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Screening Estrogen Receptor Modulators in a Paper-Based Breast Cancer Model

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Abstract

The health risks associated with acute and prolonged exposure to estrogen receptor (ER) modulators has led to a concerted effort to identify and prioritize potential disruptors present in the environment. ER agonists and antagonists are identified with end-point assays, quantifying changes in cellular proliferation or gene transactivation in monolayers of estrogen receptor alpha expressing (ER+) cells upon exposure. While these monolayer cultures can be prepared, dosed, and analyzed in a highly parallelized manner, they are unable to predict the potencies of ER modulators *in vivo* accurately. Physiologically relevant model systems that better predict tissue- or organ-level responses are needed. To address this need, we describe here a screening platform capable of quantitatively assessing ER modulators in 96 chemically isolated 3D cultures. These cultures are supported in wax-patterned paper scaffolds whose design has improved performance and throughput over previously described paper-based setups. To highlight the potential of paper-based cultures for toxicity screens, we measured the potency of known ER modulators with a luciferase-based reporter assay. We also quantified the proliferation and invasion of two ER+ cell lines in the presence of estradiol. Despite the inability of the current setup to better predict *in vivo* potencies of ER modulators than monolayer cultures, the results demonstrate the potential of this platform to support increasingly complex and physiologically relevant tissue-like structures for environmental chemical risk assessment.

Graphical Abstract

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the <http://pubs.acs.org/> at [10.1021/acs.analchem.8b02486](https://doi.org/10.1021/acs.analchem.8b02486).

Detailed schematics of the 96-zone scaffold and 96-well holder; photographs and schematics of 9-zone and single-zone scaffolds; raw luminescence values used to construct the intensity map shown in Figure 3C; invasion data for MCF7-eGFP cultures; individual dose-response curves for all of the ER transcriptional activation reference substances tested; the estrogenic response of the three negative control chemicals tested in the 3D T47D-KBluc assays; and table summarizing seeding conditions and relative standard deviations of cell number for 96-zone format (PDF)

Endocrine disrupting chemicals (EDCs) are a heterogeneous collection of synthetic and naturally occurring molecules that disrupt hormone signaling.¹ One mechanism of endocrine disruption is the interference of estrogen receptors (ERs), which regulate cellular proliferation, differentiation, and movement in a hormone-dependent manner.^{2–4} Prolonged disruption of estrogen signaling alters sex organ development⁵ and has been linked to the progression of estrogen-dependent and -independent cancers.^{6–8} Breast tissue is particularly sensitive to the carcinogenic effects of estrogen during morphogenesis and remodeling.^{9,10} These health risks have led to a concerted effort to identify potential ER modulators present in the environment for further study.¹¹

ER agonists and antagonists are identified with end-point assays, quantifying changes in cellular proliferation or gene transactivation upon exposure.^{12,13} Reporter assays that couple gene transactivation with the expression of a fluorescent protein or non-native enzyme are particularly well suited for screening applications, as they provide a single and readily quantifiable value. T47D-KBluc cells are commonly used in screening assays. These cells are an engineered variant of the estrogen receptor positive (ER+) T47D cell line that expresses luciferase in the presence of estrogenic agonists, in a dose-dependent manner.¹⁴ Two recent studies highlight the need for more physiologically relevant *in vitro* models. In one study, monolayers of T47D-KBluc cells both under- and overestimated the *in vivo* potency of specific ER modulators in Sprague–Dawley rats.¹⁵ In a separate study, the combination of *in silico* modeling and a number of cell-based screens was able to predict the bioactivity of ER modulators with accuracies of 84–93%.¹⁶ While this hybrid approach is more powerful than a single *in vitro* assay, it failed to accurately predict the biotransformation of test chemicals into an active or inactive state. Neither approach can predict specific phenotypic changes in breast tissue *in vivo*.

