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Estradiol-dependent regulation of angiopoietin expression in breast cancer cells

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ABSTRACT

Angiopoietin-1 (Ang-1) is a ligand for Tie-2 receptors and a promoter of angiogenesis. Angiogenesis plays an important role in breast cancer, as it is one of the critical events required for tumors to grow and metastasize. In this study, we investigated the influence of estradiol (E2) on the expression of angiopoietins in breast cancer cell lines. Ang-1 mRNA and protein expressions were significantly higher in estrogen receptor-negative (ERα−) breast cancer cells than in estrogen receptor-positive (ERα+) cells. Exposure of ERα+ cells to E2 resulted in further reductions of Ang-1 levels. In mouse mammary pads inoculated with breast cancer cells, both tumor size and Ang-1 production were significantly lower in ERα+ cell-derived xenografts, as compared to those derived from ERα− cells. Reduction of circulating levels of E2 by ovariectomy eliminated this response. Overall, these results indicate that Ang-1 mRNA and protein expressions: (1) negatively correlate with the level of ERα in breast cancer cell lines; (2) are downregulated by E2 in an ERα dependent manner; and (3) positively correlate with the degree of angiogenesis *in vivo*. We conclude that Ang-1 is an important modulator of growth and progression of ERα− breast cancers.

1. Introduction

Breast cancer is the most common hormone-dependent cancer in women, resulting in very high rates of mortality [1]. The hormone estrogen stimulates the growth of breast cancer tumors and women who develop the disease tend to have higher levels of circulating estrogen than those that do not. The biological effects of estradiol (E2), the most potent form of estrogen, are primarily mediated by estrogen receptor α (ERα) [2]. E2 is initially required for the survival and proliferation of normal breast epithelia, but prolonged E2 exposure promotes breast carcinogenesis [2,3]. As ERα levels gradually increase during the early stages of breast cancer, activation promotes cell survival, but also inhibits angiogenesis [4,5]. One of the mechanisms by which ERα activation inhibits angiogenesis is through downregulation of the pro-angiogenic factor vascular endothelial growth factor (VEGF) [4–7], although invasive breast cancers also express many other pro-angiogenic factors, including the recently identified angiopoietins.

The angiopoietins, including Ang-1, Ang-2 and Ang-4 in humans, are soluble ligands of endothelial cell (EC)-specific tyrosine kinase receptors, Tie-2 [8–10]. Ang-1 is the main agonist for Tie-2, hence it elicits strong angiogenic effects, including promotion of EC differentiation, tube formation, sprouting, migration and EC survival [11–13]. Ang-2 can also activate Tie-2 and promote angiogenic potentials within cells, but at relatively higher concentrations than for Ang-1 [9,14]. Ang-4, the least well-characterized angiopoietin, exerts qualitatively similar yet quantitatively weaker effects than Ang-1 [10,15]. Accumulating evidence suggests that Ang-1, Ang-2 and Ang-4 are all expressed in human breast cancer samples [16] and that angiopoietin expression correlates with lymph node invasion and short survival rates in patients with breast cancer [17]. However, despite these newly emerging roles for angiopoietins in breast cancer progression and angiogenesis, little is known about the specific role of E2 in modulating angiopoietin expression. Our group has previously reported that E2 stimulates Ang-2 mRNA expression while inhibiting Ang-1 expression in non-reproductive organs of normal rats [18]. In comparison, brain Ang-1 expression is significantly induced by E2 in overiectomized female mice [19].

In this study, we investigated the direct influence of E2 and ERα on the expression of Ang-1, Ang-2 and Ang-4 in various human breast cancer cell lines. We also examined the association between Ang-1 expression and tumor progression in a murine xenograft model of breast cancer. Our results indicate that Ang-1 mRNA and protein expressions negatively correlate with levels of ERα in breast cancer cell lines and are downregulated by E2 in an ERα dependent manner. Ang-1 mRNA and protein expres-

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sions also positively correlate with the degree of angiogenesis *in viivo*.

2. Methods

2.1. Cell cultures

Human primary mammary epithelial cells (HMECs) were obtained from Cambrex Corporation (San Diego, CA) and maintained in MEGM medium supplemented with bovine pituitary extract, human epidermal growth factor, insulin and hydrocortisone. All other cell lines and media were purchased from the American Type Culture Collection (ATCC) (Rockville, MD) and Invitrogen (Burlington, ON). MDA-MB-468, BT549 and ZR-75-1 cells were grown in RPMI 1640 supplemented with 1 mM sodium pyruvate and 10% fetal bovine serum (FBS). MCF7, BT474 and MDA-MB-231 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS. Mock transfected MDA-MB-231 cells and those transfected with a functional ERα (S30 cells) were kindly provided by Dr. C. Jordan (Northwestern University, Chicago, IL) and were maintained in Minimum Essential Medium Alpha (MEMα) and 5% FBS, under selection for neomycin resistance with geneticin (G418 sulfate) (Life Technologies, Grand Island, NY), as previously described [20]. MDA-MB-468 cells were a generous gift from Dr. B. Jean-Claude (McGill University, Montréal, QC). After reaching first confluence in 100 mm dishes, cells were subcultured following trypsin (0.25%) and ethylenediaminetetraacetic acid (EDTA) (0.01%) application at a ratio of 1:3 to 1:6, depending on cell type. For all estradiol experiments, cells were grown in stripped medium (phenol-red-free media (Invitrogen)) and charcoal-stripped FBS (sFBS (Biomeda, Foster City, CA)) to remove endogenous estrogens, and in the presence of 10 μM E2 or cyclodextrin solvent (E2 vehicle) and/or 10 μM anti-E2 tamoxifen (TAM). E2, cyclodextrin and TAM were purchased from Sigma–Aldrich Canada (Oakville, ON).

