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Vascular endothelial growth factor secreted by activated stroma enhances angiogenesis and hormone independent growth of estrogen receptor positive breast cancer

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Abstract

“Reactive” or activated stroma characterizes many malignancies including breast cancers. Recently we isolated a reactive mouse mammary gland stromal cell line called BJ3Z (1). These cells express α -smooth muscle actin (α -SMA) and stromal-cell derived factor 1 (SDF-1), and are tumorigenic when injected into mice. Here we show that *in vivo*, BJ3Z cells influence angiogenesis and proliferation of xenografted estrogen receptor (ER)-positive MCF-7 human breast cancer cell-derived solid tumors. The growth promoting effects of BJ3Z cells are equivalent to those of estradiol (E_2). BJ3Z cells also increase proliferation of normal mouse mammary luminal cells adjacent to tumors. *In vitro* BJ3Z cells reorganize and increase proliferation of co-cultured malignant MCF-7 and normal human breast MCF10A cells grown as organoids in three dimensional (3D) culture. The effects of BJ3Z cells on MCF-7 cells are equivalent to those of E_2 . In contrast, BJ3Z cells do not alter growth of highly aggressive ER-negative MDA-MB-231 human breast cancer cells. We show that BJ3Z cells secrete vascular endothelial growth factor (VEGF). Growth of MCF-7 organoids induced by BJ3Z can be inhibited by antagonists of VEGF and SDF-1. Conversely, recombinant VEGF stimulates proliferation of MCF-7 but not MDA-MB-231 organoids. We conclude that in addition to angiogenesis, VEGF released by activated stroma increases growth both of ER-positive malignant epithelial cells and of adjacent normal epithelium. Since activated stroma can substitute for E_2 and fosters hormone-independent growth of ER-positive tumors, we suggest that breast cancers exhibiting intrinsic hormone resistance may respond to anti-angiogenic therapies.

Keywords

Stroma; angiogenesis; proliferation; luminal breast cancer; VEGF; intrinsic estrogen resistance

Introduction

For several decades cancer research has focused on the genetics and tumor biology of adenocarcinomas. In the breast, such cancers originate from luminal epithelial cells that line the alveoli and ducts (2). Recent studies have demonstrated the importance of stromal influences on epithelial cells in a wide variety of normal biological processes including embryonic development and wound healing, and in malignancies including tumorigenesis and tumor growth (3–6). “Reactive” or activated stroma or desmoplasia is a common feature of many malignancies (7–9). Co-injection into mice of activated, carcinoma-associated

fibroblasts (CAF) with Ras mutant MCF-7 human breast cancer cells generates large tumors, associated with recruitment of bone marrow-derived, angiogenic endothelial progenitor cells (EPCs) (10). One mediator of this CAF effect is stromal derived factor 1 (SDF-1; CXCL12), which increases proliferation of Ras mutant MCF-7 cells by binding surface CXCR-4 receptors. CXCR-4 is expressed in a variety of breast cancer cells but is absent in normal mammary epithelial cells. SDF-1 is a chemoattractant that enhances the migratory behavior of cell lines (11). Interestingly, over-expression of SDF-1 is observed in normal lymph nodes, lungs, liver and bone marrow, the most common sites for breast cancer metastasis (11), suggesting that SDF-1 also serves a homing function for migratory tumor cells.

Angiogenesis is the physiological process by which new blood vessels are generated. Breast tumors and many other solid tumors require this fundamental step in order to grow beyond a few millimeters in diameter (12). Vascular endothelial growth factor (VEGF) is a potent and selective endothelial mitogen able to induce a rapid and complete angiogenic response in normal and malignant tissues by generating new blood vessels. In addition to being secreted by a number of different cell types, VEGF is over-expressed not only by breast cancer cells but also by activated breast stromal cells suggesting an active role for the latter in tumor growth and angiogenesis (13). Interestingly, VEGF expression in breast cancer cells can stimulate CXCR-4, linking VEGF expression to the migratory potential of cells (14) and to SDF-1 signaling.

Most breast cancers are “luminal” and express estrogen (ER) and/or progesterone (PR) receptors. The receptors identify patients whose tumors are likely to be hormone-dependent or hormone responsive and are candidates for endocrine therapies that suppress the proliferative effects of estrogens by targeting either ER or estradiol (E₂) biosynthesis. However, despite presence of the receptors, approximately half of luminal breast cancers exhibit intrinsic hormone resistance; tumors fail to respond or continue to expand at initial treatment. Such intrinsic resistance has been ascribed to a variety of factors including mutant ER, unbalanced ratios of ER coregulators, excessive growth factor signaling, and in some cases, unspecified “host” factors.

Recently we isolated and characterized an activated mouse mammary gland stromal cell line called BJ3Z, that expresses α -SMA, SDF-1 and other factors (1). We now show that *in vivo*, BJ3Z cells influence angiogenesis and proliferation of xenografted ER+ MCF-7 human breast cancer cell-derived solid tumors. The growth promoting effects of BJ3Z cells are equivalent to those of E₂. BJ3Z cells also increase proliferation of normal mouse mammary luminal cells adjacent to tumors. *In vitro* BJ3Z cells reorganize and increase proliferation of co-cultured malignant MCF-7 and normal breast MCF10A cells grown as organoids in three dimensional (3D) culture. The effects of BJ3Z cells on MCF-7 cells are equivalent to those of E₂. In contrast, BJ3Z cells do not alter growth of highly aggressive ER- MDA-MB-231 breast cancer cells. The 3D culture models faithfully replicate *in vivo* conditions. BJ3Z cells secrete VEGF and express SDF-1. Analogous to BJ3Z cells, recombinant VEGF stimulates proliferation of MCF-7 but not MDA-MB-231 organoids. We conclude that activated stroma can increase the aggressiveness and tumorigenicity of malignant epithelial cells and adjacent normal epithelium. Additionally, activated stroma can substitute for E₂ to foster hormone-independent growth of luminal tumors.

