Overexpression of Vascular Endothelial Growth Factor by MCF-7 Breast Cancer Cells Promotes Estrogen-independent Tumor Growth in Vivo

Ping Guo, Quan Fang, Huo-Quan Tao, Christopher A. Schafer, Bruce M. Fenton, Ivan Ding, Bo Hu, and Shi-Yuan Cheng

University of Pittsburgh Cancer Institute and Department of Pathology (to S-Y. C.) or Medicine (to B. H.), Research Pavilion at Hillman Cancer Center, Pittsburgh, Pennsylvania 15213-1863, and University of Rochester Cancer Center and Department of Radiation Oncology, Rochester, New York 14642 (to B. M. F. and C. A. S.)

ABSTRACT

Alteration of the phenotype of breast cancers from estrogen-dependent to estrogen-independent growth often leads to the failure of antiestrogenic tumor therapies. We report that overexpression of vascular endothelial growth factor (VEGF) by estrogen-dependent MCF-7 breast cancer cells could abolish estrogen-dependent tumor growth in ovariectomized mice. In the absence of estrogen, MCF-7 VEGF-expressing tumors with increased vessel density showed growth kinetics similar to, or even greater than, that of parental MCF-7 tumors with estrogen supplementation. Overexpression of VEGF by MCF-7 cells or treatment on parental MCF-7 cells with recombinant VEGF also stimulated cell proliferation in culture. Our data suggest that VEGF stimulation of MCF-7 tumor angiogenesis and growth is mediated by both autocrine and paracrine mechanisms.

INTRODUCTION

Progressive growth of human breast cancers is dependent on estrogen or other estrogenic hormones (1). A considerable number of estrogen-dependent breast tumors often evolve to demonstrate more aggressive and estrogen-independent growth patterns, which are responsible for frequent failures of antiestrogenic breast cancer therapies (2). Although the molecular events of breast tumorigenesis have been illustrated in great detail, the mechanisms that enable breast cancer cells to acquire the estrogen-independent growth phenotype remains largely unknown. Studies show that estrogen promotes breast cancer progression by interacting with its nuclear receptors, thus regulating a set of genes important for breast cancer growth. Accumulating evidence suggests that acquisition of estrogen-independent breast tumor growth is accompanied by constitutively increased expression of a similar set of the genes that are up-regulated by estrogen during estrogen-dependent breast tumor progression (3).

VEGF, a major angiogenic factor involved in breast cancer progression, is one of the genes that is stimulated by estrogen (4, 5). Functional estrogen-responsive elements in the gene of VEGF have been identified through which estrogen directly regulates VEGF transcription in breast cancer cells (6–8). VEGF produced by breast carcinoma cells stimulates angiogenesis through a paracrine mechanism in tumor ECs (9) and promotes cell growth by an autocrine pathway in tumor cells (10–12). In humans, VEGF exists as six alternatively spliced isoforms, of which VEGF121 and VEGF165 are the predominant isoforms (9). VEGF exerts its cellular functions by interacting with VEGF receptors, VEGFR-1, VEGFR-2, as well as a VEGF165 receptor, NRP-1. In addition to ECs, VEGF receptors are also found in breast cancer cells (10, 13). Recent studies have shown that both estrogen and VEGF regulate a similar subset of genes in promoting breast cancer progression (5). Therefore, we hypothesized that overexpression of VEGF by estrogen-dependent breast cancer cells could produce an effect on breast cancer progression (acquisition of estrogen-independent growth) similar to the growth stimulation induced by estrogen.

In this report, we show that overexpression of VEGF isoforms, VEGF121 or VEGF165, by estrogen-dependent MCF-7 breast cells stimulated breast tumor formation in an estrogen-independent fashion in ovariectomized mice, in the absence of E2 treatment. In addition, VEGF strongly stimulated neovascularization in MCF-7 tumors formed either in E2-treated or non-E2-treated mice, as well as enhanced estrogen-dependent tumor growth in E2-treated mice. Our findings suggest that up-regulation of VEGF in estrogen-dependent breast cancers contributes to the acquisition of estrogen-independent breast cancer growth by stimulating tumor angiogenesis and progression through both autocrine and paracrine mechanisms.

