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Hormonally responsive breast cancer cells in a microfluidic co-culture model as a sensor of microenvironmental activity

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Abstract

Breast cancer cell growth and therapeutic response are manipulated extrinsically by microenvironment signals. Despite recognition of the importance of the microenvironment in a variety of tumor processes, predictive measures that incorporate the activity of the surrounding cellular environment are lacking. In contrast, tumor cell biomarkers are well established in the clinic. Expression of Estrogen Receptor-alpha (ER α) is the primary defining feature of hormonally responsive tumors and is the molecular target of therapy in the most commonly diagnosed molecular subtype of breast cancer. While a number of soluble factors have been implicated in ER α activation, the complexity of signaling between the cellular microenvironment and the cancer cell implies multivariate control. The cumulative impact of the microenvironment signaling, which we define as microenvironmental activity, is more difficult to predict based on the sum of its parts. Here we tested the impact of an array of microenvironments on ER α signaling utilizing a microfluidic co-culture model. Quantitative immunofluorescence was employed to assess changes in ER α protein levels, combined with gene expression and phosphorylation status, as measures of activation. Analysis of microenvironment-induced growth under the same conditions revealed a previously undescribed correlation between growth and ER α protein down-regulation. These data suggest an expanded utility for the tumor biomarker ER α , in which the combination of dynamic regulation of ER α protein and growth in a breast cancer biosensor cell become a read-out of the microenvironmental activity.

Introduction

The microenvironment is a complex set of cellular, structural, and secreted components essential for homeostasis¹. In cancer, this tightly controlled environment is often altered in parallel or directly by the tumor^{2–4}, resulting in changes in the regulation of processes such as proliferation^{5–6}, cell survival^{7–8}, and metastasis^{9–10}. Breast cancer cell growth is clearly modulated by cellular microenvironment components, especially fibroblasts^{11–12}. Decades of research on the microenvironment's role in breast cancer have sought to identify candidate factors that stimulate tumor growth. Yet cumulatively, these studies highlight that multiple extracellular signals have redundant effects on a number of tumor processes, presenting significant challenges for developing new therapies. Candidate factors, such as Epidermal Growth Factor^{13–15}, neuregulins¹⁶, and Insulin-like Growth Factor-1^{17–19}, have

been identified using a number of approaches, from isolation from conditioned media to differential gene expression in tumors. However, the cumulative effects of biochemical properties of the extracellular environment on biological processes, or the microenvironmental activity, is challenging to understand and predict by examining the isolated effects of its parts. The complexity challenges the successful integration of measures of the microenvironment in the clinic, where biomarkers in the tumor cells are the gold standard. We postulated that biomarker dynamics in the breast cancer cells themselves might be used as a biosensor of the total microenvironmental activity. If correct, such an approach would be amenable to personalized medicine, as the patient's own biopsy samples could serve as the source of microenvironmental activity for screening.

Luminal breast cancers account for 50–80% of diagnosed breast cancer. These tumors are defined by their hormone-responsive profile, characterized by the expression of Estrogen Receptor-alpha (ER α), the primary therapeutic target in these breast cancers. The activity of this transcription factor is stimulated upon binding to its ligand, estrogen. Upon activation, the receptor targets a number of genes to either repress (ER α , *ESR1*^{20–21}) or activate (Progesterone Receptor, *PGR*^{22–23}; pS2, *TFF1*²⁴) transcription. ER α protein levels decrease upon activation through both the down-regulation of its gene expression and proteolytic degradation of the receptor²⁵. The full activity of ER α is dependent on Serine-118²⁶, which is phosphorylated upon receptor activation^{27–28}. ER α can also be activated in the absence of estrogen through such phosphorylation. Several extracellular factors have been implicated in estrogen-independent activity^{29–32}, but the cumulative effect of multivariate microenvironment signaling components on ER α signaling is likely to be more complex. Selective ER α modulators (SERMs; e.g. Tamoxifen) and aromatase inhibitors (e.g. Anastrozole), are two major classes of hormone therapy used in the treatment of ER α -positive breast cancer, and target the estrogen-dependent but not the estrogen-independent activity. It is widely considered that the estrogen-independent activity of ER α underlies therapy resistance.

Cell culture models have been instrumental in the investigation of hormone responsive breast cancer and provide insight into therapy response. The MCF-7 cell line has been used extensively to model ER α -positive breast cancer both *in vitro* and in animal xenografts. Importantly, this cell model faithfully recapitulates estrogen-dependent growth and ER α activation and regulation seen *in vivo*. MCF-7 cells accurately model responses to hormone therapies observed in the clinic, making them an ideal candidate for a biosensor model, which integrates complex extracellular signals from a cellular microenvironment to identify changes in ER α signaling and proliferation.

Passive pumping microfluidics miniaturizes cell culture vessels to a volume on the order of microliters while maintaining operation using conventional lab supplies³³. In traditional cell culture, large media volumes rapidly dilute secreted molecules. At the microscale, diffusion dominates the distribution of molecules in the media, and paracrine factors remain in close proximity to the cells, improving biological sensitivity³⁴ and control over the extracellular signaling environment^{35–36}. The high-throughput capacity facilitates testing of complex mixtures of variables (multiple co-culture pairs, pharmacologic manipulation, screening, etc.) and increased numbers of replicates³⁷. This enables direct comparisons within a panel

of co-culture conditions in a single experiment, allowing side-by-side comparison of an array of microenvironment conditions.