Materials & Methods

Cell lines

MCF-7 and MDA-MB-231 human breast cancer cells and immortalized normal human breast epithelial MCF10A cells were obtained from the ATCC or the University of Colorado Cancer Center Tissue Culture Core. All cell lines were authenticated by Single Tandem Repeat analysis

at the Sequencing Core. Transformed mouse mammary stromal BJ3Z cells were developed in our laboratory (1). Cells were passaged in minimum essential medium (MEM; Invitrogen, Carlsbad CA) containing 5% fetal bovine serum (FBS; HyClone, Logan UT), except for MCF10A which were grown in mammary epithelial growth medium (MEGM; Lonza, Walkersville MD). BJ3Z cells were tagged with red tandem-dimer Tomato (RedTomato)(15) as described (16).

Xenografts and tumor isolation

All animal procedures were performed under a protocol approved by the CU Institutional Animal Care and Use Committee. 10^6 ZsGreen tagged MCF-7 cells (16), or 10^6 RedTomato tagged BJ3Z cells or both (2×10^6 cells) were injected into mammary fat pads of ovariectomized (ovx) athymic *nu/nu* mice. 17β -Estradiol (E_2) or cellulose (C)-releasing silastic pellets were implanted subcutaneously (16). Mice were weighed and tumor areas measured weekly with digital calipers for 10–12 weeks. Two hours prior to necropsy, mice were injected intraperitoneally with 100 mg/kg Bromodeoxyuridine (BrdU) in PBS. At necropsy fluorescent tumors and metastases were visualized using Ilumatools 9900 (Lighttools Research, Encinitas, CA) and photographed with an Olympus (Melville, NY) C-5050 digital camera coupled to an Olympus SZ-61 dissecting microscope. Dissected tissues and tumors were fixed overnight in 4% paraformaldehyde, embedded into paraffin blocks and sectioned (5 μ m) for pathological review by hematoxylin and eosin (H&E) staining, and for immunohistochemistry (IHC) and other analyses.

Immunohistochemistries

For CD34, the primary antibody (1:50 dilution) (Abcam, Cambridge MA) was applied 1 hour followed by Alexa 488 Goat anti-rat secondary (1:300; Invitrogen). Other primary antibodies: Vimentin (sc-7557, Santa Cruz Biotechnology, Santa Cruz CA); Fibroblast Activation Protein (ab-53066, Abcam); α -SMA (1184-S, Epitomics, Burlingame CA); SDF-1 (MAB-350, R&D systems, Minneapolis, MN); and anti-mouse panCK (628601, BioLegend, San Diego CA). Secondary antibodies: Alexa 488 Goat anti-mouse (1:500); Alexa 488 Donkey anti-goat (1:500); Alexa 555 Goat anti-rabbit (1:400) (Invitrogen). Slides were imaged using a Nikon Eclipse E600 fluorescent microscope (Nikon Corporation, Tokyo, Japan) coupled to an RGB-MS-C MicroColor camera (CRI Inc, Boston, MA). Quantification of at least 5 fields, and 2 tumors/condition used Image Pro Plus 4.5 software (Media Cybernetics, Silver Spring MD).

CK18/BrdU or E-cadherin proliferation assay

BrdU incorporation was calculated by double staining for human CK18 (rabbit polyclonal AP1021; Calbiochem, La Jolla CA) and BrdU (mouse monoclonal #347580; Becton-Dickinson, San Jose CA). Red Alexa-555 Goat anti-rabbit and green Alexa-488 Goat anti-mouse antibodies (Invitrogen) allows their simultaneous detection (CK18/BrdU assay) after counterstaining nuclei with blue fluorescent 4'-6-Diamidino-2-phenylindole (DAPI). Proliferation rates of human cells were calculated by the ratio of BrdU+ nuclei (green) to DAPI + nuclei (blue) in CK18+ cells (red) using Image Pro, in 5–10 fields from at least 2 tumors or 4 organoids/condition. Normal luminal epithelial cells adjacent to tumors were double stained for E-cadherin (ab15148; Abcam) and BrdU, and quantified by counting BrdU+ vs. total nuclei in E-cadherin+ structures.

3D organoids

3D cultures were performed as described (17) with minor modifications. Briefly, eight-well slides were coated with 50 μ l Growth Factor Reduced Matrigel™ (BD Biosciences, Bedford MA). Cells were trypsinized and resuspended in MEM containing 5% twice Dextran Coated Charcoal (DCC)-stripped serum (DCC/MEM). Breast cancer cells (10,000) and/or BJ3Z cells

(50,000) were layered on Matrigel and cultures were maintained for ~7 days, adding fresh medium every 2/3 days. For MCF10A, 10,000 cells were resuspended in 2% Donor Horse Serum (DHS) in MEGM. To calculate proliferation indices, 7 day-old organoids were incubated with 0.25 mg/ml BrdU in 5% DCC/MEM for 1 hour. IHC for MCF-7 cells used the CK18/BrdU assay; for MCF10A and MDA-MB-231 cells, CK14 (rabbit polyclonal RB-9020-P1; Thermo, Fremont CA) and CD44 (rabbit monoclonal 1998-1; Eptomics) antibodies respectively, were substituted for CK18. A minimum of 4 fields in 4 sections were quantified; at least in triplicates.