2.2. Cell proliferation assay

MDA-MB-231 and S30 cells were plated in triplicate at a density of 10,000 cells per well in six-well plates and grown in stripped medium. Where indicated, cells were treated with E2 alone or in combination with TAM. Cells were trypsinized and counted at various time points using a Coulter counter Model ZF (Coulter Electronics, Harpenden, Hertfordshire, UK). Culture medium was replenished every second day.

2.3. Northern blotting

Total RNA (5 μg) was mixed with ethidium bromide and separated by electrophoresis on 1.2% agarose gels containing 2.2 M formaldehyde. RNA was transferred to nylon membranes and rRNA was visualized under ultraviolet light to verify equal loading. Membranes were probed with Ang-1 at 42 °C overnight in ULTRAhyb™ buffer (Ambion, Inc., Austin, TX), stripped and then re-probed with 18S. Probes were non-isotopically labeled DNA probes designed in our laboratory using RT-PCR and a PCR DIG Probe Synthesis Kit (Roche Diagnostics, Laval, QC). Ang-1 and 18S probes were designed using known sequences (accession number NM_001146, corresponding to sequences 537–935, and accession number M10098, corresponding to sequences 951–1458, respectively). Labeled DNA probes were added directly to the ULTRAhyb™ solution. Washes were performed twice at room temperature for 5 min (low stringency solution 2.0× SSC/0.1% SDS) and then twice at 42 °C for 15 min (high stringency solution 0.1× SSC/0.1% SDS). Signals were detected using a DIG Luminescent Detection Kit and CDP-*Star*, ready-to-use substrate (Roche Diagnostics). Membranes were exposed to BioMax™ Light film (Amersham Biosciences, Piscataway, NJ), at room temperature, using an intensifying screen for up to 2 h.

2.4. Real time PCR

Total RNA was collected from all cell types (90% confluent) under basal conditions. In addition, MDA-MB-231, S30, BT474 and MCF7 cells grown in stripped medium (see above) were also stimulated with E2. Prior to E2 experiments, sub-confluent cells were serum-starved for 12 h, then incubated with E2 or cyclodextrin (solvent) for 6 h. RNA was extracted with an RNeasy® kit (Qiagen, Mississauga, ON), reverse-transcribed with Superscript™II (Invitrogen) and subjected to either reverse transcription (RT) PCR or real-time PCR. For RT PCR, one tenth of synthesized cDNA was used as a template in PCR reactions containing 2.5 units Taq DNA polymerase, 50 mmol/l each dNTP, 1.5 mM $MgCl_2$, buffer provided by the manufacturer and 0.4 μM primers. Amplification cycles consisted of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. RT PCR products were analyzed on a 2% agarose gel. Three different primer pairs were used to detect regions corresponding to full length Ang-1 (1.5 kb) and 1.3 and 0.9 kb isoforms. Full length Ang-1 was detected using forward primer 5′-GGAAGTCTAGATTTCCAAAGAGGC-3′ and reverse primer 5′-CTTTATCCCATTCAGTTTTCCATG-3′, corresponding to sequences 1306–1326 bp and 1711–1734 bp, respectively, designed to give a 429 bp product [21]. Amplification of the full-length coding region of Ang-1 was accomplished using forward primer 5′-GCTGGCAGTACAATGACAGGT-3′ (identical to 5′ end) and reverse primer 5′-TCAAAAATCTAAAGGTCGAAT-3′ (complementary to 3′ end), yielding a 312 bp product [21]. Evaluation of the 1.3 kb isoform was completed using forward primer 5′-GGAATATAAAATGGTTGTATTTAA-3′ (specific for Ang-1.3 kb) paired with reverse primer 5′-TCAAAAATCTAAAGGTCGAAT-3′. The 0.9 kb isoform was generated using forward primer 5′-GTGGCTGCAAAAAGTGTTTTGC-3′ (specific for Ang-0.9 kb) and reverse primer 5′-ATCGCTTCTGACATTGCGCTT-3′, to give an amplification fragment of 595 bp [21]. Forward primer 5′-TCAGCTTGCTCCTTTCTGGAACT-3′ and reverse primer 5′-TTTACGGGCCAGATTGTAAGC-3′ were used to amplify Tie-2. 18S primers were used as internal controls. Real-time PCR was performed using a 7500 real-time PCR system and TaqMan® reagents and primers designed to amplify 18S, Ang-1, -2 and -4 (Applied Biosystems, Carlsbad, CA). Absolute copy numbers were calculated using standard curves generated by plasmids containing full length coding sequences of human Ang-1, -2 and -4.