MATERIALS AND METHODS

Cell Lines and Reagents. MCF-7 cells were obtained from American Type Culture Collection (Rockville, MD). MCF-7 cells were cultured using DMEM (Invitrogen/Life Technologies, Inc., Grand Island, NY) supplemented with 10% FBS (HyClone, Salt Lake City, UT), 10 μg/ml of insulin (Sigma, St. Louis, MO), and 1% penicillin-streptomycin. All of the other chemicals and reagents were from Sigma, Fisher Scientific (Hanover Park, IL), or Invitrogen (Carlsbad, CA).

Generation of MCF-7 Cell Lines That Stably Express VEGF or LacZ Proteins. Transfected MCF-7 cell clones that stably express VEGF or LacZ were generated by transfecting MCF-7 cells with cDNA inserts of VEGF121, VEGF165, or LacZ in a pCEP4 vector. Forty-eight h after the transfection, the cells were harvested and seeded as 500 cells/10-cm culture dish with DMEM containing 10% FBS, 10 μg/ml insulin, and hygromycin. Hygromycin-resistant clones that overexpressed VEGF121 or VEGF165 were expanded and characterized with Western Blotting analysis followed by VEGF ELISA as described previously (14). To obtain MCF-7 LacZ-overexpressing cell clones, a pool of LacZ-transfected MCF-7 cells was harvested and sorted using a fluorescence-activated cell sorter into 96-well flat-bottomed plates with 200 μl DMEM supplemented with 15% FBS. The clones that expressed exogenous β-galactosidase were expanded and identified by LacZ staining (14).

RNA Isolation and RT-PCR Analyses. Total RNA was isolated as described previously (14). Two μg of total RNA from each cell line was used for reverse transcription reactions with or without reverse transcriptase. Synthesized first-strand cDNAs were used for PCR reactions. The conditions for PCR reactions were 95°C, 3 min; 35 cycles of 95°C, 30 s; 61°C (VEGFR-1) or 51°C (VEGFR-2), 30 s; and 72°C, 1 min. An additional 10 min at 72°C was followed after the 35 cycles. The PCR products were analyzed on a 4% agarose gel. Primers for VEGFR-1 were 5’GCACCTTGGTTGGCCTGAC3’ (forward primer) and 5’GGTTGCGCGAGGATTGTTG3’ (reverse primer). Primers for VEGFR-2 were 5’TATGCTATGTGTTCCAGATA3’ (forward primer) and 5’AAGTTTCTTATGCTGATGCTT3’ (reverse primer). The

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1 The abbreviations used are: VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; NRP, neuropilin; FGF, fibroblast growth factor; ECM, extracellular matrix; EC, endothelial cell; CM, conditioned medium; IHC, immunohistochemistry; RT-PCR, reverse transcriptase-PCR; E2, 17β-estradiol; VN, vitronectin; FN, fibronectin; FBS, fetal bovine serum; BrdUrd, bromodeoxyuridine.
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**Tumorigenicity and Tissue Processing.** Human MCF-7 breast tumors were established in the mammary fat pads of ovariectomized female nude mice as described previously (15). Briefly, 1 × 10^7 of various types of MCF-7 cells were inoculated into the mammary fat pads of 7–8-week-old ovariectomized female nude mice that were or were not implanted with E2 60-day slow release pellets (Innovative Research of America, Sarasota, FL). The volumes of the tumors were measured using a caliper every fifth day. At the indicated times, the mice were sacrificed, and the tumors were removed and processed (16).

**IHC Analyses of the MCF-7 Cell-derived Tumors.** IHC analyses were performed on 5-μm cryostat tissue sections of various types of MCF-7 tumors as described previously (16). The following reagents were used for this study: rat anti-tumor CD31 antibody, its isotype control IgG2a,1 (1:1000 dilution for each antibody; BD-PharMingen, San Diego, CA), goat anti-human VN antibody (C-20; 1:200), goat anti-human FN antibody (C-20; 1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse monoclonal anti-VEGFR-1 antibody (P3H8A9; 1:100), rabbit polyclonal anti-VEGFR-2 antibody (T014; 1:200), and rabbit polyclonal anti-NRP-1 antibody, NP1EC1D1A (1:1000; Ref. 16). The secondary and tertiary antibodies were from Vector Laboratories (Burlingame, CA) or Jackson ImmunoResearch laboratories (West Grove, PA). A 3,3′-diaminobenzidine elite kit was from Dako Co. (Carpinteria, CA). Aqua block was from East Coast Biologies, Inc. (North Berwick, ME).