Previously, the culture of small quantities of cells was limited by the availability of assays with sensitivity to detect and measure various biological endpoints. Detection of changes in protein levels is made possible by the quantification of immunofluorescence images acquired under appropriate conditions^{38–39}. Additionally, image based quantification permits examination of protein distribution within a population using single-cell analysis techniques. Here we use quantitative immunofluorescence in an unbiased, microfluidic co-culture approach to a biosensor cell; that is, measuring the dynamics of ER α protein in the breast cancer cells under distinct mixtures of multivariate, extracellular signals from a diverse panel of cell lines representing. We demonstrate that decreases in ER α protein indicative of activation directly correlate with growth and therefore act as sensors for microenvironmental activity. This dynamic biomarker measurement defines subpopulations of microenvironments that generate predictions on hormone therapy response.

Methods and Materials

Cell lines and culture

Unless indicated otherwise, cells were maintained in media containing phenol red and L-glutamine supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 100 units/mL of penicillin, and 100 μ g/mL streptomycin. Reagents were from Life Technologies (Carlsbad, CA, USA) if not indicated. MCF-7eGFP, HS-5, MDA-MB-231, MEF, MDA-MB-468, HS578T, HEK293, RMF/EG, and MRC-5 cells were cultured in high glucose DMEM (Mediatech, Inc., Herndon, VA, USA). THP-1, SK-BR-3, and T47D cells were cultured in RPMI 1640. CV-1 and BT-20 cells were cultured in MEM-alpha. MCF10A cells were cultured in DMEM/F12 containing 5% horse serum (Sigma-Aldrich, St. Louis, MO, USA) and supplemented with 20ng/mL epidermal growth factor (Sigma-Aldrich), 500ng/mL hydrocortisone (BD Biosciences, San Jose, CA, USA), and 10 μ g/mL insulin (Sigma-Aldrich). MCF-7eGFP cells were generated by stable transfection of MCF-7 cells with the pEGFP-N1 and pBABEpuro plasmids using Lipofectamine 2000 according to the manufacturer's instructions, and selected with 1 μ g/mL puromycin (Sigma-Aldrich).

Co-culture and hormone treatments

Prior to all experiments, cells were incubated in estrogen-deprived, phenol red-free media supplemented with 10% charcoal dextran stripped FBS, penicillin/streptomycin, and L-glutamine for 3 days and for the duration of the experiment. For qRT-PCR assays, MCF-7 cells were cultured alone or with HS-5 cells (2:1 ratio) at a total cell concentration of 4×10^5 cells/mL in a 6-well plate (2mL total volume), and harvested after 24 hours for RNA isolation. For fluorescence growth assay, MCF-7eGFP cells were cultured alone or with various cell lines (2:1 ratio) at a total cell concentration of 3.3×10^4 cells/mL in a 96-well plate (150 μ L total volume) and cultured for a period up to 5 days. For single microchannel assays, MCF-7eGFP cells were cultured alone or with various cell lines (2:1 ratio) at a total cell number of 1.6×10^4 cells per channel (4 μ L total volume) for 24 hours. For compartmentalized microfluidic assays, 1.6×10^4 MCF-7 cells were cultured alongside

1.6×10⁴ MCF-7 or HS-5 cells for 24 hours. Where hormone treatment is indicated, vehicle (0.1% ethanol; EtOH) or 17β-estradiol (E2; Steraloids, Inc., Newport, RI, USA) was added for the last 8 hours. In the macro-culture experiments, 10nM E2 was used as a saturating dose. Microfluidic experiments were treated with an increased dose of 100nM E2 to account for (1) polydimethyl siloxane (PDMS) adsorption of steroids from the media⁴⁰ and (2) non-saturating number of molecules in the smaller media volume per cell. This dose was determined to be the lowest dose at which a consistent estrogen response could be demonstrated in these devices. Treatments with 100nM Tamoxifen (OHT; Sigma-Aldrich) and 10nM-10μM Anastrozole (Ana; Sigma-Aldrich) were performed for the 24 hour duration of the co-culture.

Fluorescence growth assay

MCF-7eGFP cells were co-cultured with the cell line array (specific cell lines described in Figure 1B) in triplicate in a white, opaque 96-well plate for 4 days. One day after seeding cells and each following day of culture, GFP fluorescence intensity was measured using a BioTek Synergy4 plate reader and Gen4 software (Winooski, VT, USA). Each day's measurement was normalized to its initial reading at day 1. The mean and standard deviation of the triplicate wells was calculated, and the Wilcoxon rank sum test was used to compare the means of three independent experiments using the Mstat program⁴¹.

qRT-PCR

Total RNA isolation, reverse transcription and qRT-PCR were performed as previously described⁴². E-cadherin mRNA (*CDH1*) served as a control for quantity of MCF-7 cells. Primer sequences are as follows: *ESR1* fwd 5'-CCTGATGATTGGTCTCGTCTG-3' rev 5'-GGCACACAACTCCTCTCC-3'; *PGR* fwd 5'-GCTGTCATTATGGTGTCTTAC-3' rev 5'-GTAGTTGTGCTGCCCTTCC-3'; *TFF1* fwd 5'-CGCCTTTGGAGCAGAGAG-3' rev 5'-ACCACAATTCTGTCTTTTACG-3'; *CDH1* fwd 5'-TATTGAAAGAGAAACAGGATGG-3' rev 5'-GGATGGTGTAAAGCGATGG-3'. Relative RNA levels were calculated using the delta C_t method⁴³. The Wilcoxon signed rank test was used to determine significance using the Mstat program⁴⁴.