2.5. Immunoblotting

Adherent cells were washed twice with PBS and lysed using basic lysis buffer (pH 7.5) containing 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 150 mM NaCl, 100 mM sodium fluoride, 1 mM sodium orthovanadate, 5 mM EDTA, 1 mM phenylmethanesulfonylfluoride (PMSF), 2 μg/ml leupeptin, 5 μg/ml aprotinin and 0.5% Triton®-X 100. Cell debris and nuclei were separated by centrifugation at 14,000 × *g* at 4 °C for 5 min. Resulting supernatants (cell lysates) were used for immunoblotting. Conditioned media were concentrated 80 times using Centricon® filters with a molecular weight cut-off of 50 kDa. Cell lysates (25–80 μg protein) or concentrated media (15–100 μg protein) were heated at 100 °C for 5 min and loaded onto Tris–glycine SDS-polyacrylamide gels. Proteins were electrophoretically transferred onto polyvinylidene difluoride membranes, blocked for 1 h with 5% non-fat dry milk, and incubated overnight at 4 °C with primary monoclonal antibodies specific for Ang-1 (R&D Systems, Minneapolis, MN). Ang-1 protein was used as a positive control.

Proteins were detected with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies and ECL reagents (Chemicon, Temecula, CA). Blots were scanned with an imaging densitometer and optical densities of protein bands were quantified with Image-Pro® Plus software (MediaCybernetics, Carlsbad, CA). Predetermined molecular weight standards were used as markers. Protein concentration was measured by the Bradford method with bovine serum albumin as a standard.

2.6. Mice xenograft protocols

Normal and ovariectomized (OVX) 5-week-old BALB/c *nu.nu* female mice were obtained from Charles River, Inc. (St. Constant, QC). Before inoculation, MDA-MB-231 or S30 cells grown in serum-containing culture medium were washed with Hank's balanced buffer and centrifuged at $1500 \times g$ for 5 min. Cell pellets (5×10^5 cells/mouse) were re-suspended in 100 μl of Matrigel® (BD Labware, Oakville, ON) and saline mixture (20% Matrigel®) and injected into the mammary fat pads of mice as previously described [22]. All animals were numbered and kept separately in a temperature-controlled room on a 12-h light/dark schedule with food and water *ad libitum*. All animal studies were approved by the University Animal Care Committee (UACC) of McGill University.

2.7. Immunocytochemistry and immunohistochemistry

MDA-MB-231 and S30 cells cultured in 24-well plates were stained for Ang-1. Paraffin-embedded tumor samples were cut into 5-μm thick sections and stained for Ang-1, VEGF and CD31 (index of vessel density), using the avidin–biotin-peroxidase complex. Polyclonal VEGF and CD31 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All steps were performed at room temperature. Cells and archived tissues were treated in the same manner except that tissue sections were initially de-waxed in xylene and rehydrated through a series of ethanol-to-water gradients prior to incubation for 30 min in 1% normal goat serum (Vector Laboratories, Burlingame, CA) then overnight with a 1:100 dilution of the corresponding primary antibody, followed by incubation with biotinylated secondary antibodies for 30 min. Samples were then treated with a Vectastain® ABC-AP kit (Abcam, Cambridge, MA), developed with SIGMA*Fast*™ Red TR (Abcam)/Naphthol AS-MX phosphate (Sigma–Aldrich) containing 1 mM levamisole, counterstained with hematoxylin (Fisher Scientific Ltd., Nepean, ON) and mounted with Kaiser's glycerol jelly. For negative controls, a non-specific immunoglobulin (IgG) primary antibody was used. Images of stained cells and tissue sections were photographed with a Leica digital camera and processed using Bio-Quant image analysis software (Bio-Quant Image Analysis Corp., Nashville, TN).

2.8. Statistical analysis

All results were expressed as mean ± SEM of at least three samples. For immunocytochemistry and immunohistochemistry at least five slides per group were analyzed. Statistical comparisons were obtained using one-way analysis of variance. Probability (P) values less than 0.05 were considered significant.

3. Results

3.1. Effect of E2 on breast cancer cell growth

Effects of E2 on cell number of ERα− MDA-MB-231 and ERα+ S30 cells were assessed. Under basal conditions, significant increases in the number of MDA-MB-231 cells were seen after 5 days, as compared with S30 cells, indicating that overexpression of ERα inhibits breast cancer cell proliferation (Fig. 1). Specificity of this response was examined by culturing S30 cells in sFBS-containing stripped medium devoid of endogenous steroids. Under these conditions, a marked increase in S30 cell number was seen, where cell numbers are similar to those of MDA-MB-231 cells. Addition of E2 to S30 cells cultured in stripped medium reduced numbers to levels similar to those seen in unstimulated S30 cells. This inhibitory effect of E2 on S30 cell proliferation was reversed when cells were co-incubated with anti-E2 TAM (Fig. 1).