**Cell Proliferation Assay.** Evaluation of MCF-7 cell proliferation was performed using the Biorad cell proliferation ELISA system (Amersham Pharmacia Biotech, Piscataway, NJ). Various types of MCF-7 cells were seeded in 96-well plates at 60% confluence and maintained at 37°C in phenol red-free DMEM containing 10% charcoal-treated FBS. Twenty-four h later, the medium was changed to serum-free/phenol red-free DMEM with or without addition of stimuli for 48 h. To evaluate the cell-proliferative index of VEGF-overexpressing MCF-7 cells, nothing was added into the medium. To determine the effect of exogenous VEGF on parental MCF-7 cells or whether E2 potentiates VEGF-stimulated cell proliferation of parental MCF-7 cells, 100 ng/ml of recombinant VEGF213, VEGF165 proteins (R&D Systems, Minneapolis, MN) was included in the presence or absence of 1.0 nM of E2 (18). Then, BrdUrd was added into the wells for an additional 2 h. The cells were fixed and incubated with a peroxidase-conjugated anti-BrdUrd antibody according to the manufacturer’s instructions. The developed color was measured at 450 nm in a microtiter plate spectrophotometer. To assess the effects of ECM components on enhancing the VEGF-stimulated BrdUrd incorporation, 96-well plates were precoated with VN (400 ng/ml), FN (5.0 μg/ml), or Matrigel (1.0 μg/ml; Becton Dickinson Biosciences, Bedford, MA). The plates were kept at 4°C overnight. The coating solution was then aspirated, and the coated plates were allowed to dry. In blocking experiments, a neutralizing anti-VEGF antibody (10 μg/ml; R&D Systems) was included in the assays.

**RESULTS**

**Stable Expression of VEGF213, VEGF165 or LacZ Proteins in MCF-7 Cells.** MCF-7 cells were stably transfected with pCEP4 vectors that have a cDNA insert of VEGF213, VEGF165, or LacZ. Hygromycin-resistant clones were characterized either by Western blotting using an anti-VEGF antibody or by fluorescence sorting with an anti-VEGF antibody (10). Seventeen MCF-7 cell clones that express VEGF213 (referred to as V121), 19 MCF-7 cell clones that express VEGF165 (referred to as V165), and 6 MCF-7 cell clones that express LacZ (referred to as LacZ) were identified. Each clone of V121 or V165 cells expressed exogenous VEGF proteins at high levels (only 4 representative clones of each type are shown in Fig. 1A), whereas no VEGF was detected in either MCF-7 or LacZ cells by VEGF Western blotting. Similarly, the amounts of VEGF secreted by V121 or V165 clones into CM ranged from 288 to 421 ng/ml/10^6 cells after 48-hs of cell culture (Fig. 1B). In contrast, parental MCF-7 or LacZ cells only secreted 3–6 ng/ml/10^6 cells into the CM. Among the VEGF-expressing cell clones, clones 10 and 53 of V121 cells, and clones 35 and 37 of V165 cells were chosen for subsequent studies.

**Expression of VEGF213 or VEGF165 by MCF-7 Cells Enhanced E2-dependent Breast Tumor Growth.** To determine whether expression of VEGF213 or VEGF165 by MCF-7 cells would enhance MCF-7 breast tumor growth in vivo, MCF-7, LacZ, V121–10, V121–53, V165–35, or V165–37 cells were implanted orthotopically in ovariectomized nude mice with E2-supplementation (Fig. 2, A and B). At 45 days after implantation, 53% or 40% of E2-treated mice that received MCF-7 or LacZ cells developed tumors, with volumes of 307 ± 42.4 mm^3 (n = 6; MCF-7 tumors) or 242 ± 50.6 mm^3 (n = 6; LacZ tumors), respectively (Table 1). There were no significant differences in tumor volume or tumor formation between these two groups (P = 0.98). In contrast, with E2 treatment, expression of VEGF213 or VEGF165 not only increased the frequency of MCF-7 tumor formation to 90% (V121 tumors) or 86% (V165 tumors), but also dramatically enhanced tumor growth (1263 ± 214.3 mm^3 for V121 tumors; n = 8; P < 0.001 and 1638 ± 189.5 mm^3 for V165 tumors; n = 8; P < 0.001). In the presence of E2, VEGF165 seemed to have a stronger effect on promoting MCF-7 tumor growth than VEGF213 did (Fig. 2; Table 1; P < 0.05). Thus, with E2 treatment, expression of either VEGF213 or VEGF165 by MCF-7 cells facilitated MCF-7 cancer tumorigenesis.

