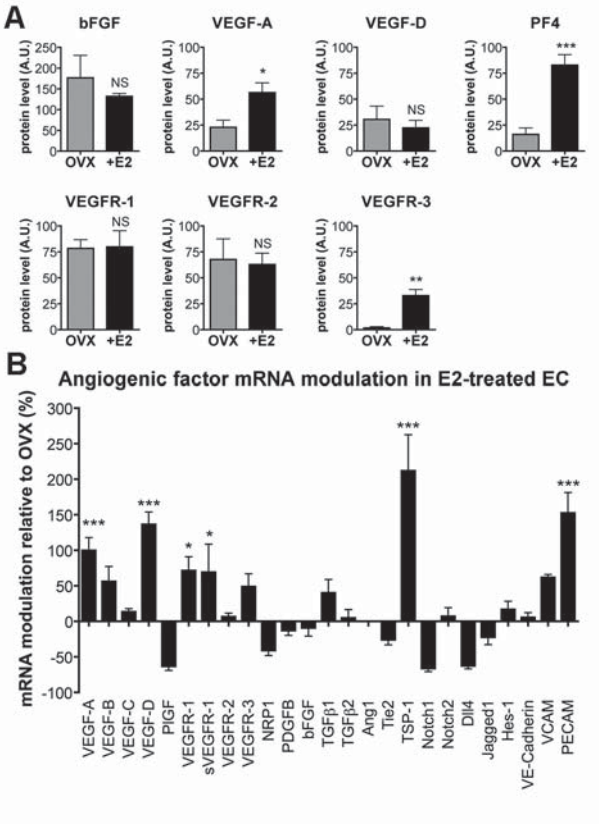

Figure 7