Hormone treatments *in vitro*

Organoids were cultured in 5% DCC/MEM for 5 days then switched to 5% DCC in phenol-red free MEM containing ethanol (1:1000 v/v) or 10 nM E₂.

Antibody array

BJ3Z organoids were cultured 2 or 4 days then switched to serum-free MEM for 48 hrs. Controls consisted of a Matrigel-coated chamber without cells. Supernatants from control or BJ3Z cells were collected, centrifuged and hybridized to a TranSignal™ mouse angiogenesis antibody array (Panomics, Redwood City CA), as instructed.

Anti VEGF and SDF-1 blockade

MCF-7/BJ3Z co-cultures were incubated 7 days with anti-mouse VEGF monoclonal antibody (VEGF MAb, R&D systems) or Avastin (Bevacizumab, Genetech Inc. San Francisco, CA) at 0.1 µg/ml, or the CXCR-4 antagonist AMD 3100 (Sigma, St Louis, MO) at 5 µg/ml, refreshed every 48 hrs. On day 7, the proliferation rate of organoids was calculated using the CK18/BrdU assay.

Recombinant (r) human VEGF

Cells were treated 7 days with 0.1, 1 or 10 ng/ml human or mouse rVEGF (R&D systems; Minneapolis, MN), refreshed every 2 days and proliferation rates calculated.

Phosphorylated-ERK (p-ERK)

Monocultured cells were treated with 10 ng/ml rVEGF or vehicle (0.1 % BSA in PBS) for 0, 10 or 30 min, and 2 or 6 hrs. p-ERK was assayed by IHC (rabbit polyclonal RA15002; Neuromics, Edina MN).

Statistical analyses

Data were analyzed with Graph Pad software using either Student's t-test or ANOVA followed by a Tukey's post-test; $p \leq 0.05$ was considered significant.

Results

BJ3Z cells are tumorigenic and influence growth and angiogenesis of MCF-7 tumors

Previously, we showed that BJ3Z stromal cells are tumorigenic when injected into mammary fat pads of nude mice (1). To track their effects *in vivo* BJ3Z cells were tagged with RedTomato fluor. Red BJ3Z cells either alone or mixed with ZsGreen tagged MCF-7 cells (16) were grown 10 weeks in the mammary glands of ovx nude mice with control (C; n=5) or E₂ (n=10) pellets (Figure 1A). ER- BJ3Z tumors are highly vascularized (top) and their growth is not E₂ dependent (right). Growth of ER+ MCF-7 cells (middle) is poor (left), unless they are exposed to E₂ (right). Under either condition, vascularization of MCF-7 tumors is minimal. Mixed MCF-7/BJ3Z tumors (bottom) grow well even in the absence of E₂ (left), and this is amplified

by E₂ supplementation (right). Mixed MCF-7/BJ3Z tumors are richly vascularized even in the absence of E₂. Weekly tumor growth rates for all conditions are shown in Figure 1B. After 10 weeks MCF-7/BJ3Z mixed tumors were larger than MCF-7 tumors. This is probably an additive effect of co-injecting two cell lines. The ratio of MCF-7 cells to BJ3Z cells in mixed tumors is increased by E₂ supplementation. Also, the E₂ supplementation to MCF-7/BJ3Z tumors significantly increases (77%) the rate of lymph node metastasis, compared to 25% metastases for E₂ alone. We previously demonstrated (16) that lymph node metastasis of solid MCF-7 tumors is size and time dependent. Activated stromal BJ3Z cells accelerate both states.

Since BJ3Z tumors exhibited increased surface vascularity, the endothelial cell marker CD34 was used to quantify intratumoral angiogenesis (Figure 1C). *In vitro*, neither MCF-7 nor BJ3Z cells express CD34. In the absence of E₂, angiogenesis in pure BJ3Z tumors and mixed BJ3Z/MCF-7 tumors is significantly higher (ANOVA $p=0.0013$) than in pure MCF-7 tumors. The same pattern is observed in the presence of E₂. Since in MCF-7 cells alone, E₂ appeared to have no effect on intratumoral blood vessel density, we conclude that E₂ does not influence tumoral vascularization; rather that BJ3Z cells stimulate recruitment of CD34+ cells into tumors.

BJ3Z cells cause an increase in MCF-7 cell proliferation in tumors and adjacent normal epithelium

Because mixed BJ3Z/MCF-7 tumors were enlarged, we examined the proliferative activity of the MCF-7 subpopulation, by quantifying BrdU incorporation (green) into CK18+ (red) malignant human epithelial cells (Figures 2A, 2B). Figure 2A, shows that in pure MCF-7 tumors, E₂ increases proliferation rate as assessed by the increased number of double CK18/BrdU-positive cells. The proliferative effects of E₂ in ER+ breast cancers are well known (16). However, Figure 2A shows that MCF-7 proliferation is increased equally by presence of BJ3Z cells, even in the absence of E₂. Quantitation of the data (Figure 2B) shows that BJ3Z cells and E₂ independently increase the proliferation rate of MCF-7 cells in a statistically significant, but non-additive manner.

The proliferative stimulation by BJ3Z cells of MCF-7 cells in the absence of E₂ prompted us to ask whether proliferation of adjacent normal mouse epithelium was similarly affected. Figure 2C shows H&E stained sections and matched serial sections stained for mouse E-cadherin (red) and BrdU (green), from cellulose treated mice bearing BJ3Z tumors, MCF-7 tumors or mixed MCF-7/BJ3Z tumors. Quantitation is shown in Figure 2D. Presence of BJ3Z cells alone or in mixed tumors markedly increased the proliferation rate of adjacent normal mouse mammary epithelial cells. Clearly, in the absence of E₂, transformed BJ3Z stromal cells are capable of increasing proliferation of both normal and malignant mammary epithelia in their vicinity.