![Fig. 1. Overexpression of VEGF213 or VEGF165 by MCF-7 breast cancer cells. A. Western blot analysis. Thirty μg of total protein from the lysates of the various types of MCF-7 cells was analyzed by immunoblotting. VEGF213 or VEGF165 ran at 18 kDa or 22 kDa with nonglycosylated (bottom band) and glycosylated forms (top band). B. VEGF ELISA analysis. The CM was collected from various types of MCF-7 cells after 48 h of cell culture and analyzed with a VEGF ELISA kit. Each bar represents the mean of three triplicates; bars, ±SE. Both experiments were performed at least two additional times with similar results.](image-url)
Expression of VEGF_{121} or VEGF_{165} in MCF-7 Cells Rendered E_{2}-independent Breast Tumor Growth. In parallel experiments, MCF-7, LacZ, V121-10, V121-53, V165-35, and V165-37 cells were inoculated separately into the mammary fat pads of ovariectomized mice without E_{2}-supplementation (Fig. 2, A and B; Table 1). Without E_{2}-treatment, neither MCF-7 nor LacZ cells formed tumors in ovariectomized mice (n = 6 in each group). In sharp contrast, 81% of the mice that received V121-10 or V121-53 cells developed tumors at 45 days after inoculation, with a volume of 830.6 ± 261 mm^3 (n = 8; P < 0.002). Also, 90% of the mice that received V165-35 or V165-37 cells formed tumors, with a volume of 391.4 ± 113.6 mm^3 (n = 10; P < 0.0001). The tumorigenicity experiments (either with or without E_{2} treatment) were independently performed a total of six times at two different institutions (the laboratory of S.Y.C., and the laboratories of I.D. and B.M.F.), with similar results. Therefore, expression of VEGF_{121} or VEGF_{165} by MCF-7 breast cancer cells rendered E_{2}-independent MCF-7 tumor formation in ovariectomized animals.

VEGF_{121} or VEGF_{165} Enhanced Neovascularization in MCF-7 Breast Tumors in Either E_{2}-treated or Non-E_{2}-treated Mice. VEGF is a potent stimulator of breast tumor angiogenesis (9). Therefore, we examined whether enhanced tumorigenicity by expression of VEGF_{121} or VEGF_{165} in MCF-7 cells elicited angiogenesis in the various types of MCF-7 tumors. The fractional area of blood vessels for E_{2}-dependent tumors derived from MCF-7 cells was similar to that of LacZ tumors. (Fig. 3A, panels a and b, and Fig. 3B; P = 0.99). In comparison with the MCF-7 tumors in mice with E_{2} treatment, the vascular fractional areas in V121 or V165 tumors with E_{2} treatment increased by 3.29-fold or 3.45-fold compared with controls (Fig. 3A, compare panels c and d with a and b; Fig. 3B; P < 0.001). To a similar extent, the vascular fractional area of V121 or V165 tumors in mice without E_{2} treatment increased by 3.25-fold and 3.77-fold, respectively (Fig. 3A, compare panels e and f with a and b; Fig. 3B; P < 0.001). Thus, overexpression of VEGF_{121} or VEGF_{165} by the MCF-7 tumors stimulated neovascularization in both E_{2}-treated and non-E_{2}-treated mice.

VEGF Receptors Were Expressed in MCF-7 Breast Tumor Cells as well as in ECs. VEGF exerts its biological functions through interaction with its functional receptors, VEGFR-1 (Flt-1), VEGFR-2 (Flk-1/KDR), as well as a VEGF165 receptor, NRP-1 (9). As shown in Figs. 2 and 3, and Table 1, expression of VEGF_{121} or VEGF_{165} enhanced MCF-7 tumor growth and angiogenesis. However, the effect of VEGF-stimulated angiogenesis alone was unlikely to be potent enough to enable E_{2}-dependent MCF-7 tumor cells to establish E_{2}-independent tumors in non-E_{2}-treated mice. We hypothesized that the VEGF produced by V121 or V165 tumor cells might have autocrine effects on MCF-7 tumor cells themselves. These autocrine effects might be similar to the effect of VEGF on cell proliferation of E_{2}-dependent T47D breast tumor cells (10) or of E_{2}-independent MDA-MB-231 breast tumor cells (11, 12). Therefore, we examined the expression of VEGFRs in MCF-7 cells and various MCF-7 tumors. In cultured cells, the expressions of VEGFR-1, VEGFR-2, and NRP-1 (data not shown) were detected in MCF-7 cells by RT-PCR analyses, although the expression levels of VEGFR-2 was low (Fig. 4A). There were no significant differences in expression levels of VEGFR-1, VEGFR-2, or NRP-1 among MCF-7, LacZ, V121, and