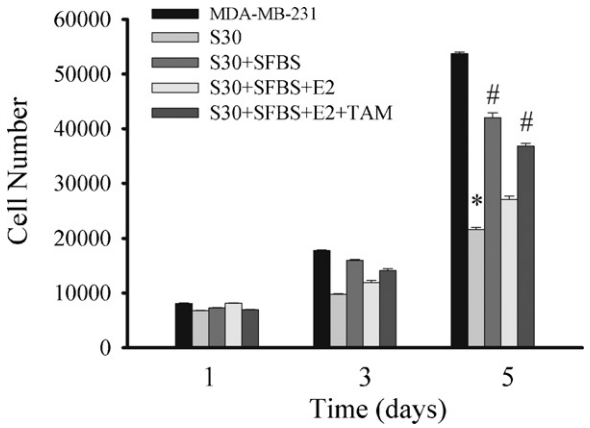

Fig. 1. Effect of E2 on breast cancer cell growth. Results are representative of two independent experiments ± SEM. *$P < 0.05$ compared with MDA-MB-231 cells. #$P < 0.05$ vs. S30 cells. sFBS = stripped medium devoid of endogenous steroids.

3.2. Expression of angiopoietins in breast cancer cells

To evaluate whether the presence of ERα correlates with angiopoietin expression, RT-PCR was performed to amplify Ang-1 mRNA in MDA-MB-231 and S30 cells, as these cells differ only by the presence of ERα in S30 cells (Fig. 2A). Three sets of Ang-1 primers, corresponding to full length (1.5 kb), 1.3 kb and 0.9 kb isoforms, were used and the sequence-specificities of these primers were confirmed by direct sequencing of the amplified products. Expression levels of full length Ang-1 and the 0.9 kb isoform were markedly higher in MDA-MB-231 cells than they were in S30 cells. Primers designed to amplify the 1.3 kb isoform did not reveal any positive bands. However, full-length Ang-1 primers yielded a faint secondary band in MDA-MB-231, corresponding to 262 bp, which appears to be the 1.3 kb isoform [21]. Tie-2 mRNA was not detected in either cell line (Fig. 2A). Northern blotting confirmed that full-length Ang-1 mRNA was significantly more abundant in MDA-MB231 cells, as compared to amounts measured in ERα+ cells such as S30, MCF7 and HMEC (Fig. 2B). Since significant Ang-2 and Ang-4 expression was not detected by northern blotting, the more sensitive real-time PCR assay was also used, which allowed for the detection of exact copy numbers of Ang-1, -2 and -4 mRNA transcripts in various breast cancer cell lines. Fig. 2C shows absolute copy numbers of angiopoietin mRNA in ERα− cell lines (MDA-MB-231, MDA-MB-468 and BT549) and in ERα+ cell lines (S30, MCF7, ZR-75-1, BT474 and the parental HMEC). Although no relationships between ERα expression and Ang-2 or Ang-4 expressions were detected, the presence of ERα correlated inversely with the abundance of Ang-1 mRNA in various cell lines, with Ang-1 mRNA being more abundantly expressed in MDA-MB-231 and MB-MB-468 cells, as compared with MCF7, HMEC, and ZR75-1 cells (Fig. 2C). In addition, absolute Ang-1 mRNA expression was relatively higher than that of Ang-2 and Ang-4 in each cell type, except for ZR75-1, where Ang-2 mRNA copy numbers were higher than for Ang-1. In all cell types except for MCF-7 cells, Ang-4 mRNA expression was negli-