Microchannel fabrication

Microchannels were cast from polydimethyl siloxane (PDMS; Sylgard 184, Dow Corning, Midland, MI, USA) using traditional soft lithography methods. Briefly, relief molds were generated through selective exposure of a UV-curable epoxy (SU-8, Microchem, Newton, MA, USA) followed by development in propylene glycol. PDMS monomer was blended with hardener at 10:1 ratio, degassed for >30 minutes in a vacuum, poured onto the mold, and cured for >3 hours at 80°C. Once cast, PDMS was peeled from the mold and bonded to pre-cleaned glass slides. PDMS/glass bonding was promoted by plasma treating in O₂ for 1 minute, contacting the PDMS and glass layers, then annealing at 135°C for >2 minutes.

Immunofluorescence

Immunofluorescence staining was performed in single or compartmentalized microfluidic devices immediately following culture. Reagent addition and washes were performed with

TBS (unless otherwise noted) by complete displacement of the original microchannel fluid content. Cells were fixed with 4% formaldehyde (Sigma-Aldrich) for 15 minutes, washed, permeabilized for 30 minutes in 0.2% Triton X-100 (Sigma-Aldrich), then washed. A 10% goat serum (Sigma-Aldrich) solution in TBST was added for 1 hour for blocking, followed by addition of primary antibody in 5% goat serum in TBST overnight at 4°C. Primary antibodies used are as follows: ER α 6F11 mouse monoclonal antibody, 1:200 (Vector Laboratories, Inc. Burlingame, CA, USA), ER α phospho-Serine-118 mouse monoclonal antibody, 1:200 (Cell Signaling, Danvers, MA, USA). Microchannels were washed with TBS, and Alexa Fluor 594 goat anti-mouse antibody in 5% goat serum in TBST was incubated for one hour. Following washing, 5 μ g/mL Hoechst 33342 was added for 30 minutes, and channels were washed and stored in TBS until microscopy was performed.

Image acquisition

Images of immunofluorescence staining were acquired using a Nikon Eclipse Ti inverted fluorescence microscope and NIS Elements software. For each experiment, individual microchannels were scanned using each excitation filter to determine optimal exposure settings for each filter such that it neither saturated highest intensity staining nor failed to detect lowest intensity staining, thus allowing the most dynamic range of detection. Once settings were established, each channel was imaged for a field of view using 15x magnification on each of three filters (for Hoechst, GFP, and Alexa Fluor 594).

Image analysis

Images were analyzed using ImageJ software⁴⁵ (NIH, Bethesda, MD, USA). All images were opened simultaneously and compiled into a stack to ensure equivalent image processing. First, background subtraction was performed using a rolling ball filter to eliminate gradual pixel intensity changes throughout each image. The bottom 2% of pixels, which contribute to low background throughout the images, was eliminated by setting minimum pixel intensity to 5. The GFP images were then isolated to create GFP masks outlining MCF-7eGFP cell position. These masks were then used to remove non MCF-7 cell staining from nuclear and target protein (i.e. ER α) images. For population analysis, the integrated intensity of nuclear and target protein images were measured and analyzed in Excel by normalizing target protein intensity to nuclear intensity as a cell number correction. Fold changes of target protein expression were calculated relative to mono-culture controls. Within each experiment, 5–10 replicates were performed, and the Wilcoxon rank sum test was performed in Mstat for statistical significance. W test statistics were then combined from independent experiments to determine significance. For single-cell analysis, particle analysis was performed on nuclear images after GFP mask subtraction to define single nuclei and exclude overlapping nuclei. The regions of interest defining individual nuclei were redirected to the ER α protein images to measure signal intensity from individual nuclei. To generate a histogram, bins of ER α staining intensity were created, and frequency of nuclei in each bin was weighted for the total number of nuclei in each condition to generate a percentage of total nuclei per bin.

Fluorescence linearity determination

To determine the linear range of fluorescence intensity in image analysis, cells in single microchannels were substituted with 1.6×10^4 beads/mL InSpeck Green fluorescent microspheres (Molecular Probes, Eugene, OR, USA) with a diameter of 6 μ m. These microspheres have known relative fluorescence intensities designed for generating calibration curves. Image acquisition and analysis were performed as described above.

Western blots

Western blots were performed using the Li-Cor Odyssey system, as per manufacturers instructions (Li-Cor, Lincoln, NE, USA). Blocking was performed in EMD Millipore Block-FL buffer (Billerica, MA, USA), which was also diluted 1:1 with TBS for use in all antibody incubations. Blots were incubated overnight with ER α H-184 rabbit polyclonal antibody (1:1,000; Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA) and E-cadherin mouse monoclonal antibody (1:1,000, BD Biosciences). IRDye 680CW anti-mouse and IRDye 800 anti-rabbit secondary antibodies (1:10,000; Li-Cor) were used after primary antibody incubation. A Li-Cor Odyssey infrared imager and software v3.0 were used to scan and quantify blots.

Results

Breast cancer cell growth in co-culture with an array of cell types

While enhanced cell growth is a defining property of cancer, the complete understanding of how the microenvironment influences growth has been technically challenging. Commonly used growth and proliferation assays, such as MTT assays and cell counting, cannot selectively measure a subpopulation of cells in a mixed culture. Assays that permit growth measurements in select cell subpopulations, such as flow cytometry, are complicated to scale to high-throughput analysis, necessitating miniaturization to the microscale. Stable expression of enhanced green fluorescent protein (eGFP) in the MCF-7eGFP cell line allows selective and technically simple growth measurement in a scalable assay. Fluorescence readings are proportional to the number of cells in a 96-well plate (Supplemental Figure 1). To probe the microenvironmental activity of a model of bone metastasis, MCF-7eGFP cells were co-cultured directly with bone marrow stromal cell line, HS-5, in estrogen-free media for 5 days, and fluorescence was measured daily. HS-5 cells significantly stimulated the growth of MCF-7eGFP cells, detectable by the third day of culture (Figure 1A). The HS-5 cell model serves as proof-of-principle that growth of hormonally responsive cells can be stimulated in a microenvironment model.

