Interaction with Endothelial Cells Is a Prerequisite for Branching Ductal-Alveolar Morphogenesis and Hyperplasia of Preneoplastic Human Breast Epithelial Cells: Regulation by Estrogen

Malathy P. V. Shekhar, Jill Werdell, and Larry Tait

Breast Cancer Program, Karmanos Cancer Institute, [M. P. V. S., J. W., L. T.] and Department of Pathology, Wayne State University School of Medicine, Detroit, Michigan 48201 [M. P. V. S., L. T.]

ABSTRACT

Although there is experimental evidence supporting the involvement of angiogenesis in the pathogenesis of breast cancer, the exact nature and effects of interaction between human breast epithelial cells (HBECs) and endothelial cells (ECs) have not been described thus far. This approach requires an assay system that permits growth and differentiation of both epithelial and endothelial cells. Here, we report the development of a three-dimensional in vitro culture system that supports growth and functional differentiation of preneoplastic HBECs and ECs and recapitulates estrogen-induced in vivo effects on angiogenesis and the proliferative potential of MCF10AT xenografts. MCF10A and MCF10AT-EIII8 (referred to as EIII8) cell lines used in this study are normal or produce preneoplastic lesions, respectively. When MCF10A or EIII8 cells are seeded on reconstituted basement membrane (Matrigel), both lines organize into a three-dimensional tubular network of cells; however, tubes produced by EIII8 cells appear multicellular in contrast to unicellular structures formed by MCF10A cells. However, when MCF10A or EIII8 cells are cocultured with human umbilical vein endothelial cells (HUVECs) on Matrigel, rather than interacting with extracellular matrix, the ECs exhibit preferential adherence to epithelial cells. Although both MCF10A and EIII8 cells provide preferential substrate for EC attachment, only EIII8 cells facilitate sustained proliferation of ECs for prolonged periods that are visualized as endothelial cell enriched spots, which express factor VIII-related antigen. At regions of endothelial-enriched spots, preneoplastic HBECs undergo branching ductal-alveolar morphogenesis that produce mucin, express cytokeratins, and proliferating cell nuclear antigen. The presence of actively proliferating and functional endothelial cells is essential for ductal-alveolar morphogenesis of preneoplastic HBECs because without ECs, the epithelial cells formed only tubular structures. This ability to establish functional ECs and ductal-alveolar morphogenesis is facilitated only by preneoplastic HBECs because normal MCF10A cells fail to elicit similar effects. Thus, a cause-effect relationship that is mutually beneficial exists between EC and preneoplastic HBECs that is critical for generation of functional vascular networks and local proliferative ductal alveolar outgrowths with invasive potential. Both these processes are augmented by estrogen, whereas antiestrogens inhibit these processes. Induction and maintenance of angiogenic phenotype is associated with up-regulation in expression of interleukin 8 and matrix metalloproteinase-2 and estrogen-induced increases in vascular endothelial growth factor and vascular endothelial growth factor receptor 2. This three-dimensional culture model offers a unique opportunity to study endothelial- and epithelial cell-specific factors that are important for ductal-alveolar morphogenesis, angiogenesis, and progression to malignant phenotype.

INTRODUCTION

Growth and formation of capillary blood vessels or neovascularization is an essential component of solid tumor growth (1, 2). Every increase in the tumor cell population must be preceded by an increase in new capillaries that converge upon the tumor, and this angiogenesis has been directly correlated with tumor growth and metastasis (3). Products derived from both tumor cells and a variety of nonneoplastic mediator systems have been implicated in this vasoproliferative response (2, 4). Several growth factors, cytokines, and extracellular matrix molecules have been reported to induce or regulate endothelial cell growth or migration in vitro (5). These include several well-characterized polypeptide growth factors, proteolytic enzymes, IFN, cyclic nucleotides, prostaglandins, heparin, lowered oxygen tension, histamine and other vasoactive amines, and several low molecular weight endothelial mitogens and chemotactic factors (5).

VEGF (6) or vascular PF (Ref. 7; VEGF/PF) is an endothelial-specific mitogen that mediates developmental, physiological, and pathological neovascularization (8). VEGF has been reported to act as a survival factor, preventing the apoptotic death of microvascular endothelial cells (9, 10). The human VEGF gene encodes a dimeric glycoprotein comprising four possible monomers as a result of differential splicing of eight exons that make up the gene product. The four VEGF subtypes are 121, 165, 189, and 206 amino acids in length (11). The smaller forms are secreted, whereas VEGF(165) and VEGF(206) are retained close to the membrane of producing cells bound to heparan proteoglycans. Receptors for VEGF, VEGFR-1 (Flt-1), and VEGFR-2 (Flk-1/KDR) bind VEGF, whereas VEGFR-3 (Flt-4) appears to be specific for VEGF-C (11). Expression of Flk-1/KDR is confined to endothelial cells, accounting for the selective nature of VEGF-induced mitogenesis (11). VEGF is expressed at high levels in a wide range of tumors and tumor cell lines (12) and is believed to be a key mediator of tumor angiogenesis (13–15) and the high blood vessel permeability characteristic of tumors (16, 17). Expression of VEGF in the uterus has been shown to be rapidly and strongly stimulated by estrogen (18), suggesting that VEGF mediates the normal, estrogen-induced increase in vascular permeability and blood vessel growth in the uterus. Similarly, expression of VEGF is rapidly induced by E2 in DMBA-induced estrogen-dependent mammary tumors (19).

Using the MCF10AT1 xenograft model for human proliferative breast disease, we have demonstrated previously that E2 exerts a growth-promoting effect on benign or premalignant ductal epithelium by enhancing the speed of transformation from simple/mild hyperplasia (grades 0/1) to atypical hyperplasia (grade 3) and ductal carcinoma...
The ECM acts locally to modulate the responsiveness of endothelial and mammary epithelial cells to external factors. Besides providing a scaffolding during capillary morphogenesis, the ECM, by virtue of its ability to mediate both biochemical and biomechanical signaling events, has been shown to exert complex local controls on the functions of endothelial cells (21) and growth, differentiation, and apoptosis of normal murine and human breast epithelial cells (22, 23). Collagenolytic degradation of endothelial and parenchymal basement membranes is an essential step in the process of tumor invasion and angiogenesis (24). Proteolysis and interruption of the basement membrane and ECM require the activation of specialized MMPs, the type IV collagenases or gelatinases, which degrade basement membrane collagen types IV and V (25). Two species of MMPs, the M₇ 72,000 species (MMP-2, gelatinase A) and the M₇ 92,000 species (MMP-9, gelatinase B), have been cloned and sequenced (25–27). Both MMP-2 and MMP-9 are secreted as latent proenzymes and require removal of an 80- and 87-amino acid NH₂-terminal domain, respectively, for activation (28, 29).