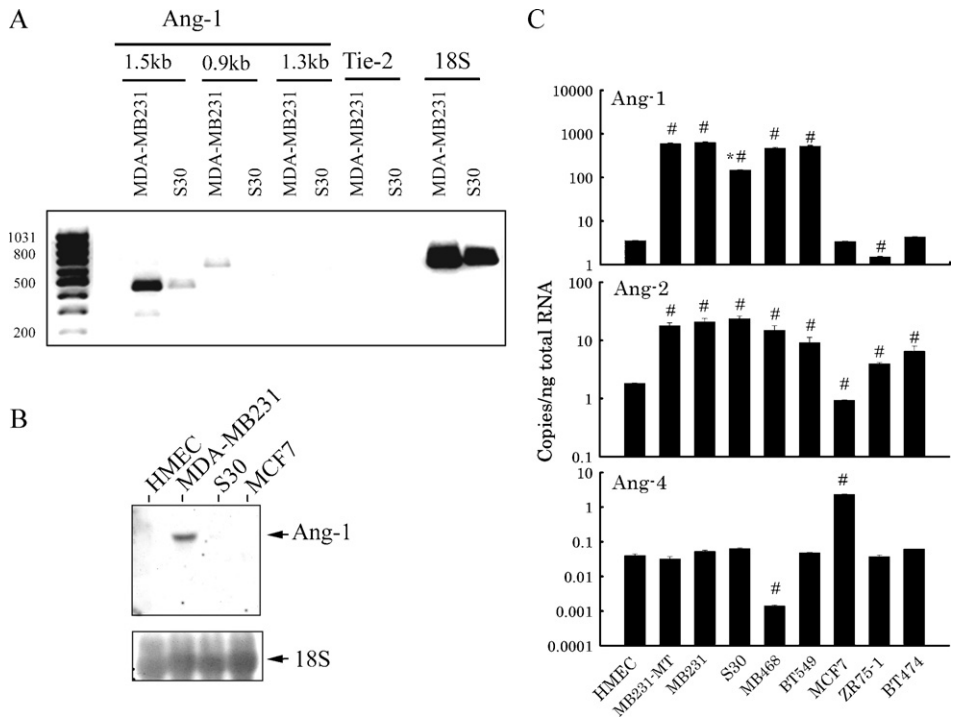

Fig. 2. Expressions of angiopoietin mRNA in breast cancer cells. (A) Expressions of Ang-1 and Tie-2 transcripts in MDA-MB-231 and S30 cells determined by RT-PCR. Ang-1 mRNA amplified using three sets of primers. (B) Northern blot detection of 1.5 kb Ang-1 mRNA transcripts in various breast cancer cells lines and primary human mammary epithelial cells (HMECs). (C) Expressions of angiopoietin mRNA levels in unstimulated breast cancer cell lines and HMECs. Results represent three experiments ± SEM. MB231-MT refers to mock transfected MDA-MB-231 cells. $^{*}P < 0.05$ represents a significant difference between S30 and MDA-MB-231 cells and $^{\#}P < 0.05$ represents a significant difference between all cell types relative to parental HMEC cells.

gible. In MCF-7 cells, more than 1 copy/ng of Ang-4 was detected (Fig. 2C).

Immunoblotting of the conditioned media of unstimulated breast cancer cells revealed that Ang-1 protein is robustly expressed in MDA-MB-231 cells, whereas it was almost undetectable in the ERα+ cell lines (HMEC, S30 and MCF7) (Fig. 3A). Likewise, immunocytochemistry analysis showed 2-fold more Ang-1 expression in MDA-MB-231 cells, as compared to S30 cells (Fig. 3B).

3.3. Regulation of angiopoietin expression by E2

Real-time PCR measurements revealed that treatment of S30 cells with E2 results in significant attenuation of Ang-1, -2 and -4 mRNA levels, indicating that expression of all angiopoietin members is E2-sensitive in ERα+ breast cancer cells (Fig. 4A). We should emphasize that treatment of MDA-MB231 cells with E2 demonstrated no significant effect on expressions of Ang-1, Ang-2 and Ang-4 mRNA (results not shown). Fig. 4B shows that E2 treatment significantly attenuated Ang-1 protein expression in ERα+ cells (S30), but had no effect on Ang-1 protein levels in ERα− cells (MDA-MB-231). In addition to studying S30 cells, inhibition of angiopoietin expression by E2 in other ERα+ breast cancer cells was verified by treating BT474 and MCF7 breast cancer cells with E2. Fig. 4C and D illustrates that E2 treatment significantly attenuated mRNA expression of the three angiopoietins in BT474 and that of Ang-1 and Ang-2 in MCF7 cells. Both of these cell lines are ERα+. These results confirm that E2 treatment exerts a negative influence on angiopoietin expression in ERα+ breast cancer cells.

3.4. Effects of E2 on in vivo tumor growth

The effects of E2 on tumor growth were examined using a xenograft model of breast cancer where both MDA-MB-231 and S30 cells were injected into the mammary fat pads of normal female BALB/c *nu.nu* mice. Animals that received MDA-MB-231 cells developed palpable tumors by week 5, which continued to grow for the following 4 weeks. However, experimental animals that were inoculated with S30 cells at various time points grew tumors of significantly lower volumes (approximately 2.5-fold), as compared with MDA-MB-231 xenografts, as previously reported [22]. The role of E2 in altering tumor growth in the S30 xenografts was further investigated by comparing tumor volumes between normal (non-OVX) and ovariectomized (OVX) animals. In OVX animals, tumor volume was significantly higher (approximately 2-fold), as compared to non-OVX animals that were inoculated with S30 cells. At the conclusion of experiments, animals were sacrificed and primary tumors from all groups were removed and embedded in paraffin. Archived samples from control and experimental animals were subjected to immunohistochemical analysis for Ang-1, VEGF and CD31. Expression of Ang-1 protein was significantly higher in tumors derived from MDA-MB-231 recipients, as compared with S30-derived xenografts (Fig. 5). Analysis of S30 tumors in OVX animal revealed that Ang-1 protein expression was partly restored to levels similar to those found in MDA-MB-231 xenografts, indicating that Ang-1 expression is inhibited by endogenous production of E2 (Fig. 5). To assess whether the degree of tumor volume correlates with angiogenesis, tissues were also probed for two angiogenic markers, namely VEGF and CD31 (Fig. 5). Similar expression patterns to those of Ang-1 were obtained for these markers, as shown by lower expressions in S30 xenografts, as compared with MDA-MB-231 tumors. More importantly, these effects were reversed following E2 ablation by ovariectomy (Fig. 5).

4. Discussion

In this study, we are the first to report that: (1) Ang-1 expression is inversely correlated with the presence of ERα in breast cancer