This assay was expanded to characterize a panel of diverse cell types in co-culture with MCF-7eGFP cells. The cell lines described in Figure 1B were selected for the co-culture array to define patterns based on cell type and organ of origin in unbiased models of microenvironmental activity. Two non-human cell lines were included as further unbiased models of extracellular signaling in the induction of growth. The eleven cell lines were individually co-cultured with MCF-7eGFP cells in parallel experiments, allowing direct comparison between cell lines on the growth outcome of MCF-7 cells. A spectrum of growth stimulation was observed (Figure 1B). Surprisingly, both epithelial cells and

fibroblasts were capable of stimulating growth, without being a universal feature of either cell type. Both of the non-human cell lines, the CV-1 (African Green Monkey) and MEF (mouse) significantly stimulated growth, indicating that the source of the signaling, that is, the specific cell type, does not alone dictate the permissiveness of the microenvironmental activity. Additionally, the unbiased approach of multiple, diverse co-cultures highlights the spectral effects on growth that may result both from the expression levels of specific growth factors and the combinatorial effects of multiple factors.

Transcriptional profile of estrogen-responsive genes

In models of hormonally responsive breast cancer, such as MCF-7 cells, growth is dependent on ER α signaling activation upon estrogen treatment. The growth observed in co-culture conditions lacking estrogen and its precursors suggest that ER α may be activated independent of ligand. ER α is a transcriptional regulator of a large set of genes in response to both ligand-dependent and -independent signaling. To test whether ER α was indeed activated, direct co-culture of MCF-7eGFP cells was performed with HS-5 cells, which induced the highest growth stimulation, and qRT-PCR analysis examined the changes in expression of ER α target genes in comparison to mono-culture. As the total RNA isolated included both MCF-7eGFP and HS-5 cell RNA, E-cadherin (*CDH1*) was used as an epithelial-specific reference gene to control for total MCF-7eGFP RNA in the sample. This analysis revealed a decrease in the gene encoding ER α (*ESR1*), which is consistent with a previously reported macrophage co-culture model⁴⁶. Additionally, the transcriptional profile demonstrated an increase in expression of genes encoding the Progesterone Receptor (*PGR*), and pS2 (*TFF1*) (Figure 2), which are dependent on ER α activation for expression. *ESR1*, *PGR*, and *TFF1* genes were not expressed in HS-5 cells, thus represent differential expression in the MCF-7eGFP cells only.

ER α protein dynamics in heterotypic cell culture

Clinically, ER α expression in breast cancers predicts the dependence of the tumor on estrogen for growth. ER α expression, however, is dynamic in response to activation, either in response to estrogen or ligand-independent signals. The gene expression profile of ER α target genes observed in MCF-7 co-cultures with bone marrow stromal cells indicates ER α activation. Thus, we would expect that target gene expression changes coincide with additional markers of activation, such as ER α protein down-regulation^{20–21, 25, 47–48} and phosphorylation²⁷. Microfluidic device culture and image analysis were optimized to assess ER α expression and signaling (Figure 3A). Two specific devices are used here: the single microchannels and the two-channel compartmentalized device. Single microchannels directly co-culture cells in a total volume of 4 μ L and were the predominant devices used here for their maximal sensitivity and high-throughput. The two-channel compartmentalized device, with a total volume of 40 μ L, was used specifically to test the requirement of cell contact for the biological endpoints.

The ability to detect changes in protein levels by image analysis was validated using two methods. First, a dilution of MCF-7eGFP cells in single microchannels demonstrates that eGFP fluorescence intensity is linearly related to cell number, similar to growth assays (Supplemental Figure 2A). Second, InSpeck microsphere beads with established relative

intensity levels demonstrate that protein dynamics can also be detected to a 10^2 order of magnitude change with reasonable linearity (Supplemental Figure 2B). The workflow for a typical protein quantification assay by image analysis is summarized in Figure 3B. Proteins of interest are first labeled by standard immunofluorescence, and microscopy exposure settings maximizing the dynamic range are selected. Following background subtraction, image intensities were measured using ImageJ software. As with growth assays, the expression of eGFP provides an internal normalization and spatial identification of MCF-7eGFP cells, permitting measurement of target signal selectively from MCF-7 cells in heterotypic cultures.

To specifically model the interaction between breast cancer cells and the relevant stromal microenvironments of both primary and metastatic breast cancer, MCF-7 cells were co-cultured with bone marrow HS-5 cells, mammary fibroblast cell line RMF/EG, and lung fibroblast cell line MRC-5 for 24 hours in single microchannels, treated with or without 100nM 17 β -estradiol (E2) for the last 8 hours, then stained for ER α protein (representative images in Figure 4A). Surprisingly, quantification of ER α protein levels detected a significant decrease in all three co-culture conditions, as well as in response to the E2 positive control, relative to mono-cultured cells (Figure 4B). An additional ER α -positive cell line, T47D, similarly down-regulated ER α protein levels upon co-culture with HS-5 cells, which was demonstrated in traditional macro-scale culture and Western blot (Supplemental Figure 3). A further decrease in MCF-7 ER α levels upon treatment with E2 was seen only in co-culture with RMF/EG cells, although HS-5 cells displayed a similar trend. Treatment of MRC-5 co-cultures, however, resulted in an intermediate decrease between E2-treated MCF-7eGFP cells and MRC-5 co-culture. This could suggest that the co-cultures differ in the signaling pathways used to activate ER α , as signaling from MRC-5 cells appears to preclude estrogen-dependent activation of the receptor, whereas HS-5 and RMF/EG signaling appears to overlap with estrogen signaling. In the following experiments, HS-5 cells were more closely examined as the most robust model for inducing ER α , as it is an ideal model for looking more specifically at ER α signaling and a useful reference point for experiments that expand on the co-culture models examined.