Using a three-dimensional basement membrane assay system, we demonstrate the existence of a direct cause-effect relationship between endothelial and preneoplastic MCF10AT-EIII8 HBECs that is integral for generation of active angiogenesis and ductal-alveolar morphogenesis, two processes that are regulated by E₂ at the molecular and cellular levels. Furthermore, we show that the abilities to undergo ductal-alveolar morphogenesis and establish an active angiogenic process are dependent on the cellular genotype of the breast epithelial cells, because untransformed parental MCF10A breast epithelial cells lack both of these abilities. Finally, our data suggest that active angiogenesis is integral for growth and proliferative potential of “ductal-alveolar structures,” which in turn may determine the malignant phenotype.

**MATERIALS AND METHODS**

**Cell Lines.** The MCF10AT system is a xenograft model of early human breast cancer progression (30). MCF10AneoT cells are T24 Ha-ras-transformed cells derived from MCF10A human breast epithelial cells (31). MCF10A cells do not form persistent lesions in immune-deficient mice, whereas MCF10AneoT cells do (30). MCF10AneoT and lines derived by alternating in vivo transplantation and in vitro culture (MCF10ATn) are collectively known as the MCF10AT system (32). The lesions formed by lines of the MCF10AT system are composed of a heterogeneous spectrum of ductular tissues with a range of morphology that includes mild to moderate hyperplasia, atypical hyperplasia, carcinoma in situ, moderately differentiated carcinoma, and undifferentiated carcinoma, as well as histologically normal ducts (30).

Thus, the MCF10AT system provides a transplantable, xenograft model of human proliferative breast disease with proven neoplastic potential. These studies used parental MCF10A cells and the following lines of the MCF10AT xenograft model: MCF10AT1 and MCF10AT1-EIII8. MCF10AT1 represents the first transplant generation of the MCF10AT xenograft model (30). MCF10AT1-EIII8 (referred to as EIII8) cells are breast preneoplastic epithelial cells that were derived from lesions of MCF10AT1 cells arising in E₂-supplemented animals (20) and respond to E₂ with increased growth in vitro and in vivo. MCF10A and MCF10AT1-derived cells were maintained in phenol red-free DMEM-F12 medium supplemented with 0.1 μg/ml cholecalciferol, 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, 0.02 μg/ml EGF, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2.5% horse serum. Charcoal-stripped serum was not used because it reduces the proliferative capacity and/or viability of MCF10A cells, possibly because of removal of essential growth factors. The only sera used routinely were those that were unable to support growth of the estrogen-dependent cell line, MCF-7, indicating absence of biologically significant levels of E₂ or other estrogenic compounds.

**Homotypic and Heterotypic Three-dimensional Basement Membrane Culture of MCF10A, EIII8, and HUVEC Cells.** For homotypic three-dimensional cultures, 10 × 10⁴ cells were seeded as a single-cell suspension in eight-well chamber slides coated with Matrigel (Collaborative Biomedical Products, Bedford, MA) in DMEM-F12-supplemented media for MCF10A cells and its derivatives or in SFM-supplemented media for HUVEC cells. For heterotypic three-dimensional cocultures, 50 × 10⁴ MCF10A, MCF10AT1, or EIII8 cells were mixed with an equal number of HUVEC cells and seeded onto chamber slides coated with Matrigel as described for homotypic cultures. Typically, heterotypic cocultures were performed in SFM supplemented with EGF and bFGF because it allows optimal viability, growth, and three dimensional organization of both HUVEC and MCF10A cells. Heterotypic cocultures and homotypic epithelial cell cultures (MCF10A, MCF10AT1, or EIII8) were routinely maintained up to 3 weeks, and morphological development was analyzed by phase contrast microscopy.

The interaction between EIII8 and HUVEC cells was determined by prelabeling them with the fluorescent cationic membrane tracers, DiI and DiO (Molecular Probes, Inc., Eugene, Oregon), respectively, prior to coculturing. These brightly fluorescent dyes diffuse laterally within the plasma membrane, resulting in uniform staining of the entire cell, and because transfer of these probes between intact membranes is negligible and cytotoxic effects are minimal, they permit long-term cell tracking in the three-dimensional cultures.

**Preparation of Conditioned Media.** EIII8 or MCF10A cells (50 × 10⁴) were seeded alone or mixed with an equivalent number of HUVEC cells on Matrigel in SFM as described above. Cells were incubated for 6 h to attach, and media were replaced with fresh SFM. After appropriate incubation, the culture media from homotypic and heterotypic cocultures were collected, centrifuged to remove debris, and stored at −20°C. After removal of culture media, matrix containing the three-dimensional structures was either solubilized for SDS-PAGE and Western blot analysis or fixed in buffered formalin for evaluation of morphology and distribution of functional markers.

**Effect of Conditioned Medium on Proliferation of HUVEC Cells.** HUVEC cells (10 × 10⁴) were plated in SFM supplemented with EGF, fibronectin, and bFGF in 24-well plates. After 8 h of plating, various volumes of unconcentrated conditioned media prepared from homotypic EIII8 or MCF10A cultures, or heterotypic MCF10A-HUVEC or EIII8-HUVEC cocultures, were added. For the incubation experiments, polyclonal antibodies to human VEGF (recognizes the COOH terminal epitope found in VEGF₁₆₅, VEGF₁₈₉, and VEGF₂₀₆; Oncogene Science, Cambridge, MA), mouse monoclonal antibody to human Flk-1/KDR (epitope not known; Chemicon International, Inc., Tamecula, CA), or polyclonal antibody to human IL-8 (which has <5% cross-reactivity with Groα, Groβ, and Groγ; R&D Systems, Minneapolis, Minnesota) was diluted in SFM and added at 10 μg/ml. Appropriate nonimmune IgG was used at 10 μg/ml. Cultures were incubated at 37°C in 5% CO₂-95% O₂ for 5 days with medium change and supplementation of conditioned media or appropriate antibodies every other day. Cells were released by trypsinization, and viable cells, as demonstrated by trypan blue exclusion, were counted in a hemocytometer. All cell counts were done from triplicate wells, and results were expressed as the mean ± SE from three independent experiments.