inoculated with MCF-7 or LacZ cells, photographs were not taken for those mice. B, the growth kinetics of the various MCF-7 tumors. Tumor volumes were measured at the indicated times after implantation. Data are shown as the mean (bars, ± SE). Two separate clones from each type of LacZ, V121, or V165 cells were individually inoculated. The experiments included 6–10 mice in each group and were performed three separate times each at two different institutions with similar results.

Fig. 2. Overexpression of VEGF_{121} or VEGF_{165} by MCF-7 breast cancers promoted tumorigenicity in both E_{2}-treated and non-E_{2}-treated mice. One × 10^5 of the MCF-7, LacZ, V121, or V165 cells were inoculated into the mammary fat pads of mice implanted with or without 60 μ-releasing 17-β estradiol pellets. A, photographs of individual mice inoculated with various types of MCF-7 cells. Breast tumors formed by MCF-7 (a), Lac Z (b), V121 (c and e), or V165 (d and f) cells. Panels a–d, tumors established in E_{2}-treated mice. Panels e and f, tumors formed in non-E_{2}-treated mice. Arrows indicate the established MCF-7 tumors. Because no tumors were formed in the non-E_{2}-treated mice.
VEGF stimulates parental MCF-7 cell growth and whether E2 potentiates VEGF-promoted proliferation of parental MCF-7 cells in vitro, we assessed the effects of exogenous VEGF on parental MCF-7 cell proliferation in the presence or absence of E2. As shown in Fig. 5C, treatment on the parental MCF-7 cells with 100 ng/ml of recombinant VEGF121 or VEGF165 proteins caused a moderate increase of BrdUrd incorporation of the treated MCF-7 cells as compared with untreated cells (128% ± 6.1% and 150% ± 18.3%, respectively). Moreover, when the cells were treated with 1.0 nM of E2 (18) in the absence of exogenous VEGF, an increase of 230% ± 13.8% in cell growth was observed in the parental MCF-7 cells. When both VEGF and E2 are present, additional increases of the BrdUrd incorporation were seen (Fig. 5C; 269% ± 7.5% for the treatment of VEGF121 plus E2 or 275% ± 15.2% for the treatment of VEGF165 plus E2). The stimulation of cell proliferation in the presence or absence of E2 in these two sets of experiments was likely caused by the exogenous VEGF, because the neutralizing anti-VEGF antibody (10 μg/ml) inhibited the BrdUrd incorporation in both cases. Similar to the effects demonstrated in Fig. 5B, treatment with VN (Fig. 5D), FN, or Matrigel (data not shown) on the parental MCF-7 cells that were stimulated by the exogenous VEGF proteins in the absence or presence of E2 additionally potentiated the enhancements of cell proliferation. In the absence of E2, the rates of cell growth were increased from 128% ± 6.1% to 150% ± 17.6% for VEGF121-treated cells and from 150% ± 18.3% to 178% ± 10.1% for VEGF165-treated cells. In the presence of E2, the rates of BrdUrd incorporation were elevated from 269% ± 17.5% to 290% ± 21.6% for VEGF121-treated cells and from 275% ± 15.2% to 305% ± 30.2% for VEGF165-treated cells. However, there were no differences between the rates of cell growth of the E2-stimulated MCF-7 cells in the absence (230% ± 13.8%) or presence (228% ± 14.2%) of VN (Fig. 5D).