Modeling the influence of BJ3Z cells in 3D cultures

BJ3Z stromal cells influence epithelial tumors *in vivo* by increasing growth and vascularization. To define the mechanisms involved, we sought an *in vitro* model from which cell-secreted factors could be collected and analyzed. Recent studies have demonstrated that cells grown *in vitro* in a 3D matrix recapitulate essential features of solid tumors including gene expression patterns and protein function (18,19). Figure 3 shows the morphology and quantitation of BrdU incorporation of 3D organoids formed by ER+ MCF-7 cells, normal ER- human epithelial MCF10A cells, and highly aggressive ER- MDA-MB-231 human breast cancer cells, cultured alone or together with BJ3Z cells. MCF-7 cells in 3D mono-culture form dense disorganized clusters or “masses” of variable size (Figure 3A, upper panels), characterized by irregular colonies with filled centers (18). When co-cultured with BJ3Z cells (Figure 3A, lower panels), MCF-7 cells form an organized layer that encapsulates the BJ3Z cells. In these mixed MCF-7/

BJ3Z structures, proliferation of the CK18+ (red) / BrdU+ (green) MCF-7 cell subpopulation is significantly increased (Figure 3A, right panel); a pattern similar to that observed in tumors (Figure 2B). normal mammary fibroblasts do not influence MCF-7 proliferation under these conditions (data not shown). Treatment of pure MCF-7 colonies with E₂ caused the expected increase in proliferation, akin to the levels induced by BJ3Z cells and demonstrating E₂ responsiveness of MCF-7 cells in 3D conditions. Proliferative effects of BJ3Z and E₂ were similar but not additive; again reflecting the solid tumors.

Since in tumors, presence of BJ3Z cells increased proliferation of adjacent normal mammary epithelial cells (Figures 2C, 2D), we evaluated this in 3D organoids using normal human mammary epithelial MCF10A cells. In mono-culture MCF10A cells form rounded hollow structures that proliferate sluggishly (Figure 3B, upper panels), as described (17), analogous to normal mammary epithelial cells *in situ* (20). In co-culture, MCF10A cells organize into lobular structures surrounding BJ3Z cells (Figure 3B) with significantly increased proliferation (Figure 3B right panel). These *in vitro* effects of BJ3Z cells on MCF-10A cells mimic their effects on normal mammary epithelial cells *in vivo* and further validate the 3D model.

In mono-culture, the highly metastatic ER- MDA-MB-231 breast cancer cell line forms “snowflake” or “stellate” colonies with projections (18) (Figure 3C) that bridge multiple cell colonies and can organize into a continuous network. This is considered characteristic of an invasive phenotype. The morphology of MDA-MB-231 cells differs substantially from the solid organoids of MCF-7 cells. Basal proliferation levels of MDA-MB-231 cells are similar to MCF-7 cells, but unlike MCF-7 or MCF10A cells, co-culture with BJ3Z cells causes no change in proliferation (Figure 3C). In co-culture, thin layers of MDA-MB-231 cells mold themselves around a dense core of quiescent BJ3Z cells. In sum, BJ3Z cells selectively influence a subset of epithelial cells characterized by low aggressiveness.

Molecular markers of BJ3Z cells in tumors and organoids

To further characterize the properties of BJ3Z cells in tumors *vs.* 3D organoids, we examined protein expression of key molecular markers under both conditions (Figure 4A). Top panels show the morphology of BJ3Z tumors and organoids stained with H&E. Whether grown as tumors or in 3D, BJ3Z cells express classic activated fibroblast markers: Fibroblast Activation Protein (FAP) alpha, SDF-1, α -SMA and Vimentin. BJ3Z cells do not express multiple epithelial cytokeratins (PanCK). Thus, BJ3Z cells are stromal cells with similar biological properties in 3D culture and tumors. We therefore used supernatants of cells grown in 3D to probe a mouse antibody array for BJ3Z-secreted factors that may explain their influence on epithelial cells (Figure 4B). BJ3Z grown in 3D secrete high levels of VEGF and Tissue Inhibitor of Metalloproteinases-1 (TIMP-1) (Figure 4B, boxed). Both factors are expressed by activated stroma (13, 21), and we analyzed VEGF in greater detail.

BJ3Z cells enhance epithelial proliferation through VEGF and SDF-1

VEGF is secreted by BJ3Z cells in organoids, and SDF-1 is expressed in both BJ3Z tumors and organoids. Both factors increase proliferation of breast cancer cells (22,23). An anti-mouse VEGF monoclonal antibody (VEGF MAb), the humanized anti-VEGF monoclonal antibody Avastin (Bevacizumab) and AMD 3100, a blocker of the SDF-1 receptor CXCR-4, were tested for their effects on MCF-7 proliferation in BJ3Z co-cultures (Figure 5A). In the presence of BJ3Z cells, VEGF MAb and AMD 3100 significantly decreased BrdU incorporation into MCF-7 cells, suggesting that VEGF and/or SDF-1 mediate BJ3Z-induced MCF-7 cell proliferation. Avastin had no significant effect (Figure 5A) since it is highly human specific (24) and does not target murine VEGF. On the other hand human VEGF should be able to influence MCF-7 cells directly. Indeed (Figure 5B, black bars) recombinant human VEGF (rVEGF) between 0.1 and 10.0 ng/ml, significantly increased BrdU incorporation into mono-

cultured MCF-7 cell organoids. Despite similar basal proliferation levels rVEGF had no effect on BrdU incorporation into MDA-MB-231 cells (Figure 5B, black bars), perhaps explaining their unresponsiveness to BJ3Z cells. Similar results were obtained for both breast cancer cell lines using mouse rVEGF (not shown).