**Western Blot Analysis.** Analysis of expression of VEGF, IL-8, ER, and Flk-1/KDR proteins was carried out by Western analysis with the specific antibodies. Aliquots of unconcentrated conditioned media or lysates of three-dimensional cultures containing 20 or 40 μg of total protein, respectively, were collected at indicated times of culture, separated by SDS-PAGE on 12.5% (VEGF), 17% (IL-8), or 7% (ER and Flk-1/KDR) polyacrylamide gels (33), and subjected to Western blot analysis. The following antibodies to human proteins were used: rabbit polyclonal anti-VEGF antibody (recognizes the COOH terminus present in VEGF₁₆₅, VEGF₁₈₉, and VEGF₂₀₆; Oncogene...
Science), mouse monoclonal anti-ER antibody (Clone 1D5; reacts with the NH₂ terminal domain or A/B region of the receptor; Dako Corp., Carpinteria, CA), mouse monoclonal anti-Flik-1/KDR antibody (Chemicon International, Inc.), and goat polyclonal anti-IL-8 antibody (R&D Systems). Immunoreactive bands were visualized by chemiluminescence, and band intensities were quantitated with a Model 300A densitometer (Molecular Dynamics, Sunnyvale, CA).

**Effects of Estrogen on Three-dimensional Growth.** To assess growth effects of E₂ on homotypic (EIII8) or heterotypic (EIII8-HUVEC) three-dimensional cultures, 50 × 10⁴ EIII8 cells were seeded alone or mixed with an equivalent number of HUVEC cells in the appropriate medium in eight-well chamber slides coated with Matrigel as described above. Slides were incubated overnight to allow attachment of cells to surface and treated with vehicle (0.01% ethanol, v/v), pure antiestrogen ICI 182,780 alone (100 or 1000 nM; a gift from Dr. A. Wakeling, Zeneca Pharmaceuticals, Cheshire, United Kingdom), E₂ (0.1, 1, or 10 nM; Sigma Chemical Co., St. Louis, MO), or a combination of 1 nM E₂ and 100- or 1000-fold molar excess of ICI 182,780. Cultures were incubated at 37°C for 5 days, after which cell viability was measured with the MTS kit according to the manufacturer’s directions (Promega Corp., Madison, WI). Measurements were made from triplicate sets of wells for each treatment. Background absorbance was corrected by preparing triplicate sets of wells containing only Matrigel (“no cell”) and same volumes of culture medium and MTS reagent as in experimental wells. Average absorbances from “no cell” wells were subtracted from sample absorbance values containing cells to yield corrected absorbance, and the results represent the average ± SE of triplicate samples. Each experiment was repeated at least three times. Because the MTS assays performed here differ from routine MTS assays in that they are done on three-dimensional cultures growing in Matrigel rather than monolayers, results of MTS assays were validated by performing direct cell counts of viable cells in the three-dimensional cultures. For this, the medium was removed, wells were rinsed with PBS, and Matrigel was digested with dispase for 2 h at 37°C. The digested material was centrifuged at 4000 × g for 10 min, and the pellet was treated with trypsin to recover single cells from the three-dimensional tubular structures. The number of viable cells was determined by trypan blue exclusion in a hemocytometer. Results were expressed as mean ± SE from three independent experiments.

**Morphological Evaluation.** For histological evaluation, three-dimensional cocultures were fixed in buffered formalin and embedded in paraffin, and 4-μm sections were stained with H&E. For immunohistochemical evaluation of mammary epithelial and endothelial cell functional markers, sections were incubated with monoclonal antibodies directed against the following human proteins: pan-cytokeratin 5/6/8/18 is a cocktail of monoclonal antibodies designed to recognize epithelial cells and their tumors (Novocastra Laboratories, Ltd., Newcastle upon Tyne, United Kingdom); muc-1 glycoprotein is a mammary type apomucin also known as milk fat globule membrane antigen (Novocastra Laboratories, Ltd.); cd31 or PECAM-1 (Dako Corp.); factor VIII-related antigen (Dako Corp.); and rabbit polyclonal antibody against P53 (Novocastra Laboratories, Ltd.); Ras (Novocastra Laboratories, Ltd.); mouse monoclonal anti-ER antibody (Clone 1D5; reacts with the NH₂ terminal domain or A/B region of the receptor; Dako Corp., Carpinteria, CA), mouse monoclonal anti-Flik-1/KDR antibody (Chemicon International, Inc.), and goat polyclonal anti-IL-8 antibody (R&D Systems). Immunoreactive bands were visualized by chemiluminescence, and band intensities were quantitated with a Model 300A densitometer (Molecular Dynamics, Sunnyvale, CA).

**Gelatin Zymography.** The activity of gelatinolytic enzymes in conditioned media of MCF10A, EIII8, HUVEC, MCF10A-HUVEC, or EIII8-HUVEC three-dimensional cultures was detected by electrophoresis in 7.5% (w/v) polyacrylamide gels containing gelatin at a final concentration of 0.6 mg/ml. Aliquots of conditioned media containing equivalent amounts of protein were mixed with SDS-sample buffer (33) and electrophoresed under nonreducing conditions. After electrophoresis, the gel was soaked for 10 min in 2.5% Triton X-100/10 mM Tris-HCl (pH 8.0) at room temperature, rinsed, and incubated at 37°C for 16 h in 5 mM CaCl₂/50 mM Tris-HCl (pH 8.0). Gels were stained with 0.1% Coomassie Brilliant Blue R250 and destained. The purified precursor form of MMP-2 (pro-MMP-2; gift from Dr. Herman Friedman, Wayne State University, Detroit, MI) was activated with 1 mM 4-aminophenylmercuric acetate and used as a positive control for activated MMP-2 form (34).

**Statistical Analysis.** Data were analyzed with an ANOVA. Specific differences among treatments were examined using the Student’s t test. Statistical significance was determined using the Student’s t test with P < 0.05 considered as statistically significant.

**RESULTS**

**Three-Dimensional Basement Membrane Coculture of EIII8 Cells with HUVEC Cells Recapitulate the Phenotypic Characteristics of Preneoplastic Breast Tissue in Vivo: Effects of Estrogen on Three-dimensional Growth of Homotypic Cultures.** When MCF10A cells or ras-transformed MCF10A AT1 or EIII8 cells are seeded on Matrigel, within 24 h all of the lines organize into a three-dimensional tubular network of cells that are arranged in a duct-like pattern around a central space (Fig. 1, a–d). However, profound differences between the duct-like structures of EIII8 cells and MCF10A cells become evident after about 4 days in culture. Tubes produced by EIII8 cells appear multicellular in contrast to the unicellular structures formed by MCF10A cells. The lack of tubular thickening is not attributable to loss of viability of MCF10A cells because these tubular structures are stable and persist indefinitely as those formed by EIII8 cells. Rather, this difference is attributable to the difference in proliferative capacities of EIII8 and MCF10A cells on Matrigel (Fig. 1, compare a and b with c and d), although the two cell lines have similar doubling times (~19 h) on tissue culture plastic (data not shown). Because MCF10A AT1 cells exhibit growth characteristics intermediate to those of MCF10A and EIII8 cells, most of our work used EIII8 cells. A major difference between MCF10A and EIII8 cells was the growth-stimulatory effects of E₂ on EIII8 three-dimensional structures. Treatment with 1 or 10 nM E₂ resulted not only in marked thickening of tubes over those of control cultures but also in the formation of several new “central spaces” and “connecting bridges” (Fig. 2, compare b and d with a). These effects could be blocked by a 100-fold molar excess of the pure antiestrogen, ICI 182,780, indicating the specificity of E₂-induced growth effects (Fig. 2c).