All co-cultures were conducted in media stripped of all steroid molecules, including precursors of estradiol to avoid estrogen synthesis. To test the possibility of ligand-dependent activation further, MCF-7 co-cultures with HS-5 were treated with the competitive inhibitor for estrogen binding, Tamoxifen (OHT), which did not preclude the down-regulation of ER α in HS-5 co-cultures (Supplemental Figure 4A), indicating that estrogen is not stimulating the ER α signaling. Additionally, treatment with the aromatase inhibitor Anastrozole (Ana), which blocks the enzyme responsible for converting androgens into estrogens, did not recover ER α expression in HS-5 co-cultures (Supplemental Figure 4B), again, indicating that estrogens synthesized by the aromatase-expressing HS-5 cells are not stimulating the ER α pathway.

HS-5 and MDA-MB-231 cells demonstrated high growth stimulation and ER α down-regulation, and were therefore selected as sensitive models for further analysis. The two-channel compartmentalized device, allows diffusion-based communication between two cell culture regions through diffusion ports measuring 10 μ m high and 100 μ m wide. The utility of

this device is analogous to commercially available membrane inserts, and further benefits from the ability to image both culture regions in the same plane with enhanced sensitivity³⁴. Co-culture in compartmentalized microfluidic devices demonstrates that down-regulation of ER α protein does not require heterotypic cell-cell contact between MCF-7eGFP cells and MDA-MB-231 cells, and must occur through soluble factors (Figure 4C). After 24 hours of co-culture with HS-5 cells in single microchannels, immunofluorescence image analysis of MCF-7eGFP cells detects an increase in the level of phosphorylation of ER α at Serine-118 (S118), a site essential for full activity of the receptor^{26, 49}(Figure 4D). Note that the levels of phosphorylated S118 are not normalized to total ER α protein, and thus reflect a large shift in the proportion of phosphorylated ER α protein since the total ER α levels are decreased at this time point. The total ER α antibody used in immunofluorescence staining does not discriminate between unphosphorylated and phosphorylated ER α ⁵⁰. ER α down-regulation was hereafter selected as a surrogate marker of ER α activation in following co-culture assays based on the transcriptional profile of ER α activation and the presence of phosphorylation consistent with receptor activation.

Single-cell analysis to examine intra-population ER α distribution

Using single-cell analysis, protein levels can be quantified per cell, providing information on the distribution of protein expression within a population. Following measurement of ER α staining within the boundaries created by nuclear staining, a histogram of ER α staining intensity describes the distribution of ER α protein within a population (Figure 5). To account for variations in MCF-7 cell number in each culture condition, the frequency of nuclei expressing each ER α expression level was normalized to the total number of particles in each condition, and therefore the area under each raw curve is equal to one. The height and width of the histogram therefore describe the degree of heterogeneity in expression, where a taller, narrower peak describes more homogenous expression in the population. Surprisingly, we found that monocultured MCF-7 cells had some degree of heterogeneity in ER α protein expression per cell, despite the clonal origin of cell lines. Upon co-culture with HS-5 cells, the ER α protein levels not only decrease, but become more homogenous within the co-cultured population. Similar trends were observed with CV-1 cells (data not shown), which also demonstrated a high growth stimulation of MCF-7 cells.

Relating ER α protein levels to growth in co-culture

While the cell panel did not reveal patterns of microenvironmental activity based on cell type or organ origin on growth of MCF-7eGFP in co-culture, combining ER α protein dynamics as a measure of ER α activity with growth outcome infers the role of ER α signaling in growth stimulation from the microenvironmental activity. ER α protein in MCF-7eGFP cells co-cultured with the cell panel in single microchannel devices was quantified by immunofluorescence image analysis. As in the growth data, a spectrum of changes in ER α protein levels was observed with no clear pattern based on cell type or original organ site (Figure 6A). However, when ER α levels were plotted against their paired growth effect, a significant correlation was observed (Figure 6B). Intriguingly, two data points, representing HS-5 and MEF co-cultures, lie farthest above the line of best fit. Additional axes were superimposed on the plot to distinguish samples that had significant changes in growth (horizontal axis) and/or ER α down-regulation (vertical axis). These axes

form four quadrants that lead to distinct patterns of growth. Those samples without growth or ER α changes suggest that the microenvironmental activity of these co-culture pairs is inert in terms of these parameters. In the upper-left quadrant, ER α down-regulation correlates with growth stimulation in the MCF-7 cells, which would lead to the hypothesis that in these co-cultures the microenvironmental activity promotes ligand-independent activation of the receptor, which is driving the proliferation. Interestingly, one co-culture falls in the upper-right quadrant, suggesting that MEF cells may stimulate the growth of MCF-7 cells by an alternative, ER α -independent pathway. Encouragingly, no cell lines lie in the quadrant that would suggest that ER α activity is not related to growth.