Among three VEGF receptors, VEGFR-2 (KDR/Flk-1) is expressed by endothelial cells and is responsible for the proliferative activity of VEGF (25). VEGFR-2 activation leads to Raf-1/MEK and phosphorylated extracellular-signal regulated kinase (p-ERK) upregulation (26). Since both MCF-7 and MDA-MB-231 cells express comparable levels of VEGFR-2 (27) and total ERK (28), we investigated their p-ERK expression in response to rVEGF (Figure 5C). Basal p-ERK levels were low in MCF-7 organoids, but rVEGF rapidly (10 min) augmented their levels. In contrast, p-ERK levels are constitutively high in MDA-MB-231, which are unchanged by rVEGF. MDA-MB-231 cells possess both K-Ras and B-Raf mutations that trigger p-ERK overexpression (29,30), which may explain their unresponsiveness to VEGF and BJ3Z cells.

Discussion

BJ3Z cells and tumor angiogenesis

The microenvironment of breast adenocarcinomas is composed of a fibrous extracellular matrix, together with non-cancerous adipocytes, fibroblasts, inflammatory cells, etc. collectively called stroma. This microenvironment plays a critical role in cancer cell growth, metastatic potential, and possibly in determining the destination of metastatic cells and the outcome of therapies. Fibroblasts are the major stromal cell types and in tumors they acquire an “activated” phenotype characterized by increased proliferation and motility and heightened expression levels of α -SMA (31) and other factors. Under these conditions fibroblasts are generally called carcinoma associated fibroblasts (CAFs). Here we report that activated malignant mouse stromal BJ3Z cells (1), either alone, or mixed with MCF-7 cells, generate highly vascularized solid tumors containing increased intratumoral CD34+ vascular endothelial cells; both indicative of enhanced angiogenesis. Such tumors may be highly aggressive and metastatic. Since neither BJ3Z cells nor MCF-7 cells express CD34, we hypothesize that upregulation of CD34 positive cells in tumors containing BJ3Z cells, is due to recruitment of vascular endothelial cells in response to VEGF secretion. It is postulated that endothelial precursor cells originate in bone marrow and travel to the tumor mass *via* the circulation, attracted there by VEGF (32,33). Endothelial precursors may also arise from nearby capillaries (33). Orimo *et al.* (10) showed that co-injection of human CAFs with Ras mutant MCF-7 cells generated highly vascularized tumors. They proposed that the increased angiogenesis was caused by CAF expression of SDF-1 leading to recruitment of endothelial progenitor cells from bone marrow. Additionally, SDF-1 expression by CAFs increased proliferation of MCF-7 by direct activation of the SDF-1 receptor CXCR-4. Both VEGF and SDF-1 pathways are activated in our BJ3Z containing tumor models (Figure 5A) and could explain the angiogenesis we observe in mixed MCF-7 tumors (Figure 2C, 2D), in the absence of E₂ supplementation or introduction of Ras mutations.

Activated stroma and normal epithelia

It is widely recognized that the stroma plays a key role in normal tissue development and homeostasis (34). In fact, many years ago, normal stroma was shown to suppress expression of the neoplastic phenotype (35,36). Conversely, malignant stroma actively influences breast carcinogenesis (37,38). Here we demonstrate that presence of BJ3Z cells in tumors increases the proliferative activity of adjacent normal epithelium. Similarly we show that co-culture of BJ3Z cells with normal human breast epithelial MCF10A cells in organoids, strongly increases proliferation of the normal cells, suggesting an active role for malignant stroma in influencing

the behavior of normal epithelium. Indeed, irradiated or N-nitrosourea treated (both carcinogenic events) stroma transplanted into cleared mammary fat pads induce tumors in co-transplanted normal mammary epithelial cells (39,40). We speculate that in normal epithelium, malignant stroma influences tumorigenesis by increasing the proliferation, raising the likelihood of mutagenesis. In the early stages of cancer, malignant stromal cells would increase proliferation and inhibit apoptosis of nearby carcinoma cells, stimulate angiogenesis, promote acquisition of *de novo* mutations and foster aggressive behavior.

VEGF, SDF-1 and Intrinsic hormone resistance

3D culture systems have become accepted alternatives to the use of animal models (41). They have proven to be valuable tools to study the behavior of cells and the signaling pathways governing epithelial-stromal interactions in an environment that closely resembles the microenvironment of cells in tissues (42–44). Here we demonstrate that BJ3Z cells express a similar pattern of molecular markers in tumors and 3D organoids; that co-culturing BJ3Z cells *in vitro* or in solid tumors caused a similar increase in MCF-7 proliferation; that BJ3Z cells induce similar increases in proliferation of normal mammary cells in organoids and tumors; and that BJ3Z cells can substitute for the proliferative effects of E₂ in tumors and organoids. Our data point to a role for SDF-1 and VEGF for these effects. Previous studies have shown that VEGF regulates CXCR-4 levels suggesting that the two factors act cooperatively in breast cancers (14). Both factors have been previously associated with metastatic breast cancers: CXCR-4 activation is implicated in metastasis to the bone and lung (11), and a recent study identified a VEGF gene “signature” composed of 13 related genes as a strong prognostic indicator in breast cancers (45). We hypothesize that VEGF secretion by BJ3Z exerts a dual action in tumors: enhancing the recruitment of CD34-expressing cells leading to endothelial cell proliferation and neovascularization; and directly activating breast cancer cell proliferation. The latter effect of VEGF has been previously observed in MCF-7 cells engineered to over-express VEGF (22). In our studies, VEGF is supplied by stromal cells. Interestingly, unlike ER+ MCF-7 cells, highly aggressive ER- MDA-MB-231 cells are insensitive to BJ3Z cells or to treatment with VEGF. A related study, also using organoids, showed that MDA-MB-231 cell proliferation, unlike that of ER+ cell lines, is unaffected by presence of human bone marrow stromal cells (46). The authors postulated that ER- and/or triple negative basal breast cancers like those represented by MDA-MB-231, have acquired multiple K-Ras, B-Raf and p53 mutations (30,47) allowing E₂ independent growth. This could explain the disappointing efficacy of long term anti-angiogenic or anti-VEGF directed therapies like Bevacizumab on overall survival of late-stage HER2 negative, triple negative disease (48,49). Most clinical trials of anti-angiogenic therapies focus on combinations with chemotherapeutic agents targeting recurrent metastatic breast cancers that have been previously unresponsive to one or more rounds of chemotherapy (50,51). Our data indicate that angiogenesis can substitute for the proliferative effects of E₂. Therefore, counterintuitively anti-angiogenic therapies might be especially successful in ER+ luminal breast cancers that exhibit intrinsic resistance to endocrine therapies.