Finally, we determined whether VN or FN was expressed in the various types of MCF-7 tumors. High levels of expression of VN (Fig. 5E) or FN (data not shown) were detected in all of the types of MCF-7 tumors that were established in either E2-treated or non-E2-treated mice. Taken together, these data suggest that in the absence of E2, VEGF stimulates mitogenesis of MCF-7 tumor cells by an autocrine mechanism, and ECM components promote VEGF-stimulated MCF-7 tumor cell proliferation. Furthermore, treatment on the parental MCF-7 cells with E2 additionally potentiates the VEGF-promoted cell proliferation, and ECM components also augment VEGF-stimulated cell growth under this condition.

DISCUSSION

During the past 3 decades, several model systems have been established for studying the process whereby initially estrogen-dependent breast cancer cells acquire estrogen resistance or estrogen-independent growth. Overexpression of FGF-1 (15), FGF-4 (19), or e-Jun (20) by MCF-7 cells enabled E2-independent MCF-7 tumor growth, and stimulated tumor invasion and metastases in ovariectomized mice. However, in vivo, estrogen treatments of mice had no effects on tumor growth (FGF-1 or e-Jun expressing MCF-7 cells; Refs. 15, 20) and suppressed tumor formation (FGF-4 expressing MCF-7 cells; Ref. 19). On the other hand, E2-independent, but E2-responsive MCF-7 sublines were also isolated by selecting adriamycin-resistant cell clones (21) or from established MCF-7 tumors in ovariectomized mice (3). Although these two MCF-7 cell sublines formed E2-independent breast tumors in mice, little was known about the mechanisms that conferred E2 independence in these two model systems. In the present study, we examined the consequences of overexpression of VEGF by MCF-7 tumors in ovariectomized nude mice with or without E2 treatment. Expression of VEGF121 or VEGF165 by MCF-7 cells rendered E2-independent tumor formation. The growth rates of V121
or V165 tumors were similar to those of tumors derived from estrogen-independent MCF-7 sublines (3) or of the parental, estrogen-dependent MCF-7 tumors in E2-treated mice (Fig. 2; Table 1). The V121 and V165 tumors remained responsive to estrogen stimulation in vivo, which was also consistent with the response of breast tumors derived from the estrogen-independent MCF-7 sublines (3). In addition, VEGF stimulation (either by overexpression of VEGF in MCF-7 tumor cells or by treatments on parental MCF-7 cells with exogenous VEGF proteins) increased mitogenesis of MCF-7 cells in vitro. The enhancements of MCF-7 cell proliferation by VEGF were additionally potentiated by E2 as well as by ECM components. Thus, the estrogen-independent but estrogen-responsive phenotype may exemplify a natural progression of development of estrogen-independent growth in breast cancers.

VEGF is a major angiogenic factor in breast tumor progression. This factor is expressed at high levels in breast cancer specimens compared with normal breast tissue, and suppression of VEGF function inhibits breast tumor formation (22). Our results show that expression of VEGF isoforms, VEGF121 or VEGF165, by MCF-7 cells at high levels stimulated both E2-independent and E2-dependent breast tumor growth in mice. Moreover, we also obtained several V121 or V165 cell clones that secreted VEGF at lower levels. These low VEGF-expressing cell clones did not show enhancement of tumor growth in E2-treated mice nor formed tumors in non-E2-treated mice (data not shown). This observation is in agreement with two separate studies reported previously (23, 24). With lower expression levels of the VEGF isoforms by MCF-7 cells, moderate augmentation on

Fig. 3. VEGF121 or VEGF165 stimulated angiogenesis in MCF-7 breast cancers in both E2-treated and non-E2-treated mice. A, IHC stains of tumor vessels with a monoclonal anti-CD31 antibody. Breast tumors formed by MCF-7 (a), Lac Z (b), V121 (c and e), or V165 (d and f) cells. Panels a–d, tumors established in E2-treated mice. Panels e and f, tumors developed in non-E2-treated mice. Because no tumors were formed in the mice that received MCF-7 or LacZ cells without E2 supplementation, no analysis was done. Arrows indicate blood vessels. Four to 8 individual tumor samples of each class from each in vivo experiment were analyzed, and the experiments were repeated at least two additional times with similar results. Original magnification: ×200. B, quantitative analysis of the increased fractional vascular area in VEGF-expressing MCF-7 tumors (17). Representative IHC stains from the various MCF-7 tumors are shown in A. Data are means; bars, ±SD. Numbers above each bar indicate the numbers of mice analyzed in each group. Numbers in the parentheses under the X-axis are the differences (in folds) of the VEGF expressing tumors in comparison with parental MCF-7 tumors.
E2-dependent breast tumor growth in ovariectomized mice was seen (23). In another study, although MCF-7 VEGF165-expressing tumors responded vigorously to estrogen stimulation in promoting tumorigenesis, the V165 cells could only form E2-independent tumors in ovariectomized mice when implanted with Matrigel (24). Thus, threshold levels of VEGF expression in breast cancer cells may be critical for the acquisition of the estrogen-independent phenotype in human breast cancers. This hypothesis is clinically relevant because high levels of VEGF proteins could be detected in primary breast cancer specimens, especially in hypoxic regions (22).