Discussion

Clinically, ER α expression in tumor cells is used as a predictive measure of the effectiveness of hormonal therapies that target and inactivate ER α . In contrast to early stage breast cancer, metastatic tumors exist in unique non-native environments such as bone, liver, pleura, and brain. These unnatural contexts generate specific microenvironments that interact with tumor cells via complex and varying signaling events, making it challenging to assess cumulative effects. Here, we show a correlation between the degree of growth stimulation and ER α down-regulation upon co-culture with a cell line array, which has a number of novel implications. While the outcomes of ER α protein down-regulation and growth stimulation are well known responses to ER α activation, the surprisingly linear relationship between these two outcomes in response to diverse signaling inputs has not been realized previously. This finding implies that dynamic measure of ER α protein levels may have utility as a sensor to predict how cell-extrinsic signaling modulates growth of hormonally-responsive breast cancer cells. Further, the data indicate that the microenvironmental activity may initiate growth through ligand-independent, and potentially SERM-refractory, mechanisms. Thus, the expansion of ER α from a tumor biomarker to a dynamic microenvironment biosensor capitalizes on our current knowledge and incorporates a functional measure of microenvironmental activity, which could better inform therapy decisions.

The novel approaches utilized were instrumental in allowing detection of the correlation between ER α and growth. The small scale of microfluidic culture enabled high-throughput operation amenable to the cell culture array, allowing a number of replicates and conditions with fewer resources than traditional culture scales. The use of image analysis in measuring protein levels aids in the downstream detection of endpoints with distinct advantages over other approaches. In comparison to traditional approaches such as Western blots, image analysis is more scalable to high-throughput assays without significantly adding to the workload and time to analysis. The spatial information provided by image analysis provides an additional level of information to incorporate into analysis. Here we have described how this aids in selective detection of the target protein using cell markers and masking techniques and the ability to measure protein at the cellular level. Additional utilities of the spatial parameter may include detection of gradient effects within a population in response to a given signal and co-localization of additional proteins, such as proliferation markers, to a single cell. Ultimately, the combination of the microfluidic system with canonical model

of hormonally responsive breast cancer, MCF-7 cells, creates an assay of the microenvironmental activity of the breast cancer cellular context.

Closer examination of the ER α vs. growth plot reveals that two heterotypic cell culture conditions appear to lie farther from the general trend. Interestingly, both conditions demonstrate disproportionate growth stimulation when compared to the level of ER α down-regulation, consistent with an ER α -independent mode of growth. This could occur through stimulation of alternative signaling pathways for growth, bypassing the requirement for ER α . These outliers may therefore represent a microenvironmental activity that is hormone therapy refractive. *In vitro* testing is underway to determine whether ER α is necessary for growth stimulated by microenvironmental activity and if ER α dynamics predict hormone therapy response. Our growth and ER α protein assays are ideal for studies using human primary cells^{51–52}, in which the microenvironmental activity surrounding the tumor could be sampled in co-culture with MCF-7 cells, serving as biomarker cells for ER α activation. The results presented here serve as a proof-of-principle for the feasibility of such assays. Such an approach may be linked to clinical responses in the future to test predictions of therapy response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Nelson CM, Bissell MJ. *Annu Rev Cell Dev Biol.* 2006; 22:287–309. [PubMed: 16824016]
2. Moinfar F, Man YG, Arnould L, Bratthauer GL, Ratschek M, Tavassoli FA. *Cancer Res.* 2000; 60:2562–6. [PubMed: 10811140]
3. Hanahan D, Weinberg RA. *Cell.* 2011; 144:646–74. [PubMed: 21376230]
4. Bissell MJ, Hines WC. *Nat Med.* 2011; 17:320–9. [PubMed: 21383745]
5. Camps JL, Chang SM, Hsu TC, Freeman MR, Hong SJ, Zhau HE, von Eschenbach AC, Chung LW. *Proc Natl Acad Sci U S A.* 1990; 87:75–9. [PubMed: 2296606]
6. van Roozendaal CE, van Ooijen B, Klijn JG, Claassen C, Eggermont AM, Henzen-Logmans SC, Foekens JA. *Br J Cancer.* 1992; 65:77–81. [PubMed: 1733444]
7. Neville-Webbe HL, Cross NA, Eaton CL, Nyambo R, Evans CA, Coleman RE, Holen I. *Breast Cancer Res Treat.* 2004; 86:269–79. [PubMed: 15567943]
8. Chen Q, Zhang XH, Massague J. *Cancer Cell.* 2011; 20:538–49. [PubMed: 22014578]
9. Karnoub AE, Dash AB, Vo AP, Sullivan A, Brooks MW, Bell GW, Richardson AL, Polyak K, Tubo R, Weinberg RA. *Nature.* 2007; 449:557–563. [PubMed: 17914389]
10. Corcoran KE, Trzaska KA, Fernandes H, Bryan M, Torga M, Srinivas V, Packman K, Patel PS, Rameshwar P. *PLoS One.* 2008; 3:e2563. [PubMed: 18575622]
11. Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL, Weinberg RA. *Cell.* 2005; 121:335–48. [PubMed: 15882617]