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References

1. Jacobsen BM, Harrell JC, Jedlicka P, Borges VF, Varella-Garcia M, Horwitz KB. Spontaneous fusion with, and transformation of mouse stroma by, malignant human breast cancer epithelium. *Cancer Res* 2006;66:8274–9. [PubMed: 16912208]

2. Nandi S, Guzman RC, Yang J. Hormones and mammary carcinogenesis in mice, rats, and humans: a unifying hypothesis. *Proc Natl Acad Sci U S A* 1995;92:3650–7. [PubMed: 7731959]
3. Woodward TL, Xie JW, Haslam SZ. The role of mammary stroma in modulating the proliferative response to ovarian hormones in the normal mammary gland. *J Mammary Gland Biol Neoplasia* 1998;3:117–31. [PubMed: 10819522]
4. Bissell MJ, Radisky D. Putting tumours in context. *Nat Rev Cancer* 2001;1:46–54. [PubMed: 11900251]
5. Weaver VM, Gilbert P. Watch thy neighbor: cancer is a communal affair. *J Cell Sci* 2004;117:1287–90. [PubMed: 15020668]
6. Troester MA, Lee MH, Carter M, et al. Activation of host wound responses in breast cancer microenvironment. *Clin Cancer Res* 2009;15:7020–8. [PubMed: 19887484]
7. Tuxhorn JA, Ayala GE, Rowley DR. Reactive stroma in prostate cancer progression. *J Urol* 2001;166:2472–83. [PubMed: 11696814]
8. Walker RA. The complexities of breast cancer desmoplasia. *Breast Cancer Res* 2001;3:143–5. [PubMed: 11305947]
9. Korc M. Pancreatic cancer-associated stroma production. *Am J Surg* 2007;194:S84–6. [PubMed: 17903452]
10. Orimo A, Gupta PB, Sgroi DC, et al. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell* 2005;121:335–48. [PubMed: 15882617]
11. Muller A, Homey B, Soto H, et al. Involvement of chemokine receptors in breast cancer metastasis. *Nature* 2001;410:50–6. [PubMed: 11242036]
12. Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* 1995;1:27–31. [PubMed: 7584949]
13. Brown LF, Guidi AJ, Schnitt SJ, et al. Vascular stroma formation in carcinoma in situ, invasive carcinoma, and metastatic carcinoma of the breast. *Clin Cancer Res* 1999;5:1041–56. [PubMed: 10353737]
14. Bachelder RE, Wendt MA, Mercurio AM. Vascular endothelial growth factor promotes breast carcinoma invasion in an autocrine manner by regulating the chemokine receptor CXCR4. *Cancer Res* 2002;62:7203–6. [PubMed: 12499259]
15. Shaner NC, Steinbach PA, Tsien RY. A guide to choosing fluorescent proteins. *Nat Methods* 2005;2:905–9. [PubMed: 16299475]
16. Harrell JC, Dye WW, Allred DC, et al. Estrogen receptor positive breast cancer metastasis: altered hormonal sensitivity and tumor aggressiveness in lymphatic vessels and lymph nodes. *Cancer Res* 2006;66:9308–15. [PubMed: 16982776]
17. Debnath J, Muthuswamy SK, Brugge JS. Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. *Methods* 2003;30:256–68. [PubMed: 12798140]
18. Kenny PA, Lee GY, Myers CA, et al. The morphologies of breast cancer cell lines in three-dimensional assays correlate with their profiles of gene expression. *Mol Oncol* 2007;1:84–96. [PubMed: 18516279]
19. Weigelt B, Bissell MJ. Unraveling the microenvironmental influences on the normal mammary gland and breast cancer. *Semin Cancer Biol* 2008;18:311–21. [PubMed: 18455428]
20. Moraes RC, Zhang X, Harrington N, et al. Constitutive activation of smoothened (SMO) in mammary glands of transgenic mice leads to increased proliferation, altered differentiation and ductal dysplasia. *Development* 2007;134:1231–42. [PubMed: 17287253]
21. Jones JL, Glynn P, Walker RA. Expression of MMP-2 and MMP-9, their inhibitors, and the activator MT1-MMP in primary breast carcinomas. *J Pathol* 1999;189:161–8. [PubMed: 10547569]
22. Guo P, Fang Q, Tao HQ, et al. Overexpression of vascular endothelial growth factor by MCF-7 breast cancer cells promotes estrogen-independent tumor growth in vivo. *Cancer Res* 2003;63:4684–91. [PubMed: 12907650]
23. Korach KS, Emmen JM, Walker VR, et al. Update on animal models developed for analyses of estrogen receptor biological activity. *J Steroid Biochem Mol Biol* 2003;86:387–91. [PubMed: 14623535]