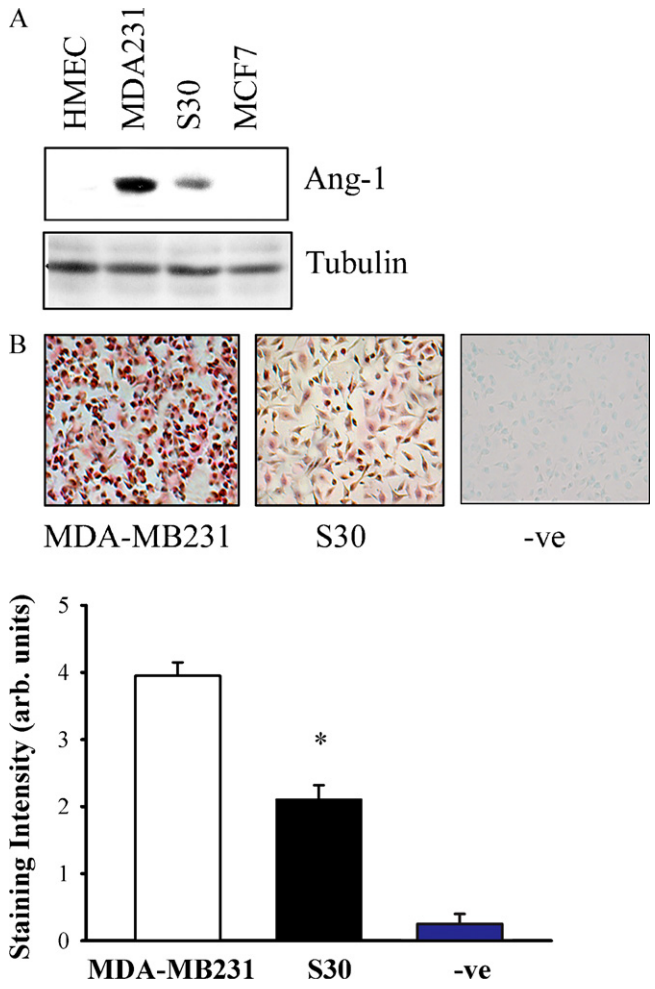

Fig. 3. Expressions of Ang-1 protein in breast cancer cells. (A) Immunoblot detection of Ang-1 protein in concentrated culture media of HMEC and breast cancer cell lines. (B) Immunocytochemical analysis of the expression of Ang-1 protein in MDA-MB-231 and S30 cells. −ve refers to negative control. Bars represent mean of three experiments ± SEM. $^{*}P < 0.05$ vs. MDA-MB-231 cells.

cell lines; (2) mRNA expressions of Ang-1, Ang-2 and Ang-4 are inhibited by E2 treatment in ERα+ breast cancer cells; and (3) *in vivo* expression of Ang-1 is significantly elevated along with markers of angiogenesis in ERα− xenografts, as compared with ERα+ xenografts, and that reduction of circulating E2 levels by ovariectomy in animals with ERα+ xenografts results in elevated Ang-1 expressions.

It is has been well established that the switch from an ERα+ to an ERα− phenotype correlates with increased breast cancer invasiveness and lethality [2]. With this study, we demonstrate that cell growth and tumor volume are inhibited in breast cancer cells transfected with ERα (S30), as compared to ERα− counterparts (MDA-MB-231), both in culture and in murine mammary xenografts. In addition, these inhibitory effects of ERα are partly reversed upon E2 depletion, which is in accordance with several previous reports [4,22]. The anti-invasive role of E2 occurs mainly when levels of ERα reach relatively high levels, which leads to simultaneous upregulation of anti-angiogenic factors, such as α1-anti-chymotrypsin, and downregulation of pro-angiogenic factors, including VEGF [4,5,22,23].

There has been increasing evidence that E2 regulates angiopoietin expression, however, the differential influence of E2 on Ang-1 vs. Ang-2 expressions varies considerably between studies. In non-reproductive rat tissues, our group [18] has reported that E2 administration reduces Ang-1 expression while inducing Ang-2 expression. Subsequent studies have revealed that in primate endometrium, Ang-1 expression varies considerably during the menstrual cycle [24] and that acute administration of E2 induces endometrial Ang-1 expression [25]. In contrast, in pregnant baboons subjected to prolonged exposure of the estrogen precursor androstenedione, placental Ang-1 expression is significantly attenuated but has no influence on Ang-2 levels [26]. In humans, Lee et al. [27] have described significant reductions in Ang-1 levels, as well as upregulation of Ang-2 expression, in endometrial biopsies that have been taken following chorionic gonadatrophin injection.

Little information is as yet available regarding the influence of E2 on angiopoietin expression in cancerous tissues. Currie et al. [16] have detected Ang-1, Ang-2 and Ang-4 expressions in malignant breast tissues and have described a significant correlation between Ang-4 expression and ER, and a negative correlation between Ang-1 expression and thymidine phosphorylase (promoter of angiogenesis) expression. These authors have also reported that E2 treatment of MCF7 cells has no influence on Ang-4 expression.

Our study is the first to investigate the expression of all three human angiopoietins in breast cancer, under both basal conditions and in response to E2. Our results reveal that under basal conditions, only Ang-1 expression is inversely correlated with ERα expression in breast cancer cell lines and tissues. We also found that following acute E2 exposure, Ang-1, -2 and -4 mRNA expressions are all downregulated in an ERα dependent manner, except for that of Ang-4 expression in MCF7 cells, which, as Currie et al. have previously reported, is unresponsive to E2 treatment.

We did not investigate the exact mechanisms through which E2 regulates angiopoietin expression in ERα+ breast cancer cells; however, we speculate that E2 may inhibit angiopoietin expression through decreased transcription of angiopoietin genes. Upon hormone binding, ERs exert their effects by interacting with DNA elements in target gene promoters, either directly or through interactions with other transcription factors [28]. Although molecular analyses of human Ang-1 and Ang-2 promoters have not uncovered the presence of any of the abundant palindromic estrogen response elements that bind to ER with relatively high affinity and specificity [29,30], there is evidence that ER interacts directly with various transcription factors, particularly AP-1, which is composed of heterodimers of the Jun/Fos family of proteins [31]. Both Ang-1 and Ang-2 promoters possess abundant AP-1 binding sites. No information is as yet available regarding the molecular structure of Ang-4 promoter.