In contrast to stable tubular networks formed by the breast epithelial cell lines, similar cultures of HUVECs in Matrigel resulted in formation of tubes within 24 h that remained stable only for an additional 48–72 h and disintegrated by day 5 of culture (Fig. 1, e and f).

![Fig. 1. Phase contrast morphology of cells in three-dimensional Matrigel culture. a and b, MCF10A cells at 24 h and 10 days, respectively; c and d, EIII8 cells at 24 h and 10 days, respectively; e and f, HUVEC cells at 24 h and 5 days, respectively.](cancerres.aacrjournals.org)
and EIII8 cells provided “soil” for endothelial cell attachment, only EIII8 cells sustained active proliferation of endothelial cells for >3 weeks. This is evident from the remarkable difference in sizes and capillary outgrowths of “endothelial enriched spots” formed between EIII8-HUVEC and MCF10A-HUVEC cocultures (Fig. 3, compare a, b and d, e). The “endothelial cell enriched spots” present on MCF10A-induced tubular networks remained viable for only ~1 week, although the epithelial framework persisted (data not shown). These results suggest major differences in the angiotropic response between normal and transformed human breast epithelial cells, i.e., whereas MCF10A cells switch from a “pro-angiogenic” to an “anti-angiogenic” phenotype, preneoplastic EIII8 cells remained turned-on in the “pro-angiogenic” state. Another interesting feature unique to EIII8-HUVEC three-dimensional cultures and not observed in MCF10A-HUVEC cocultures is the development within 2–3 days of coculture of “branching end buds” or “ductular-alveolar outgrowths” in close proximity with “endothelial enriched spots” (Fig. 3, d and e; Fig. 4). Treatment with 1 nm E2 enhanced both growth of “endothelial cell enriched spots” and “ductal-alveolar outgrowths” over those of control cultures, as seen by an increase in size of both “spots” and ductal branches in 5-day-old cocultures (Fig. 3, compare d and e). Although no sera and only phenol red-free media were used, the magnitude and specificity of E2-mediated stimulatory effects on angiotropic response and ductal-alveolar morphogenesis/growth became more obvious when cultures were treated with a combination of 1 nm E2 and a 100-fold molar excess of 4(OH)-tamoxifen (data not shown) or the pure antiestrogen ICI 182,780. By day 5, these cultures showed dramatic inhibition of both endothelial sprouting and associated ductal-alveolar morphogenesis (Fig. 3f) that disintegrated by days 10–14 of culture (Fig. 3f, inset). These data suggest that the presence of contaminating estrogen in the culture media probably contributed to endothelial cell growth and ductal-alveolar morphogenesis observed in control cultures (Fig. 3d). It is interesting to note that although addition of E2 to MCF10A-HUVEC cocultures had no significant influence on growth and proliferation of endothelial or epithelial cells (Fig. 3, a and b), addition of ICI 182,780 abolished formation of

**Fig. 2.** Phase contrast morphology of EIII8 cells in three-dimensional Matrigel culture after treatment with estrogen. a, cultures treated with 100 nm ICI 182,780; b and d, cultures treated with 1 or 10 nm E2, respectively; c, cultures treated with a combination of 1 nm E2 and a 100-fold molar excess of ICI 182,780. All cultures represent morphologies at 5 days of culture. Note the remarkable differences in contrast between cultures exposed to ICI 182,780 (a and c) and E2 (b and d). Also, note that addition of ICI 182,780 significantly inhibits epithelial multilayering induced by E2. Treatment with E2 also induces formation of several new central spaces and connecting bridges (b and d). Magnification, ×100.

**Heterotypic Cultures and Effects of E2 on Growth.** We compared the abilities of normal MCF10A and preneoplastic EIII8 cells to support and maintain endothelial cell growth. When heterotypic cocultures of MCF10A or EIII8 cells were set up with HUVEC cells, the tubular networks observed with the homotypic cultures (Figs. 1 and 2) were preserved. However, although equal numbers of epithelial and endothelial cells were seeded, the tubular frameworks were comprised mainly of epithelial cells, whereas endothelial cells demonstrated preferential affinity to attach and proliferate at certain sites of the tubular framework. These regions, referred to as “endothelial cell enriched spots,” became prominent at ~2–3 days of culture (Fig. 3, a, b, d, and e). This distribution pattern of the two cell types in the three-dimensional structures was confirmed in cocultures of EIII8 and HUVEC cells that were prelabeled with DiI and DiO, respectively (Fig. 4, a and b). It is interesting to note that although both MCF10A and EIII8 cells sustained active proliferation of endothelial cells for >3 weeks, the tubular networks observed with the homotypic cultures (Figs. 1 and 2) were preserved. However, although equal numbers of epithelial and endothelial cells were seeded, the tubular frameworks were comprised mainly of epithelial cells, whereas endothelial cells demonstrated preferential affinity to attach and proliferate at certain sites of the tubular framework. These regions, referred to as “endothelial cell enriched spots,” became prominent at ~2–3 days of culture (Fig. 3, a, b, d, and e). This distribution pattern of the two cell types in the three-dimensional structures was confirmed in cocultures of EIII8 and HUVEC cells that were prelabeled with DiI and DiO, respectively (Fig. 4, a and b). It is interesting to note that although both MCF10A