24. Ferrara N, Hillan KJ, Novotny W. Bevacizumab (Avastin), a humanized anti-VEGF monoclonal antibody for cancer therapy. *Biochem Biophys Res Commun* 2005;333:328–35. [PubMed: 15961063]
25. Cebe-Suarez S, Zehnder-Fjallman A, Ballmer-Hofer K. The role of VEGF receptors in angiogenesis; complex partnerships. *Cell Mol Life Sci* 2006;63:601–15. [PubMed: 16465447]
26. Zachary I. Vascular endothelial growth factor and anti-angiogenic peptides as therapeutic and investigational molecules. *IDrugs* 2003;6:224–31. [PubMed: 12789611]
27. Lee TH, Seng S, Sekine M, et al. Vascular endothelial growth factor mediates intracrine survival in human breast carcinoma cells through internally expressed VEGFR1/FLT1. *PLoS Med* 2007;4:e186. [PubMed: 17550303]
28. Wen YJ, Mancino A, Pashov A, Whitehead T, Stanley J, Kieber-Emmons T. Antigen binding of human IgG Fabs mediate ERK-associated proliferation of human breast cancer cells. *DNA Cell Biol* 2005;24:73–84. [PubMed: 15699628]
29. Seddighzadeh M, Zhou JN, Kronenwett U, et al. ERK signalling in metastatic human MDA-MB-231 breast carcinoma cells is adapted to obtain high urokinase expression and rapid cell proliferation. *Clin Exp Metastasis* 1999;17:649–54. [PubMed: 10919709]
30. Hollestelle A, Elstrodt F, Nagel JH, Kallemeijn WW, Schutte M. Phosphatidylinositol-3-OH kinase or RAS pathway mutations in human breast cancer cell lines. *Mol Cancer Res* 2007;5:195–201. [PubMed: 17314276]
31. Ronnov-Jessen L, Petersen OW. Induction of alpha-smooth muscle actin by transforming growth factor-beta 1 in quiescent human breast gland fibroblasts. Implications for myofibroblast generation in breast neoplasia. *Lab Invest* 1993;68:696–707. [PubMed: 8515656]
32. Li B, Sharpe EE, Maupin AB, et al. VEGF and PlGF promote adult vasculogenesis by enhancing EPC recruitment and vessel formation at the site of tumor neovascularization. *FASEB J* 2006;20:1495–7. [PubMed: 16754748]
33. Weinberg, RA. *The biology of cancer*. Garland Science; New York: 2007.
34. Wiseman BS, Werb Z. Stromal effects on mammary gland development and breast cancer. *Science* 2002;296:1046–9. [PubMed: 12004111]
35. DeCosse JJ, Gossens CL, Kuzma JF, Unsworth BR. Breast cancer: induction of differentiation by embryonic tissue. *Science* 1973;181:1057–8. [PubMed: 4269415]
36. Cooper M, Pinkus H. Intrauterine transplantation of rat basal cell carcinoma as a model for reversion of malignant to benign growth. *Cancer Res* 1977;37:2544–52. [PubMed: 872081]
37. Bhowmick NA, Moses HL. Tumor-stroma interactions. *Curr Opin Genet Dev* 2005;15:97–101. [PubMed: 15661539]
38. Tlsty TD, Hein PW. Know thy neighbor: stromal cells can contribute oncogenic signals. *Curr Opin Genet Dev* 2001;11:54–9. [PubMed: 11163151]
39. Maffini MV, Soto AM, Calabro JM, Ucci AA, Sonnenschein C. The stroma as a crucial target in rat mammary gland carcinogenesis. *J Cell Sci* 2004;117:1495–502. [PubMed: 14996910]
40. Barcellos-Hoff MH, Ravani SA. Irradiated mammary gland stroma promotes the expression of tumorigenic potential by unirradiated epithelial cells. *Cancer Res* 2000;60:1254–60. [PubMed: 10728684]
41. Kim JB, Stein R, O'Hare MJ. Three-dimensional in vitro tissue culture models of breast cancer-- a review. *Breast Cancer Res Treat* 2004;85:281–91. [PubMed: 15111767]
42. Li ML, Aggeler J, Farson DA, Hatier C, Hassell J, Bissell MJ. Influence of a reconstituted basement membrane and its components on casein gene expression and secretion in mouse mammary epithelial cells. *Proc Natl Acad Sci U S A* 1987;84:136–40. [PubMed: 3467345]
43. Kunz-Schughart LA, Heyder P, Schroeder J, Knuechel R. A heterologous 3-D coculture model of breast tumor cells and fibroblasts to study tumor-associated fibroblast differentiation. *Exp Cell Res* 2001;266:74–86. [PubMed: 11339826]
44. Schmeichel KL, Bissell MJ. Modeling tissue-specific signaling and organ function in three dimensions. *J Cell Sci* 2003;116:2377–88. [PubMed: 12766184]
45. Hu Z, Fan C, Livasy C, et al. A compact VEGF signature associated with distant metastases and poor outcomes. *BMC Med* 2009;7:9. [PubMed: 19291283]