An essential first step in generating more predictive *in vitro* assays is the incorporation of tissue-like architectures, provided by three-dimensional (3D) cultures. When placed in preformed scaffolds or suspended in biomimetic hydrogels, cells that were initially cultured as monolayers adopt morphologies, growth rates, and transcript profiles that are more representative of those found *in vivo*.^{17–20} Mammary-derived cell lines form microtissues that mimic luminal structures in biomimetic hydrogels. When exposed to 17 β -estradiol (E2), bisphenol A (BPA), or propylparaben, MCF-12A microtissues in Matrigel increased in both number and size.²¹ When exposed to E2, MCF7 microtissues cultured on agarose hydrogels had decreased luminal areas and increased overall sizes.²²

In their current state, many 3D culture platforms are not compatible with the highly parallelized setups needed to screen libraries of potential toxicants.^{23–25} Paper-based cultures offer many unique advantages for screening applications.^{26–29} First, tissue-like structures can be reproducibly formed using equipment common to any tissue culture lab. In these cultures, cells suspended in a hydrogel are pipetted directly into wax-patterned sheets of paper. The paper scaffolds provide mechanical support to the cell-laden gels, permitting repeated manipulation without fear of cracking or breaking. Second, pipetting cell suspensions into the scaffolds bypasses the prolonged culture periods required to generate multicellular aggregates or spheroids. Lastly, the scaffolds support end-point and information-rich readouts, such as viability assays, microscopic imaging, qPCR, and mass spectrometry.^{30–33}

To screen multiple ER modulators in parallel, we wax-patterned sheets of Whatman 105 lens paper with 96 culture zones. We also fabricated an acrylic holder that ensured each of the 96 cultures were chemically isolated throughout the experiment. Compared to previous 96-zone formats,²⁷ our setup requires smaller numbers of cells and can prevent chemical crosstalk between wells. The data sets generated in this work show the paper platform can support quantitative assays including dose–response analyses of known ER agonists and antagonists, cellular proliferation, and cellular invasion.

METHODS

Reagents.

All reagents were used as received unless otherwise stated. Ammonium perchlorate, bisphenol A (BPA), cycloheximide, diethylstilbestrol (DES), 17 β -estradiol (E2), genistein, 4-hydroxytamoxifen (4-OHT), methoxychlor, 4-nonylphenol (4-NP), and sodium azide were purchased from Sigma-Aldrich. ICI 182 780 (ICI) was purchased from Tocris Bioscience; 2,2-bis(4-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) was purchased from Toronto Research Chemicals. Cell culture medium and additives were purchased from Gibco, except for collagen I (Corning), DMSO (Fisher Scientific), and fetal bovine serum (FBS, VWR). CellTiter-Glo, One-Glo, and 5 $\times$ reporter lysis buffer were purchased from Promega.

Scaffold and Holder Preparation.

Paper scaffolds were prepared as previously detailed.²⁹ Sheets of Whatman 105 lens paper were wax-patterned with a ColorQube 8650 printer (Xerox) and sterilized under UV light overnight. Three designs were used in this study: (1) a single-zone scaffold, which fits into the well of 96-well plate; (2) a 9-zone scaffold, which fits into the well of a 12-well plate; and (3) a 96-zone scaffold, which matches the dimensions and spacing of a standard well plate. In each design, the zones were 3.3 mm in diameter. Detailed schematics and photographs of each scaffold are in the Supporting Information (Figures S1 and S2).

The 96-well holder developed in this work consisted of three components (Figure 1), each of which was laser cut (Universal Laser Systems, ILS9.75) from sheets of cast acrylic or food-grade silicone rubber 40A. These materials, as well as stainless steel screws and washers,

were purchased from McMaster Carr. Detailed schematics of the 96-well holder (Figure S3) are in the Supporting Information.

Cell Culture.