In ERα− cells lines, the most abundantly expressed angiopoietin is Ang-1, followed by Ang-2, while Ang-4 mRNA expression is very low. This suggests that Ang-1 is the primary angiopoietin that is expressed in ERα− cells. Unlike the situation for Ang-1, mRNA expressions of Ang-2 and Ang-4 do not correlate with ERα status. This is particularly true with respect to Ang-4, as its expression is extremely low in all cell types, save MCF7 cells. In addition, we observed that the basal Ang-4/Ang-1 mRNA ratio in MCF7 is much higher than in other breast cancer cells, suggesting that Ang-4 may function as the main Tie-2 receptor agonist in MCF7 cells. A similar suggestion has been made by Currie et al. [16].

Although we did not directly investigate the roles of Ang-1 in breast cancer, we report, for the first time, that expressions of Ang-1 and two angiogenic markers, VEGF and CD31, are significantly lower in S30 tumor xenografts as compared to those generated by MDA-MB-231 xenografts. Although several studies have recently been published which address the functional roles of Ang-1 in breast cancer, the main conclusions of these studies are contradictory. Hayes et al. [32], for instance, report that Ang-1 overexpression inhibits breast tumor growth by stabilizing the vasculature. Others, however, have used relatively lower concentrations of exogenous Ang-1 or have targeted inhibition of

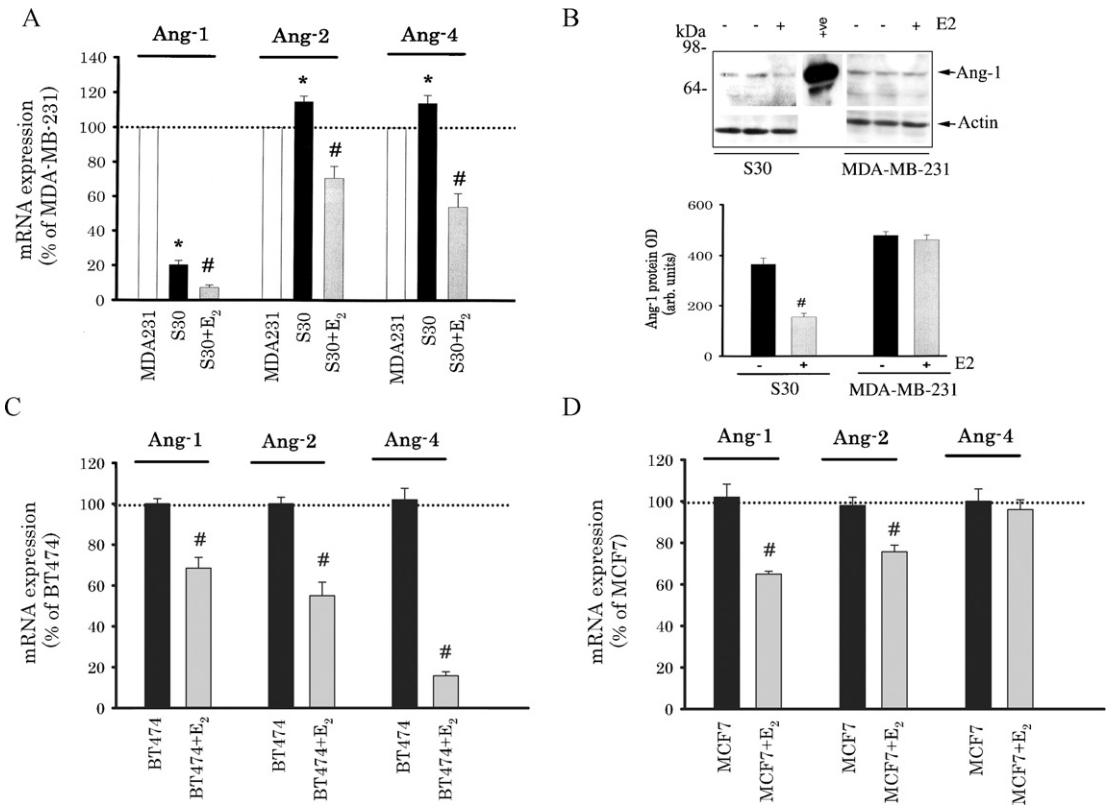

Fig. 4. Regulation of angiopoietin expressions by E2 in breast cancer cells. (A) Change in angiopoietin mRNA levels in response to E2 treatment in S30 cells. $^{*}P < 0.05$ compared with MDA-MB-231 cells. $^{\#}P < 0.05$ compared with S30 cells without E2. (B) Immunoblots and mean values of Ang-1 protein optical densities in S30 and MDA-MB-231 cells with or without E2 treatment. Results are representative of three experiments ± SEM. $^{\#}P < 0.05$ compared with control (no E2 treatment) values. +ve refers to positive control. (C and D) Change in angiopoietin mRNA levels in response to E2 treatment in BT474 and MCF7 cells. $\#P < 0.05$ vs. cells without E2.