**Fig. 3.** Phase contrast morphology of heterotypic EIII8-HUVEC or MCF10A-HUVEC cocultures in Matrigel at day 5. MCF10A-HUVEC or EIII8-HUVEC cultures were treated with vehicle (0.01% ethanol, v/v; a and d, respectively), 1 nm E2 (b and e, respectively), or a combination of 1 nm E2 and a 100-fold molar excess of ICI 182,780 (c and f, respectively). Note the difference in the sizes of “endothelial cell enriched spots” (open arrows) formed on EIII8 and MCF10A epithelia. Also, note the specific association of “ductal-alveolar outgrowths” (arrows) with “endothelial cell enriched spots” in EIII8-HUVEC cultures (d and e), and the specific inhibition by ICI 182,780 of ductal-alveolar growth and endothelial cell enriched spots in EIII8-HUVEC (f) and endothelial cell growth in MCF10A-HUVEC cultures (c). Inset in f, results of exposure to ICI 182,780 for 14 days. Bar, 100 μm.
Quantitative Assessment of Estrogen Effects on 3-Dimensional Growth of Heterotypic (EIII8-HUVEC) Cultures. Because exposure to E2 confers pronounced growth enhancement of EIII8 cells both in homotypic and heterotypic cultures (Figs. 2 and 3), we measured the effects of E2 on cell proliferation in heterotypic three-dimensional cocultures by both MTS and trypan blue dye exclusion assays of dispase-treated cultures. By both assays, E2 elicited a dose-dependent induction of growth at concentrations \( \geq 1 \mu M \); a 2-fold increase in growth over control cultures was observed with 10 nM E2 (\( P, 0.01 \); Fig. 5). This induction of growth by E2 occurs through the ER-dependent pathway, because the proliferation effects of E2 were abolished by inclusion of 100-fold molar excess of the pure antiesrogen, ICI 182,780 (\( P, 0.001 \); Fig. 5). Only the results of MTS assay are shown in Fig. 5, because results of both assays were in good agreement.

Only Conditioned Media from Heterotypic Cocultures Have Endothelial Cell Growth Stimulatory Activity. To determine whether the stimulatory effects on endothelial cell proliferation observed in EIII8-HUVEC cell cocultures are attributable to soluble factor(s) secreted by EIII8 cells, we examined the effects of conditioned media prepared from homotypic (EIII8 or MCF10A) or heterotypic (EIII8-HUVEC or MCF10A-HUVEC) three-dimensional cultures on HUVEC cell proliferation. Addition of up to 50 \( \mu l \) of conditioned media from homotypic EIII8 (Fig. 6), MCF10A (data not shown), or heterotypic MCF10A-HUVEC (Fig. 6) cultures were ineffective in stimulating growth of HUVEC cells over that of control cultures. In contrast, conditioned media from heterotypic EIII8-HUVEC cocultures elicited a significant dose-dependent induction of growth at volumes \( \geq 5 \mu l \) (\( P = 0.02 \)), and addition of 20 \( \mu l \) of conditioned medium elicited a 3-fold increase in HUVEC cell proliferation over that of control cultures (\( P < 0.001 \); Fig. 6). These results indicate that secretion of growth-stimulatory activity into the culture media requires intimate interaction between epithelial and endothelial cells. Because the cytokine, IL-8 (35–38), and the endothelium-specific mitogen, VEGF, have been demonstrated to function as survival and anti-apoptotic factors for endothelial cells, we tested the effects of antibodies to VEGF, its receptor, Flk-1/KDR, and IL-8 on conditioned media-induced growth of HUVEC cells. Because expres-

Fig. 4. Phase contrast micrographs of three-dimensional EIII8-HUVEC cocultures. a and b, cocultures established with DiI- and DiO-prelabeled EIII8 and HUVEC cells, respectively. Note that ductal-alveolar outgrowths are comprised of epithelial cells (a), whereas DiO-labeled endothelial cells are concentrated at this region as a spot (b). Note the presence of immature buds at day 5 of culture (c and d) that have developed into distinct and well-formed buds by day 10 (e and f). The dark areas in close association with alveolar structures represent the endothelial enriched sites. Bar, 100 \( \mu m \) (a, b, c, e) and 50 \( \mu m \) (d and f).

Fig. 5. Regulation of three-dimensional growth of EIII8-HUVEC cultures by estrogen. Growth was quantitated by MTS assay on day 5 of culture. Control wells received additions of vehicle (0.01% ethanol (v/v)). Treatments included E2 at 0.1, 1, or 10 nM; ICI 182,780 at 100 or 1000 nM; and combinations of 1 nM E2 with 100-fold or 1000-fold excess of ICI 182,780. Results obtained from three independent experiments performed in triplicate are expressed as mean; bars. SE. *, doses of compounds that increased cell growth significantly over nonhormone-treated control (\( P < 0.01 \)); **, doses of ICI 182,780 that significantly decreased cell growth induced by E2 (\( P < 0.001 \)).
Effects of antibodies on CM-induced HUVEC cell proliferation were from EIII8, MCF10A-HUVEC, or EIII8-HUVEC cultures, respectively. Significant decrease in cell proliferation induced by anti-IL-8 antibody relative to cultures treated with normal IgG. Because no differences in cell numbers were observed between cultures treated with mouse or rabbit normal IgG, the results are grouped together. Results obtained from three independent experiments performed in triplicate are expressed as means; bars, SE. *, cell proliferation that is significantly increased by CM from EIII8-HUVEC cocultures over untreated control (P < 0.001). **, significant decrease in cell proliferation caused by addition of anti-VEGF or anti-Flk-1 antibodies relative to cultures treated with normal IgG (P < 0.001). ***, significant decrease in cell proliferation induced by anti-IL-8 antibody as compared with cultures treated with normal IgG (P < 0.02).

Fig. 6. Effect of conditioned medium (CM) from MCF10A-HUVEC, EIII8-HUVEC, or EIII8 cultures on proliferation of HUVEC cells. HUVEC cells were treated with 50 or 1–20 μl of uncentrurated CM from EIII8, MCF10A-HUVEC, or EIII8-HUVEC cultures, respectively. Effects of antibodies on CM-induced HUVEC cell proliferation were tested in cultures treated with a combination of 20 μl of CM from EIII8-HUVEC cultures and 10 μg/ml of anti-VEGF, anti-Flk-1, or anti-IL-8 antibodies or an equivalent amount of mouse or rabbit normal IgG. Because no differences in cell numbers were observed between cultures treated with mouse or rabbit normal IgG, the results are grouped together. Results obtained from three independent experiments performed in triplicate are expressed as means; bars, SE. *, cell proliferation that is significantly increased by CM from EIII8-HUVEC cocultures over untreated control (P < 0.001). **, significant decrease in cell proliferation caused by addition of VEGF or anti-Flk-1 antibodies relative to cultures treated with normal IgG (P < 0.001). ***, significant decrease in cell proliferation induced by anti-IL-8 antibody as compared with cultures treated with normal IgG (P < 0.02).

sition of Flk-1/KDR is confined to endothelial cells, effects of neutralization of Flk-1/KDR with its antibody would indicate the selective nature of VEGF-induced mitogenesis. The addition of 10 μg/ml of antibodies to either VEGF or its receptor, Flk-1/KDR, abolished conditioned media-induced HUVEC cell proliferation (P < 0.001), whereas the addition of similar amounts of polyclonal anti-human IL-8 antibody evoked only 40% inhibition of growth (P < 0.02; Fig. 6). Inclusion of equivalent amounts of the corresponding normal IgG had no effect on conditioned media-stimulated growth (Fig. 6). This inability of anti-IL-8 antibody to evoke a greater degree of endothelial cell growth inhibition is not attributable to incomplete neutralization of IL-8 in the conditioned media because the addition of higher amounts (up to 25 μg/ml) of IL-8-specific antibody failed to increase the magnitude of growth inhibition (data not shown). Our results suggest that although IL-8 is an important endothelial cell survival factor in our assays, it is not as potent as VEGF. Support for this assumption is also provided by the equally potent inhibition of growth by anti-Flk-1/KDR antibody as by VEGF antibody.