The T47D-KBluc cell line was kindly provided by Dr. Vickie Wilson. The MCF7 cell line (ATCC) was engineered to constitutively express enhanced green fluorescent protein (MCF7-eGFP), as detailed previously.³¹ Both cell lines were maintained in phenol red-free DMEM medium supplemented with 10% FBS, 4.5 g/L D-glucose, 4 mM L-glutamine, 25 mM HEPES, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 0.05 mg/mL gentamicin. The T47D-KBluc culture medium was also supplemented with 0.5 mg/mL Geneticin. Cells were maintained at 37 °C in a 5% CO₂ environment. Culture medium was exchanged every 2–3 days. The T47D-KBluc cells were passed at a 1:10 dilution when cultures reached confluency. The MCF7-eGFP cells were passed at a 1:5 dilution when cultures reached 80% confluency.

T47D-KBluc Transactivation Assay.

The T47D-KBluc cells were placed in withdrawal medium for 3 days prior to being suspended in collagen I (1.2 mg/mL) at a final concentration of 40 000 cells/ μ L. The withdrawal medium was phenol red-free DMEM supplemented with 10% charcoal-stripped FBS, 4.5 g/L D-glucose, 4 mM L-glutamine, 25 mM HEPES, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 0.05 mg/mL gentamicin. Each paper scaffold zone was seeded with 40 000 cells, incubated overnight in withdrawal medium, and dosed with a test chemical.

Stock solutions of each test chemical were prepared at 1,000x their working concentration in either ethanol or DMSO and stored at 4 °C in amber glass vials. Prior to dosing, stock solutions were diluted in withdrawal medium. A minimum of six concentrations was prepared for each dose–response curve. These dilutions were at least 3-fold differences in concentration and centered around the literature-reported EC₅₀ value. Cell-containing scaffolds were dosed for 24 h. Luciferase activity and cellular viability were assessed with One-Glo or CellTiter-Glo reagents, respectively, according to the manufacturer's protocol. Luminescence values were recorded on a SpectraMax M5 plate reader.

Proliferation Assays.

T47D-KBluc or MCF7-eGFP cells were seeded at either 500 cells/well in a 96-well plate or 2 000 cells/zone in a single-zone scaffold. The cells were treated with either vehicle (0.1% ethanol) or 10 nM E2, and cell numbers determined with the CellTiter-Glo assay. Culture medium was exchanged every 2 days during the course of this experiment.

ELISAs.

Cell-containing paper scaffolds were placed in ice-cold RIPA buffer. The resulting lysates were clarified by centrifugation and protein content analyzed by ELISA. ER α concentration was measured with a commercial kit (R&D Systems, DYC-5715). β -actin concentration was measured with a sandwich assay consisting of a capture antibody (R&D Systems, AF4000) and an HRP-linked detection antibody (Santa Cruz Biotechnology, sc-47778).

Paper-Based Invasion Assays.

Invasion assays were performed as detailed previously.²⁹ Seven-layer invasion stacks were assembled by sandwiching a cell-laden scaffold between six scaffolds containing only collagen. The zones of the cell-laden sheet contained 40 000 estrogen-deprived MCF7-eGFP cells suspended in 1 μ L of collagen. Each zone of the remaining scaffolds was seeded with 1 μ L of collagen. The invasion stacks were placed in a holder that limited nutrient exchange to the top and bottom of the stack. The assembled stack was incubated for 48 h in withdrawal medium containing vehicle (0.1% ethanol) or 10 nM E2. Upon destacking, the number of cells in each layer of the invasion stack was determined from fluorescence images obtained on a Typhoon 9400 scanner. Images were analyzed with ImageJ as detailed previously.²⁹

Calculations and Statistical Analyses.

All data sets were analyzed with GraphPad Prism v 7.0b. A Student's t-test was used to compare two groups, a *p*-value of <0.05 was considered significant. Dose–response curves were generated according to the procedure described by Conley et al.¹⁵ Briefly, raw luminescence values were normalized to a vehicle control, log₁₀ transformed to correct for heterogeneity of variance, and converted to the percentage of maximum E2 response (% of E2_{max}). E2_{max} was determined with 100 pM E2. Dose–response data points were fit with a 4-parametric logistic regression curve, with the bottom of the curve constrained to zero.