12. Bauer M, Su G, Beebe DJ, Friedl A. *Integr Biol*. 2010; 2:371–8.
13. Hofland LJ, van der Burg B, van Eijck CH, Sprij DM, van Koetsveld PM, Lamberts SW. *Int J Cancer*. 1995; 60:93–9. [PubMed: 7529213]
14. Briand P, Lundholt BK, Skouv J, Lykkesfeldt AE. *Mol Cell Endocrinol*. 1999; 153:1–9. [PubMed: 10459848]
15. Loi S, Sotiriou C, Haibe-Kains B, Lallemand F, Conus NM, Piccart MJ, Speed TP, McArthur GA. *BMC Med Genomics*. 2009; 2:37. [PubMed: 19552798]
16. Kung W, David F, Langen H, Weyer KA, Schlaeger EJ, Lahm HW, Silber E, Mueller H, Eppenberger U. *Biochem Biophys Res Commun*. 1994; 202:1357–65. [PubMed: 8060314]
17. Stewart AJ, Johnson MD, May FE, Westley BR. *J Biol Chem*. 1990; 265:21172–8. [PubMed: 2174437]
18. Bartucci M, Morelli C, Mauro L, Ando S, Surmacz E. *Cancer Res*. 2001; 61:6747–54. [PubMed: 11559546]
19. Fox EM, Miller TW, Balko JM, Kuba MG, Sanchez V, Smith RA, Liu S, Gonzalez-Angulo AM, Mills GB, Ye F, Shyr Y, Manning HC, Buck E, Arteaga CL. *Cancer Res*. 2011; 71:6773–84. [PubMed: 21908557]
20. Read LD, Greene GL, Katzenellenbogen BS. *Mol Endocrinol*. 1989; 3:295–304. [PubMed: 2785242]
21. Pink JJ, Jordan VC. *Cancer Res*. 1996; 56:2321–30. [PubMed: 8625307]
22. Nardulli AM, Greene GL, O'Malley BW, Katzenellenbogen BS. *Endocrinology*. 1988; 122:935–44. [PubMed: 3342760]
23. Schultz JR, Petz LN, Nardulli AM. *Mol Cell Endocrinol*. 2003; 201:165–75. [PubMed: 12706304]
24. Kim J, Petz LN, Ziegler YS, Wood JR, Potthoff SJ, Nardulli AM. *J Steroid Biochem Mol Biol*. 2000; 74:157–68. [PubMed: 11162921]
25. Alarid ET, Bakopoulos N, Solodin N. *Mol Endocrinol*. 1999; 13:1522–34. [PubMed: 10478843]
26. Le Goff P, Montano MM, Schodin DJ, Katzenellenbogen BS. *J Biol Chem*. 1994; 269:4458–66. [PubMed: 8308015]
27. Ali S, Metzger D, Bornert JM, Chambon P. *EMBO J*. 1993; 12:1153–60. [PubMed: 8458328]
28. Bunone G, Briand PA, Mikscek RJ, Picard D. *EMBO J*. 1996; 15:2174–83. [PubMed: 8641283]
29. Smith CL. *Biol Reprod*. 1998; 58:627–32. [PubMed: 9510949]
30. Nicholson RI, Hutcheson IR, Britton D, Knowlden JM, Jones HE, Harper ME, Hiscox SE, Barrow D, Gee JM. *J Steroid Biochem Mol Biol*. 2005; 93:257–62. [PubMed: 15860268]
31. Baron S, Escande A, Alberola G, Bystricky K, Balaguer P, Richard-Foy H. *J Biol Chem*. 2007; 282:11732–41. [PubMed: 17317669]
32. Sauve K, Lepage J, Sanchez M, Heveker N, Tremblay A. *Cancer Res*. 2009; 69:5793–800. [PubMed: 19584281]
33. Walker GM, Beebe DJ. *Lab Chip*. 2002; 2:131–4. [PubMed: 15100822]
34. Domenech M, Yu H, Warrick J, Badders NM, Meyvantsson I, Alexander CM, Beebe DJ. *Integr Biol*. 2009; 1:267.
35. Walker GM, Zeringue HC, Beebe DJ. *Lab Chip*. 2004; 4:91–7. [PubMed: 15052346]
36. Young EW, Beebe DJ. *Chem Soc Rev*. 2010; 39:1036–48. [PubMed: 20179823]
37. Meyvantsson I, Beebe DJ. *Annu Rev Anal Chem*. 2008; 1:423–49.
38. Ortiz De Solorzano C, Costes S, Callahan DE, Parvin B, Barcellos-Hoff MH. *Microsc Res Tech*. 2002; 59:119–27. [PubMed: 12373722]
39. Puccinelli JP, Su X, Beebe DJ. *Journal of the Association for Laboratory Automation*. 2010; 15:25–32. [PubMed: 20209121]
40. Regehr KJ, Domenech M, Koepsel JT, Carver KC, Ellison-Zelski SJ, Murphy WL, Schuler LA, Alarid ET, Beebe DJ. *Lab Chip*. 2009; 9:2132. [PubMed: 19606288]
41. Drinkwater NR, Denniston C. *Statistical Problems in Genetics and Molecular Biology*. Createspace. 2011
42. Valley CC, Solodin NM, Powers GL, Ellison SJ, Alarid ET. *J Mol Endocrinol*. 2008; 40:23–34. [PubMed: 18096994]

43. Livak KJ, Schmittgen TD. *Methods*. 2001; 25:402–8. [PubMed: 11846609]
44. Yuan JS, Reed A, Chen F, Stewart CN Jr. *BMC Bioinformatics*. 2006; 7:85. [PubMed: 16504059]
45. Abramoff MD, Magelhaes PJ, Ram SJ. *Biophotonics Int*. 2004; 11:36–42.
46. Stossi F, Madak-Erdogan Z, Katzenellenbogen BS. *Oncogene*. 2012; 31:1825–34. [PubMed: 21860415]
47. Nawaz Z, Lonard DM, Dennis AP, Smith CL, O'Malley BW. *Proc Natl Acad Sci U S A*. 1999; 96:1858–62. [PubMed: 10051559]
48. El Khissiini A, Leclercq G. *FEBS Lett*. 1999; 448:160–6. [PubMed: 10217432]
49. Joel PB, Traish AM, Lannigan DA. *J Biol Chem*. 1998; 273:13317–23. [PubMed: 9582378]
50. Al-Dhaheeri MH, Rowan BG. *Nucl Recept Signal*. 2006; 4:e007. [PubMed: 16741565]
51. Yu H, Alexander CM, Beebe DJ. *Lab Chip*. 2007; 7:388–91. [PubMed: 17330172]
52. Young EW, Pak C, Kahl BS, Yang DT, Callander NS, Miyamoto S, Beebe DJ. *Blood*. 2012; 119:e76–85. [PubMed: 22262772]