Heterotypic EIII8-HUVEC 3-Dimensional Structures Express Epithelial and Endothelial Cell Function Markers. Results of Figs. 3, 4, and 6 demonstrate clearly that in heterotypic EIII8-HUVEC cocultures, not only do both endothelial and epithelial cell populations remain proliferative but that there is an intimate interaction that is mutually beneficial. This is evident from the colocalization of branching “ductal-alveolar outgrowths” with “endothelial cell enriched spots” (Figs. 3 and 4). Histological evaluation of H&E-stained paraffin-embedded sections of three-dimensional cocultures revealed the presence of multilayered epithelium at several regions of the tubular framework with branching end buds (resembling finger-like projections) invading into the surrounding ECM with coincident ECM degradation (Fig. 7A). To confirm the proliferative potential of the three-dimensional structures and to provide biochemical evidence for breast epithelial and endothelial cell growth and function, we used immunohistochemistry to examine the distribution of epithelial (cytokeratins, muc-1), endothelial (cd31, factor VIII-related antigen), and proliferation (PCNA) markers in 10-day-old cocultures. Although positive cytoplasmic immunoreactivity to pan-cytokeratins was observed in the majority of cells as expected (Fig. 7C), muc-1 glycoprotein expression was predominantly localized in the lumen or lumen-forming areas of epithelium (Fig. 7D). These data confirm that the main tubular network is indeed comprised of breast epithelial cells that in three-dimensional cultures synthesize and secrete mucin, a characteristic feature of epithelial glandular differentiation (39). In contrast to widespread distribution of cytokeratin immunoreactivity, reactivity to the endothelial cell marker cd31 is restricted to areas on the tubular framework that probably correspond with “endothelial cell enriched spots” (Fig. 7E). Expression of factor VIII-related antigen is localized to “endothelial cell enriched spots,” whereas the epithelial branching end buds in its immediate vicinity and tubular framework are negative (Fig. 7F). It is interesting to note that the majority of nuclei, particularly in the branching end buds, demonstrate positive nuclear immunoreactivity to anti-PCNA antibody, corroborating the high proliferative capacity of the cells in the three-dimensional structures (Fig. 7B).

Expression of VEGF, IL-8, ER, and Flk-1 Proteins Is Up-Regulated in EIII8-HUVEC Cell Cocultures. We have shown that unlike normal MCF10A cells, preneoplastic EIII8 cells provide a “good soil” for optimal survival, proliferation, and functioning of endothelial cells. This ability appears to be facilitated, at least in part, by secretion of endothelial cell growth-stimulatory factors, VEGF and IL-8, into the culture media (Fig. 6). Densitometric analysis of the steady-state levels of VEGF protein in the culture media of homotypic (EIII8, MCF10A, or HUVEC) and heterotypic (EIII8-HUVEC or MCF10A-HUVEC) three-dimensional cultures showed the presence of ~20-fold higher levels of VEGF165 in culture media of EIII8-HUVEC cultures when compared with corresponding fractions from homotypic EIII8 or MCF10A, or MCF10A-HUVEC cultures (Fig. 8A). Because the antibody used for Western analysis recognizes the COOH terminal epitope that is present in VEGF165, VEGF189, and VEGF206 but not in VEGF121 (11), alterations in relative levels of VEGF121 have not been determined. Densitometric analysis of VEGF165 in culture media of EIII8-HUVEC three-dimensional cultures at various times of culture revealed that although control samples maintain a constant level of VEGF165 from days 1 to 10 of culture, treatment with 1 nM E2 induces 4–8-fold increase in VEGF165 levels over those of control cultures by days 5 and 10 of culture, respectively (Fig. 8B). The role of estrogen in regulation of VEGF expression became more apparent when addition of pure antiestrogen,ICI 182,780, at 100-fold molar excess of E2 abolished the E2-stimulated increase in VEGF165 levels, returning them to control levels (Fig. 8B). Analysis of expression of the cytokine, IL-8, in culture media of HUVEC (Fig. 8C, Lane 1) or MCF10A-HUVEC three-dimensional cultures (Fig. 8C, Lanes 2 and 3) showed the presence of only trace amounts of IL-8. In contrast, levels of IL-8 are significantly

INTERACTING WITH THE EIIII8 THREE-DIMENSIONAL MODEL FOR DUCTAL-ALVEOLAR MORPHOGENESIS

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up-regulated (>10-fold) in culture media of corresponding 5- and 10-day-old EIII8-HUVEC cocultures (Fig. 8C, Lanes 4 and 7). Interestingly, loss of IL-8 expression in 10-day-old MCF10A-HUVEC cocultures (Fig. 8C, Lane 3) is coincident with loss of viability and stability of endothelial-enriched sites on MCF10A epithelium. In contrast to the inductive effects of estrogen on VEGF expression, E2 does not appear to regulate IL-8 expression (Fig. 8C, Lanes 5 and 8), and addition of 100-fold molar excess of the pure antiestrogen, ICI 182,780, had no effect on IL-8 levels (Fig. 8C, Lanes 6 and 9).