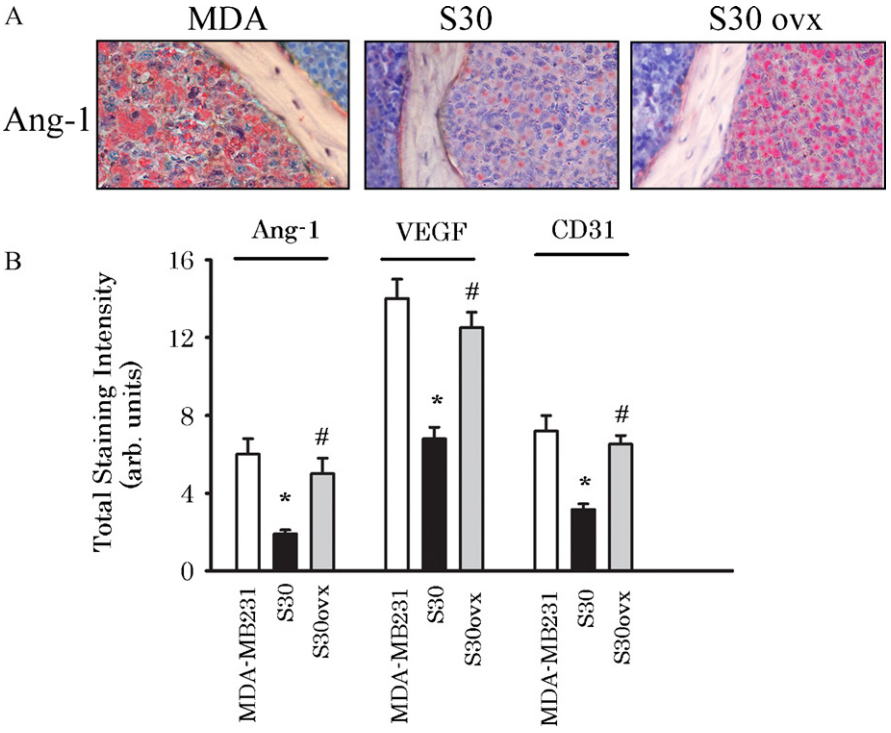

Fig. 5. Effects of E2 on *in vivo* tumor growth. Representative Ang-1 immunostaining (red color, A) and mean ± SEM of Ang-1, VEGF and CD31 staining intensity (B) in tumor samples from animals inoculated with MDA-MB-231, S-30 and S-30 cells into OVX animals. $^{*}P < 0.05$ vs. MDA-MB-231-derived tumors. $^{\#}P < 0.05$ vs. S30-derived tumors. (For interpretation of the references to color in text, the reader is referred to the web version of the article.)

Ang-1 expression by siRNA to demonstrate that Ang-1 actually promotes tumor angiogenesis and that this effect is potentiated by the presence of VEGF [33–35]. These contradictory findings can be attributed, in part, to the possibility that overexpression of Ang-1 may mask the effects of other angiogenic factors, such as VEGF, thereby shifting the angiogenic balance toward greater vessel stabilization. This usually occurs in the late stages of breast cancers. In contrast, in earlier stages, enhanced VEGF production causes strong EC proliferation and migration. As such, the positive correlation that we observed between Ang-1 and VEGF expression in the larger tumors, along with the increased vessel density evidenced by CD31 staining, suggests that Ang-1 may cooperate in a positive manner with VEGF to enhance breast cancer angiogenesis.

One major limitation of this study is worth noting, namely, that we have only assessed Ang-1 protein levels, but not those of Ang-2 and Ang-4, in cells and tissues. This is because protein levels of Ang-2 and Ang-4 were significantly lower than those of Ang-1. In addition, commercially available anti-Ang-2 and Ang-4 antibodies showed significant cross reactivity with Ang-1 protein when tested in several pilot experiments. This lack of Ang-2 and Ang-4 protein measurements limits our discussion solely to the data on mRNA expressions of these two angiopoietins. However, the relatively low levels of Ang-2 and Ang-4 proteins reinforce our conclusion that Ang-1 is the main angiopoietin under hormonal control in breast cancer.

In summary, for the first time, we have demonstrated that Ang-1 expression is inversely related to ERα status in breast cancer cells, and positively correlates with levels of VEGF and CD31 in breast cancer xenografts. These results strongly suggest that the expression of Ang-1 by breast cancer cells is hormonally regulated and that Ang-1 levels might be used as a prognosis marker, along with VEGF. Finally, our results also suggest that targeted inhibition of Ang-1 expression, combined with VEGF blockade, might be more effective in inhibiting breast cancer angiogenesis and growth than would inhibition of the VEGF pathway alone. This proposal is supported by recent studies that reveal that inhibition of VEGF signaling alone fails to enhance overall survival of breast cancer patients and that this is partly due to the upregulation of other angiogenic factors, including Ang-1 [36]. Given then, that Ang-1 appears to be an important modulator of growth and progression of ERα− breast cancers, simultaneous blockade of VEGF and Ang-1 signaling represents a new and promising therapeutic approach to the treatment of late stage breast cancer patients.

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