It has been established that estrogen stimulates the expression of VEGF in breast cancers. In vivo, E2 treatment augments VEGF expression in E2-induced rat mammary cancer (25). In vitro, E2 directly regulates VEGF transcription by acting on the estrogen-responsive elements in the VEGF gene in breast cancer cells (6–8). On the other hand, E2 and VEGF appear to regulate several common genes that are critical for breast cancer progression. Both E2 and VEGF up-regulate the expression of cyclin D1, nuclear factor κB, and Bcl-2 in breast cancer cells (E2) and ECs (VEGF; Refs. 26–31).

Furthermore, earlier studies have shown that factors induced by E2 in E2-dependent MCF-7 cells could partially replace E2 to promote breast tumor growth (32). Our data corroborate these observations. Both in vivo and in vitro, VEGF promoted E2-independent MCF-7 tumor cell growth, and treatment of E2 additionally potentiated the VEGF-stimulated MCF-7 cell proliferation. The enhancement of MCF-7 cell growth by E2 was at least partially through the up-regulation of VEGF. We observed that E2 slightly increased VEGF secretion from the MCF-7 cells (data not shown; Ref. 8), and the neutralizing anti-VEGF antibody partially inhibited E2-stimulated cell proliferation in the absence of exogenous VEGF (Fig. 5, C and D).

The effects on the VEGF-stimulated MCF-7 cell proliferation potentiated by E2 in vitro was not as strong as that in mice probably because in vivo, in addition to stimulation of MCF-7 tumor cells, VEGF expressed at high levels strongly promoted vessel growth in tumors. Increased neovascularization (Fig. 3) in VEGF-expressing MCF-7 tumors also stimulated E2-promoted tumor growth. Together, our results demonstrate that during the progression of E2-dependent breast cancers, up-regulation or constitutive expression of VEGF is critical for the acquisition of estrogen independence.

### Figure 4

**A** RT-PCR analysis on the expression of VEGFRs in human dermal ECs, MCF-7 cells, and human U87MG glioma cells with (+) or without (−) reverse transcriptase. The length of the PCR product of VEGF-1 or VEGF-2 was 441 bp or 473 bp, respectively. The arrow indicates a weak VEGFR-2 cDNA fragment detected in MCF-7 cells. N, negative control. The experiments were done three times with identical results. **B** Expression of VEGFR-1 in various MCF-7 tumors. Panels a–h, IHC analysis of various types of established MCF-7 tumors using a mouse monoclonal anti-VEGFR-1 antibody. Breast tumors formed by MCF-7 (a), Lac Z (b), V121 (c and e), and V165 (d and f) cells. Panels a–d, tumors established in E2-treated mice. Panels e and f, tumors formed in non-E2-treated mice. Arrows indicate blood vessels that were positively stained by the anti-VEGFR-1 antibody. Arrows show tumor cells that expressed VEGFR-1. Six to 8 individual tumor samples of each group from each in vivo experiment were analyzed each time and the experiments were repeated at least two additional times with similar staining patterns. Original magnification: ×400.