RESULTS

Fabrication and Assembly of the 96-Zone Screening Platform.

To ensure each of the tissue-like cultures in the wax-patterned paper scaffolds remained chemically isolated during incubation and analysis, we prepared the device shown in Figure 1. This device consisted of (1) an acrylic 96-well insert, (2) a silicone rubber gasket, (3) paper scaffolds wax-patterned with 96 culture zones, and (4) an acrylic base plate with a solvent-bonded acrylic border.³⁴ Each component of the assembled device was held in conformal contact with 15 stainless steel screws, which compressed the 96-well insert against the acrylic base plate. The assembled device had the same dimensions as a commercial well plate, allowing us to seed the paper scaffolds with a multichannel pipet and perform automated analyses with a commercial plate reader.

To overcome the chemical crosstalk between neighboring cultures that limited a previously published 96-zone paper format,²⁷ we re-engineered the paper scaffolds. First, we replaced the Whatman 115 paper (200 μ m thick) with Whatman 105 paper (40 μ m thick). While both paper scaffolds can be patterned, it was easier to ensure the wax fully penetrated the thinner scaffolds to provide a uniform barrier between each zone. Second, we decreased the diameter of the culture zones from 6.9 mm to 3.3 mm. This decrease in size increased the amount of wax separating each cell-containing region and reduced the volume of cell-laden gel required to fill each zone from 4 to 1 μ L.

Evaluating the 96-Zone Screening Platform.

A successful screening platform depended on our ability to (1) prevent chemical crosstalk between neighboring wells and (2) consistently seed the same number of cells into each

zone of the paper scaffold. To assess crosstalk, paper scaffolds seeded with cell-free collagen were presoaked in culture medium and then assembled into the 96-well holder. Each well of the device was loaded with either fluorescein-containing or dye-free culture medium, as shown in Figure 2A. A comparison of the fluorescence intensity of each well at the start of the experiment and after a 24 h incubation (Figure 2B) showed there was no change in the mean fluorescence intensity of the dye-containing or dye-free wells; the spread of these data sets was unchanged, and there were no outliers.

Relying on previously published results from our laboratory, we compared a narrow set of seeding parameters to determine which most affected our ability to seed the paper scaffolds reproducibly. In each experiment, the scaffolds were seeded with MCF7-eGFP cells using a multichannel pipet. Once assembled into the 96-zone device, the cell-containing paper scaffolds were imaged through the optically transparent acrylic base plate with a Typhoon scanner (Figure 3A). The relative standard deviations (RSDs) in fluorescence intensity of the cell-containing wells were used to make comparisons between each seeding condition. We have shown previously that eGFP fluorescence intensity and cell number are correlated.³¹

In the first comparison, we seeded two 96-zone scaffolds with 0.5 μL of collagen containing either 40 000 or 70 000 cells. In the second comparison, we seeded two 96-zone scaffolds with 40 000 cells suspended in either 0.5 or 1.0 μL of collagen. We found that 1.0 μL of collagen containing 40 000 cells yielded the most consistent results, with an RSD of 12.7% across the entire plate. Comparisons of cell number by row and column (Figure 3B) showed there was no bias in seeding, with RSD values across a single seeding event (by column) ranging from 10.6 to 21.4% and RSD values across multiple seeding events with the same pipet tip (by row) ranging from 8.6 to 19.9%. The deviations associated with seeding parameter are summarized in Table S1. The remainder of the experiments described used 40 000 cells suspended in 1.0 μL of collagen.

Screening Estrogen Receptor Agonism and Antagonism in 96-Zone Holders.