Analysis of levels of Flk-1/KDR and ER proteins in the corresponding matrix fractions of homotypic (HUVEC) and heterotypic (MCF10A-HUVEC or EIII8-HUVEC) three-dimensional cultures revealed the presence of the endothelial cell-specific receptor, Flk-1/KDR, in HUVEC cells as well as in MCF10A-HUVEC and EIII8-HUVEC cocultures (Fig. 8C). However, levels of Flk-1/KDR protein are significantly up-regulated (~12-fold) in 5-day-old EIII8-HUVEC cocultures (Fig. 8C, Lane 4) over those expressed in corresponding MCF10A-HUVEC cocultures (Fig. 8C, Lane 2) or homotypic cocultures.
The gels are representative of three independent experiments. Arrow, EIII8-HUVEC, or MCF10A-HUVEC cultures or at 2 days of HUVEC cell cultures were analyzed on gelatin-embedded substrate gels.

of ICI 182,780 at 100-fold molar excess of E2 caused heterotypic (EIII8-HUVEC or MCF10A-HUVEC) cultures or in HUVEC cells at 48 h of culture. Analyzed on days 1, 5, and 10 of culture. Culture media were collected at indicated times from cultures treated with vehicle (0.01% ethanol, v/v), 1 nM E2, or a combination of 1 nM E2 and a 100-fold molar excess of ICI 182,780. C, steady-state levels of Flik-1 and ER or IL-8 were analyzed in matrix and corresponding culture media, respectively, from HUVEC cultures on day 2 (Lane 1), MCF10A-HUVEC (Lanes 2 and 3), or EIII8-HUVEC (Lanes 4–9) cultures on days 5 (Lanes 2 and Lanes 4–6) and 10 (Lane 3 and Lanes 7–9). Cultures were treated with vehicle (Lanes 1–4 and 7), 1 nM E2 (Lanes 5 and 8), or with a combination of 1 nM E2 and a 100-fold molar excess of ICI 182,780 (Lanes 6 and 9). Positions of Flik-1, wild-type ER, Mr 42,000 ER-reactive band, and IL-8 are indicated. D, zymographic analysis of gelatinases secreted from HUVEC (Lane 2), EIII8 (Lane 3), MCF10A (Lane 4), EIII8-HUVEC (Lane 5), and MCF10A-HUVEC (Lane 6) cultures. Lane 1, activated MMP-2 used as positive control. Conditioned media prepared from 5-day-old EIII8, MCF10A, EIII8-HUVEC, or MCF10A-HUVEC cultures or at 2 days of HUVEC cell cultures were analyzed on gelatin-embedded substrate gels. Arrow, position of the active form of MMP-2. The gels are representative of three independent experiments.

HUVEC cultures (Fig. 8C, Lane 1). Although the levels of Flik-1/KDR protein in control and E2-treated 5-day-old EIII8-HUVEC cultures appear to be equally up-regulated (Fig. 8C, Lanes 4 and 5), addition of ICI 182,780 at 100-fold molar excess of E2 caused ~80% reduction in Flik-1/KDR protein levels (Fig. 8C, compare Lanes 5 and 6). Although the levels of VEGF165 (Fig. 8B) and IL-8 (Fig. 8C) proteins remained elevated in 5- and 10-day-old EIII8-HUVEC cultures, Flik-1/KDR protein levels in 10-day-old EIII8-HUVEC cocultures declined by ~75% and were no longer regulated by E2 or ICI 182,780 (Fig. 8C, Lanes 7–9). It is interesting to note that the pattern of expression of Flik-1/KDR protein parallels the expression pattern of ER in EIII8-HUVEC cocultures. HUVEC cells are ER positive (40), whereas MCF10A cells are ER negative (41), and as expected, a constant level of wild-type ER protein (Mr 67,000), probably originating from HUVEC cells, is detected both in homotypic HUVEC cocultures (Fig. 8C, Lane 1) and heterotypic MCF10A-HUVEC three-dimensional cultures (Fig. 8C, Lanes 2 and 3). As observed in the case of Flik-1/KDR protein expression, levels of wild-type ER protein are enhanced ~25-fold in heterotypic EIII8-HUVEC cocultures relative to those in HUVEC or MCF10A-HUVEC cultures (Fig. 8C, Lanes 4 and 5). Treatment with ICI 182,780 at 100-fold molar excess of E2 resulted in significant reduction in amounts of wild-type ER with concomitant appearance of a Mr 42,000 protein that is immunoreactive with the anti-ER antibody (Fig. 8C, compare Lanes 5 and 6). It is not yet clear whether the M, 42,000 (Fig. 8C, Lanes 6–9) and the M, 26,000 bands (Fig. 8C, Lanes 4 and 5) represent variant or truncated forms of ER (42). In 10-day-old EIII8-HUVEC cocultures, the Mr 67,000 wild-type ER band is either undetectable or present in only trace amounts, whereas the Mr 42,000 band represents the major immunoreactive ER band (Fig. 8C, Lanes 7–9). It is not yet clear whether this shift in ER protein expression from Mr 67,000 to Mr 42,000 species reflects an alteration in hormonal sensitivity or responsiveness of the EIII8-HUVEC three-dimensional cultures.

Expression of an Active Form of MMP-2 Is Enhanced in EIII8-HUVEC Cocultures. Degradation and remodeling of ECM are essential processes for angiogenesis and involve the MMP/tissue inhibitor of metalloproteinases family of proteases (43–45). MMP-mediated matrix remodeling also appears to promote the growth of tumor cells, possibly by facilitating the proliferation and migration of endothelial cells and the neovasculature of tumor tissue (46). Results of histological evaluation of three-dimensional cultures have shown that in the presence of endothelial cells, EIII8 cells have acquired the ability to invade and degrade the surrounding ECM (Fig. 7A). MMPs, including MMP-2 (47, 48) and MMP-9 (49, 50), have been shown to play major roles in degradation of ECM in tumor invasion. To identify the gelatinolytic activity of three-dimensional cultures, we performed gelatin zymography of conditioned media from homotypic (HUVEC, EIII8, or MCF10A) and heterotypic (EIII8-HUVEC or MCF10A-HUVEC) cultures. Although the latent or pro-form of MMP-2 (Mr 72,000) was detected in all samples at low levels, the active form of
MMP-2 (M, 62,000) was present at significantly elevated levels only in the conditioned media of EIII8-HUVEC cocultures (Fig. 8D, Lane 5). No difference in the amounts of proteins corresponding to M, 92,000 (MMP-9) and M, 96,000 bands was observed (Fig. 8D). This lack of regulation of MMP-9 levels suggests either the presence of contaminating MMP-9 that is secreted from Matrigel during culture or the lack of regulation of MMP-9 levels suggests either the presence of contaminating MMP-9 that is secreted from Matrigel during culture or the lack of regulation of MMP-9 levels suggests either the presence of contaminating MMP-9 that is secreted from Matrigel during culture or the lack of regulation of MMP-9 levels or lack of regulation of MMP-9 levels or lack of regulation of MMP-9 levels or lack of regulation of MMP-9 levels or lack of regulation of MMP-9 levels or lack of regulation of MMP-9 levels.