46. Sasser AK, Mundy BL, Smith KM, et al. Human bone marrow stromal cells enhance breast cancer cell growth rates in a cell line-dependent manner when evaluated in 3D tumor environments. *Cancer Lett* 2007;254:255–64. [PubMed: 17467167]
47. Bartek J, Iggo R, Gannon J, Lane DP. Genetic and immunochemical analysis of mutant p53 in human breast cancer cell lines. *Oncogene* 1990;5:893–9. [PubMed: 1694291]
48. Boudreau N, Myers C. Breast cancer-induced angiogenesis: multiple mechanisms and the role of the microenvironment. *Breast Cancer Res* 2003;5:140–6. [PubMed: 12793895]
49. Marty M, Pivot X. The potential of anti-vascular endothelial growth factor therapy in metastatic breast cancer: clinical experience with anti-angiogenic agents, focusing on bevacizumab. *Eur J Cancer* 2008;44:912–20. [PubMed: 18396037]
50. Ramaswamy B, Elias AD, Kelbick NT, et al. Phase II trial of bevacizumab in combination with weekly docetaxel in metastatic breast cancer patients. *Clin Cancer Res* 2006;12:3124–9. [PubMed: 16707611]
51. Miller K, Wang M, Gralow J, et al. Paclitaxel plus bevacizumab versus paclitaxel alone for metastatic breast cancer. *N Engl J Med* 2007;357:2666–76. [PubMed: 18160686]

Figure 1. BJ3Z cells are tumorigenic, stimulate angiogenesis and promote estrogen-independent tumor growth

A. mammary glands of ovx nude mice were injected with RedTomato tagged BJ3Z cells (top panels), ZsGreen tagged MCF-7 cells (central panels) or co-injected with both BJ3Z and MCF-7 cells (bottom panels). Mice were implanted with cellulose control (C; left) or 17 β -Estradiol (E₂) releasing silastic pellets. Tumors were photographed and representative pictures are shown. B. Tumor growth was monitored for 10 weeks for C (n=5) and E₂ (n=10) for each tumor type. Statistically significant differences (* p<0.05) were calculated by two-way ANOVA. C. Neovascularization of tumors was quantified by IHC of fluorescent CD34 positive cells in tumors, in the absence or presence of E₂; * p<0.05.

Figure 2. BJ3Z cells enhance proliferation of MCF-7 cells and of adjacent normal mammary tissue in the absence of E₂

A. Ovx nude mice were implanted in mammary glands with MCF-7 cells alone, or MCF-7/BJ3Z cell mixtures. Mice were supplemented with cellulose (C) or E₂-releasing pellets. Tumors were grown for 10 weeks. Slides were stained by IHC against CK18 (red) and BrdU (green). Magnification bar is 50μm. **B.** BrdU incorporation was quantified; * and # p<0.05 tested by ANOVA, followed by Tukey's post-test. **C.** Tumors were grown as above in mice receiving BJ3Z cells only, MCF-7 cells only or BJ3Z/MCF-7 cell mixtures; all in the absence of E₂. Slides from tumors were stained with H&E (left). Serial sections were stained by IHC for normal E-cadherin positive cells (red) and BrdU (green nuclei). **D.** Quantification of BrdU incorporation; * p<0.05.

Figure 3. B3Z cells enhance proliferation of MCF-7 and MCF10A but not MDA-MB-231 in 3D organoids

Eight-well slides were coated with Growth Factor Reduced Matrigel™. MCF-7, MCF10A, MDA-MB-231 and B3Z cells were plated on Matrigel alone or in the combinations shown and grown for 7 days. Organoids were paraffin embedded, and sections were cut for IHC. **A.** Left panels, light microscopy images of organoids at 7 days. Magnification bar is 100μm. Middle panels show dual IHC against CK18 (red) and BrdU (green) for MCF-7 cells; **B.** CK14 (red) and BrdU (green) for MCF10A cells; and **C.** CD44 (red) and BrdU (green) for MDA-MB-231 cells. Magnification bar is 50μm. Right panels show quantification of BrdU incorporation; * p<0.05.

Figure 4. BJ3Z cells grown in tumors and 3D organoids show similar expression profiles for stromal makers and secrete VEGF and TIMP-1
A. BJ3Z cells were grown *in vivo* as solid tumors in the mammary glands of ovx nude mice (tumor), or as 3D organoids *in vitro*. Sections of tumors and organoids were stained by IHC for Fibroblast Activation Protein (FAP); alpha-smooth muscle actin (α -SMA); stromal derived factor 1 (SDF-1); vimentin and with an anti-mouse epithelial panCK. Magnification bar is 50 μ m. B. BJ3Z cells were cultured in 3D as described for 2 days, then switched to serum-free MEM for 48 hrs. Controls consisted of a Matrigel-coated chamber without cells. Supernatants from control or BJ3Z cells were collected and hybridized to a TranSignal™ mouse angiogenesis antibody array. Lower panel lists the arrayed factors.

Figure 5. BJ3Z cells enhance proliferation of MCF-7 cells through VEGF and SDF-1; VEGF activates p-ERK

MCF-7 or MDA-MB-231 cells were grown alone or together with BJ3Z cells as 3D organoids. **A.** MCF-7/BJ3Z co-cultures were incubated with a blocking anti-mouse VEGF monoclonal antibody (VEGF MAb) or Avastin (Bevacizumab) or with the CXCR-4 blocker AMD 3100 for 7 days. On day 7, cells were processed as described and the proliferation rate was calculated using the CK18/BrdU proliferation assay; * $p < 0.05$. **B.** BrdU incorporation in MCF-7 and MDA-MB-231 monocultures treated with rVEGF for 7 days; * $p < 0.05$. **C.** Seven day-old MCF-7 or MDA-MB-231 organoids were treated 10 min with vehicle (control) or rVEGF then processed and stained by IHC against p-ERK; magnification bar is 50 μ m.