To ensure there was no crosstalk between wells during the transactivation assays, we dosed T47D-KBluc cells with a checkerboard pattern of 1 nM E2 (a potent ER agonist) and 1 μM ICI (a potent ER antagonist). After a 24 h incubation, the cells were lysed in the assembled device and luciferase activity quantified with the One-Glo assay. A heat map of luciferase activity had the expected pattern (Figure 3C) of ICI-dosed wells having little luciferase activity and the E2-dosed wells having significant luciferase activity. Luminescence values for each well are displayed in Figure S4.

To confirm the ICI was not diffusing into neighboring wells, we compared the luciferase fold-induction in the 96-zone device to single-zone scaffolds treated with 1 nM E2 (Figure 3D). The single zones were placed directly into the wells of a commercial well plate, ensuring there was no chemical crosstalk. The average fold induction (mean $\pm$ SEM) for the 96-zone and single-zone setups were statistically indistinguishable: 33.0 ± 1.8 and 34.0 ± 2.4 , respectively. Luciferase fold-induction was calculated in both setups by dividing the relative luminescence intensity from E2-containing wells by the relative luminescence intensity for ICI-containing wells.

Comparing the Estrogen Responsiveness of 2D and 3D Cultures.

To determine if placing the T47D-KBluc cells in a collagen matrix altered their responsiveness to estrogenic compounds, we dosed both monolayer and 3D formats with increasing concentrations of E2 (Figure 4A). Both formats yielded indistinguishable EC₅₀ values: 5.5×10^{-12} M for monolayers and 5.47×10^{-12} M for 3D cultures. The maximum log-fold induction in the monolayer cultures was 1.33-fold greater than in the 3D format. To ensure these differences were not caused by a decreased expression or an increased degradation of ER α , we quantified protein levels in both culture formats with a sandwich ELISA. To account for differences in cell number, we normalized the ER α intensities to β -actin. The 3D cultures had significantly more ER α than their monolayer analogues for both the E2-withdrawn ($p = 0.0044$) and E2-dosed ($p = 0.0043$) conditions (Figure 4B). ER α levels decreased by 55% in both formats when exposed to 10 nM E2.

We also compared E2-induced proliferation between monolayer and 3D culture formats. Compared to vehicle-exposed cells, the proliferation rates of T47D and MCF7 cells in the paper scaffolds increased when exposed to 10 nM E2 (Figure 4C–F). In T47D cells, this increase was not significantly different from that observed in E2-dosed monolayers: a 2.51-fold increase in 3D and a 2.44-fold increase in 2D. In MCF7 cells, there was a significant difference between the two formats: a 2.47-fold increase in 3D and a 3.98-fold increase in 2D.

Previous studies showed estrogenic compounds promoted the migration of both ER+ and ER – cell lines in scratch assays.^{35,36} To assess the ability of E2 to induce cellular invasion through an extracellular matrix, we quantified movement with a paper-based invasion assay.^{29,32} Here, MCF7-eGFP cells seeded in a 9-zone paper scaffold were sandwiched between six collagen-laden scaffolds. The assembled paper stacks were incubated for 48 h in culture medium containing either the vehicle (0.1% ethanol), 10 pM E2, or 10 nM E2. Following incubation, the scaffolds were peeled apart, and the fluorescence intensity of each zone imaged on a Typhoon scanner. While we observed invasion in each setup (Figure S5), exposure to E2 did not significantly increase the number of invasive cells.

Screening a Library of Known ER Reference Chemicals in Paper-Based Cultures.

We screened 12 reference substances from the ER transactivation assay validation list developed by the National Toxicology Program Interagency Center for Evaluation of Alternative Toxicological Methods.³⁷ Our library contained ER agonists and antagonists of varying potency and chemical structure (Table 1) as well as three negative controls. Initial screens were performed in single zones. The cell-laden zones were placed in a commercial polystyrene 96-well plate, incubated for 24 h in medium containing increasing concentrations of a library member, and ER transactivation quantified with the One-Glo assay.