DISCUSSION

Despite wide agreement about the involvement of estrogen in the etiology of breast cancer, there is uncertainty as to its precise role(s) in the biology of breast cancer development. In this report, we describe an in vitro assay system that allows exploration of the interactions between HBEcs and endothelial cells on reconstituted basement membrane and show that distinct patterns of angiogenesis permit discrimination between normal (or benign) and premalignant mammary epithelial cells. Results from this study show that estrogen exerts a direct and early effect on mammary carcinogenesis by stimulating proliferation of both endothelial cells (an important stromal component) and premalignant epithelial cells. This assay system is unique from those reported by others in that morphogenesis of ductal-alveolar units resembling terminal ductal lobular units occurs de novo (from single cells) rather than from organoids of primary cultures or simple organization from single cells into spherical structures with acini. When normal MCF10A or preneoplastic EIII8 cells are cocultured with HUVEC cells on a layer of Matrigel, all cell types in both cocultures rapidly organize into interconnected tubes, with the endothelial cells migrating on the epithelium. However, although further differentiation of endothelial cells into complex three-dimensional networks is observed in both MCF10A- and EIII8-HUVEC cocultures, stable and sustained proliferation of endothelial networks is observed only in EIII8-HUVEC cocultures. Although the endothelial cells grow on top of epithelial cells, stable and functional three-dimensional vascular networks (established by factor VIII expression) develop only at specific sites on the EIII8 epithelium. It is not yet clear whether the occurrence of three-dimensional vascular networks at specific sites is induced by a subpopulation of EIII8 cells (with morphological, genetic, or functional alterations) that are committed progenitors of cancers. This is possible because like its parental line, MCF10AT1 (20), lesions arising from EIII8 cells in immune-deficient mice exhibit a heterogeneous spectrum of advanced histological grades of progression (atypical hyperplasia, carcinoma in situ, and invasive carcinoma) besides simple ducts, with prominent angiogenesis. A fraction of preneoplastic MCF10A cells exhibit angiogenesis and ductal-alveolar outgrowths, whereas normal MCF10A cells are ER negative (41, 57).

We have shown previously that estrogen enhances preneoplastic progression of MCF10AT1 cells in vivo as lesions of MCF10AT1 (20), or EIII8 cells 4 harvested from estrogen-supplemented animals exhibit rapid growth and advanced histological grades of progression with prominent angiogenesis as compared with simple or moderate hyperplasia without atypia or angiogenesis in control unsupplemented animals. The importance of estrogen in regulation of the angiogenic response in vivo is recapitulated in our in vitro assays, because estrogen specifically stimulates growth of both three-dimensional vascular networks and ductal-alveolar outgrowths, and these processes are blocked or significantly inhibited by the pure antiestrogen ICI 182,780.

Endothelial cells have been found to possess ER (40), and estrogen has been reported to increase endothelial cell proliferation (58). In our system, much of the E2/ER-mediated effects on angiogenesis appear to emanate from its stimulatory effects on expression of angiogenesis-regulating factors, VEGF and its receptor, Flk-1/KDR (VEGFR-2). These data are consistent with previous reports that estrogen influences VEGF/PF mRNA expression in the uterus (18) and in the well-vascularized, DMBA-induced, hormone-dependent rat mammary tumors (59). Fukuda et al. (60) observed that the growth of capillary endothelial cells in the DMBA-induced tumors is estrogen-dependent, and that treatment of DMBA-exposed rats with E2 after ovarioectomy prevents tumor necrosis and maintains high rates of endothelial cell proliferation. Similarly, estrogen-induced angiogenesis in rat pituitary tumors is associated with E2-mediated increases in the expression of both ligand and its receptor, VEGF and Flk-1/KDR, respectively, suggesting an important role for estrogen in the initial step of angiogenesis regulation (61). In contrast to effects of estrogen on VEGF and Flk-1/KDR expression in our system, steady-state levels of the cytokine, IL-8, an important endothelial cell survival factor are unaffected by estrogen and remain elevated throughout the culture period.

It is interesting to note that in 10-day-old cultures, although high levels of IL-8 and VEGF cells are maintained, Flk-1/KDR protein levels are significantly reduced but maintained at a steady level that is unaffected by exposure to either estrogen or E2 in culture. Although the significance of this is not yet clear, this alteration in regulation of Flk-1/KDR protein is correlated with a dramatic down-regulation in levels of the M, 67,000 wild-type ER and concomitant appearance of a prominent M, 42,000 anti-ER immunoreactive pro-
tein. It remains to be established whether the $M_r$ 42,000 protein represents a form of variant ER or is simply a protein that is cross-reactive with an NH$_2$ terminus-specific anti-ER antibody. A plethora of studies have reported the detection by reverse transcription-PCR of ER mRNA splice variants in normal and cancerous human breast tissues, the biological and clinical significance of which might be significant but remain to be established because of a lack of evidence for their existence at the protein level (62, 63). However, it is intriguing to speculate that such a shift in ER status, coupled with alterations in sensitivity of Flik-1/KDR expression to estrogen and ICI 182,780, may signify a switch from an estrogen-responsive to an estrogen-insensitive phase of angiogenesis and mark the beginning of "new autonomous growth."

Expression of angiogenic activity is a predictable property of many preneoplastic cells and may represent one of the earliest indications that a cell population has become committed to malignancy (64). Results from our novel in vitro assay system reinforce the concept that like tumor cells, preneoplastic breast epithelial cells actively produce diffusible angiogenic factors and cytokines that directly activate endothelial cells, stimulating them to sprout and initiate development of three-dimensional vascular networks, which in turn induce development of ductal-alveolar outgrowths with capacity to invade and degrade the surrounding ECM. The characteristic expression of activated MMP-2 observed only in preneoplastic EIII8-HUVEC cocultures fortifies the importance of proteolytic enzymes in the release of angiogenic factor secreted in ECM (65, 66). The direct correlation observed between growth and development of vascular networks and ductal-alveolar outgrowths with coincident ECM remodeling suggests that increased gelatinolytic activity secreted by epithelial and/or endothelial cells may facilitate release of angiogenic growth factors locally from ECM.

In summary, we have established a novel, physiologically relevant in vitro model system that not only recapitulates several important aspects of estrogen-induced growth and preneoplastic progression of MCF10AT1 cells in vivo but also demonstrates for the first time the integral role endothelial cells play in ductal-alveolar morphogenesis and proliferation of preneoplastic HBECs. This assay system will provide a unique tool to explore systematically the expression of growth-regulatory molecules that determine epithelium-specific and endothelium-specific requirements for angiogenesis and progression of preneoplastic breast disease.

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Malathy P. V. Shekhar, Jill Werdell and Larry Tait


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