**Fig. 4.** VEGFR-1 is expressed in MCF-7 breast cancer cells in vitro and in vivo. A, RT-PCR analysis on the expression of VEGFRs in human dermal ECs, MCF-7 cells, and human U87MG glioma cells with (+) or without (−) reverse transcriptase. The length of the PCR product of VEGF-1 or VEGF-2 was 441 bp or 473 bp, respectively. The arrow indicates a weak VEGFR-2 cDNA fragment detected in MCF-7 cells. N, negative control. The experiments were done three times with identical results. B, expression of VEGFR-1 in various MCF-7 tumors. Panels a–h, IHC analysis of various types of established MCF-7 tumors using a mouse monoclonal anti-VEGFR-1 antibody. Breast tumors formed by MCF-7 (a), Lac Z (b), V121 (c and e), and V165 (d and f) cells. Panels a–d, tumors established in E2-treated mice. Panels e and f, tumors formed in non-E2-treated mice. Arrows indicate blood vessels that were positively stained by the anti-VEGFR-1 antibody. Arrows show tumor cells that expressed VEGFR-1. Six to 8 individual tumor samples of each group from each in vivo experiment were analyzed each time and the experiments were repeated at least two additional times with similar staining patterns. Original magnification: ×400.
Fig. 5. E2 and VN potentiate MCF-7 cell proliferation stimulated by VEGF. **A** and **B**, cell proliferation assay of MCF-7, LacZ, V121, or V165 cells using a BrdUrd cell proliferation ELISA system. In some samples, a neutralizing anti-VEGF antibody was included. **A**, mitogenic activities of the various MCF-7 cells in noncoated 96-well plates. **B**, mitogenic activities of the various MCF-7 cells in 96-well plates coated with 400 ng/ml of VN. The increased proliferation was calculated as the percentage of that of parental MCF-7 cells. **C**, mitogenic activities of the parental MCF-7 cells that were stimulated with recombinant VEGF121 or VEGF165 (100 ng/ml each) in the absence or presence of 1.0 μM of E2 in noncoated 96-well plates. **D**, mitogenic activities of the parental MCF-7 cells that were stimulated with recombinant VEGF121 or VEGF165 (100 ng/ml each) in the absence or presence of 1 μM of E2 in 96-well plates coated with 400 ng/ml of VN. The increased proliferation was calculated as the percentage of that of parental MCF-7 cells. * denotes statistically significant (P < 0.002). The assays were performed three times using different cell clones of various cell passages with similar results. **E**, expression of VN in various types of established MCF-7 breast tumors. Breast tumors formed by MCF-7 (**a**), LacZ (**b**), V121 (**c** and **e**), and V165 (**d** and **f**) cells. **Panels a–d**, tumors established in E2-treated mice. **Panels e** and **f**, tumors formed in non-E2-treated mice. **Arrows** indicate tumor cells that were positively stained by the anti-VN antibody. Six to 8 tumor samples of each group of each in vivo experiment were analyzed, and the experiments were repeated at least two additional times with similar results. Original magnification: ×400.
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to the treatment of human breast cancers.

In addition to stimulating breast tumor angiogenesis by a paracrine mechanism, VEGF also promotes breast cancer cell growth, survival, and invasion by an autocrine pathway. Studies have shown that a VEGF165 receptor, NRP-1, mediates both VEGF-stimulated survival and invasion for E2-independent breast cancer cells (10–12). In E2-dependent T47D breast cancer cells, VEGFR-1 and VEGFR-2 are responsible for VEGF-stimulated mitogenic and migratory responses (10). In our studies, MCF-7 cells express VEGFR-1 and NRP-1 at high levels, both in vitro and in vivo, whereas the expression of VEGFR-2 was only detected in tumor E2Cs, but not in the carcinoma cells of the MCF-7 tumors. Because both VEGF121 and VEGF165 stimulated E2-dependent and E2-independent MCF-7 breast tumor growth, and VEGF121 does not bind to NRP-1 (13), we propose that augmentation of E2-dependent MCF-7 tumor growth and acquisition of E2-independence are likely to be mediated by VEGFR-1, at least in V121 tumors.

In summary, we have shown that overexpression of VEGF by human MCF-7 breast cancer cells not only enhances E2-dependent tumor growth, but also enables E2-independent tumor formation in vivo. The stimulation by VEGF of MCF-7 tumor growth is through both a paracrine effect on tumor angiogenesis and an autocrine effect on tumor cell proliferation. Our data suggest that if VEGF is expressed at high levels in breast cancer cells, it could partially replace E2 stimulation of breast cancer growth. Our results of VEGF-stimulated E2-dependent or E2-independent breast cancer growth provides an excellent system to investigate the mechanisms of acquisition of estrogen-independent growth by estrogen-dependent breast cancers. Our findings also indicate the need for targeting the VEGF/VEGFR pathway and other signaling pathways as well as estrogen-ablution in the treatment of human breast cancers.

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Ping Guo, Quan Fang, Huo-Quan Tao, et al.


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