Insight, Innovation, Integration

The *biological insight* is the concept that a tumor biomarker can be used to predict the activity of the surrounding microenvironment. We show here that ER α protein dynamics, a component of the ER α activation mechanism, correlates with the growth-promoting activity in the context of a cellular microenvironment. Secondly, this study highlights the added value of using the dynamics of biomarkers to evaluate complex intercellular interactions like those encountered in tumors. The *innovation* is the application of microfluidics and immunofluorescence-based measurement techniques to test the effect of a panel of multivariate signaling systems on the dynamics of ER α in tumor cells. The *integration* of microfluidics and ER α dynamics in response to complex extracellular signaling enables prediction of environment's influence on growth.

Figure 1.

MCF-7eGFP cell growth in co-culture. MCF-7 eGFP cells were directly co-cultured with various cell lines in steroid-free media in a 96-well plate. Fluorescence was first measured after 24 hours, and each day following to day 4. Each well's fluorescence was normalized to its day 1 value, and each condition was run in triplicate. (A) Initial co-culture of MCF-7eGFP cells with HS-5 cells and in mono-culture. Error bars represent standard deviation of 3 independent experiments. Statistical significance at each time point is represented by * ($p < 0.05$ versus monoculture). (B) Summary of cell lines co-cultured in parallel with MCF-7eGFP cells. Fold change in growth represents fluorescence from MCF-7eGFP cells at day 4 relative to initial measurement. Statistical significance ($p < 0.05$) was determined after 3 independent experiments

Figure 2.

Transcriptional profile of ER α target genes in response to co-culture. MCF-7eGFP cells in mono-culture or in direct co-culture with HS-5 cells in 6-well plates were harvested after 24 hours and RNA was isolated and reverse transcribed. Genes for ER α (*ESR1*), Progesterone Receptor (*PGR*), pS2 (*TFF1*), and E-cadherin (*CDH1*), which served as an MCF-7 cell-specific reference gene, were quantified by qRT-PCR. Error bars represent standard deviation of 4–5 independent experiments. Statistical significance is represented by * ($p < 0.05$ versus monoculture).

Figure 3.

Schematic of microfluidic devices and quantitative immunofluorescence procedure. (A) Microfluidic devices used were the single microchannels and two-channel compartmentalized microchannels. (B) Work flow of immunofluorescence image analysis at population and single-cell level.

Figure 4.

ERα protein expression and phosphorylation in co-culture. (A) Representative immunofluorescence images of ERα and Hoechst (nuclear) staining from single microchannels. (B) Quantified ERα immunofluorescence relative to mono-cultured, vehicle-treated MCF-7eGFP cells. MCF-7eGFP cells were cultured alone (mono) or with bone marrow stromal cells (HS-5), human mammary fibroblasts (RMF/EG), or lung fibroblasts (MRC-5) for 24 hours in single microchannels and treated for the last 8 hours with 100nM 17β-estradiol (E2) or vehicle (EtOH). In each of three experiments, 5–10 internal replicates were performed. (C) MCF-7eGFP cells cultured with or without HS-5 cells in two-channel compartmentalized microfluidic devices for 24 hours were stained for ERα protein and analyzed as in B. (D) MCF-7eGFP cells were cultured with or without HS-5 cells for 20 hours in single microchannels and treated with 100nM 17β-estradiol (E2) or vehicle (EtOH) for the last 30 minutes. Staining of ERα Serine-118 phosphorylation in MCF-7eGFP cells was quantified by immunofluorescence image analysis as in Figure 3B. The means of each experiment were averaged, and error bars represent standard error of the mean between the three independent experiments. Statistical significance is represented by symbols: * = versus mono-culture, vehicle-treated MCF-7eGFP ($p < 0.05$), ‡ = versus respective vehicle-treated co-culture ($p < 0.05$).

Single cell analysis:

Use particle analysis to
identify individual MCF-7
nuclei

Measure ER α per nuclei

Figure 5.

Population distribution of ER α protein in mono-cultured and HS-5 co-cultured MCF-7eGFP cells from single microchannels.

Single-cell analysis of immunofluorescence images was performed to identify individual MCF-7eGFP nuclei as individual particles and ER α intensity per nuclei was measured. Four representative image views per condition were processed, encompassing a total of 400–700 cells per condition, where the specific number of cells (n) for each condition is specified in the histogram. To allow direct comparison between conditions for determination of heterogeneity, the frequencies of events per bin (i.e. image intensity interval) were weighted by n for the respective condition, resulting in an area under the curve of 1. The raw curves were then modeled using Log-normal peak fits, shown as shaded curves.

Figure 6.

ERα protein quantification in MCF-7eGFP cell panel co-culture. (A) MCF-7eGFP cells were co-cultured with the cell panel described in Figure 1 for 24 hours in single microchannels, and stained for ERα protein. Immunofluorescence image analysis was performed as in Figure 3B. Statistical significance is represented by * ($p < 0.05$ versus mono-culture). (B) ERα protein level was plotted against its respective fold growth in Figure 1B. Spearman's correlation test was used to determine correlation between the two parameters, and the R^2 value is derived from the linear best-fit line. Quadrants were superimposed by creating axes between significant and non-significant differences in growth and ERα down-regulation.