For the ER agonists, we observed classic dose–response relationships (Figure 5A and Figure S6).^{14,38} The EC₅₀ values and estradiol equivalency factors (EEFs) of each compound matched those obtained in monolayer cultures (Table 2). The synthetic estrogen DES was the most potent compound tested with an EEF of 2.90. The EEF values of the other

compounds ranged from 9.40×10^{-7} for the pro-estrogenic molecule, methoxychlor, to 2.29×10^{-4} for the commercial detergent, 4-nonylphenol. The ER antagonists inhibited E2-induced luciferase expression in a dose-dependent manner (Figure 5B), with IC_{50} values of 1.27×10^{-9} for ICI and 1.91×10^{-8} nM for 4-hydroxytamoxifen. There was an insignificant induction of luciferase expression across all tested concentrations of the negative control compounds (Figure S7) ammonium perchlorate, cycloheximide, and sodium azide.

We also prepared a 96-zone assay containing the following in triplicate: vehicle controls, 100 pM E2 positive controls, and dose-response curves spanning six concentrations for DES, E2, genistein, and cycloheximide. The EC_{50} values, efficacy, and overall shape of the dose-response curves for E2 were statistically indistinguishable between the single- and 96-zone formats (Figure 5C). The EC_{50} values for DES and genistein also matched those obtained in the single zones (Figure 5D). As expected, the cycloheximide did not induce significant luciferase expression across all tested concentrations.

DISCUSSION

3D Cultures Support Cell-Based Assays for Screening ER Modulators.

Both the single- and 96-zone scaffolds support the preparation and analysis of 3D tissue-like structures with tools commonly found in tissue culture laboratories. With the single-zone format, small numbers of samples can be screened in commercially available 96-well plates. With the 96-zone format, scaffolds can be loaded with a multichannel pipet or a liquid handling robot. This increased throughput format requires the fabrication of specialized holders, which can be prepared readily in a MakerSpace or facility with access to a laser cutter. Both the single- and 96-zone formats are amenable to image-based readouts, in-plate analyses of cell lysate, and offline analyses of protein or gene expression.

The equal variances between the monolayer and paper-based formats for each E2 concentration across the entire dose-response curve (Figure 4A) highlight the reproducible nature in which the paper scaffolds can be seeded and dosed. While placing the T47D-KBluc cells in collagen did not improve their ability to estimate potency *in vivo*, the muted response of these cells to E2 does suggest the 3D environment is affecting cellular response and phenotype. The statistically indistinguishable EC_{50} and efficacy values for the single- and 96-zone formats further support cellular-level changes caused by the 3D environment and that the muted response is not due to chemical crosstalk between wells nor is it due to nonspecific adsorption to the 96-well device components.

We found that a decrease in $ER\alpha$ expression does not cause this muted response in a 3D environment. A recent study, which supports our findings, showed a decreased trans-activation of ER-regulated gene targets in MCF7 cells when they were placed in an agarose hydrogel.¹⁹ Related studies have also found that placing cancer cell lines in a 3D environment increased their innate resistance to chemotherapeutics through differential expression of growth factor receptors and drug efflux pumps.^{19,39,40}

Paper-Based Platform Can Support Multiple Analyses.

In addition to the gene transactivation assays, we also measured cellular proliferation and invasion in the paper scaffolds. These assays are complementary because they require the activation of multiple signaling pathways, which might not be reflected when focusing solely on the trans-activation of estrogen response element (ERE)-regulated genes. This ability to support multiple readouts further supports the continued development of the paper platform.

We were able to interrogate the cells in the paper scaffolds with a number of biochemical assays, demonstrated by our ability to perform both luciferase-based assays and ELISAs. Such assays are important to elucidate the transcript- or protein-level changes caused by E2 exposure. In a recent report, Vantangoli et al. used a custom PCR array to profile gene expression of MCF7 cells exposed to 1 nM E2 for 24 h.²² In monolayer cultures, E2 exposure increased the expression of five transcripts, of which two were direct ER targets, *PGR* and *PDZK1*. Microtissues prepared in soft agar were more responsive to E2 exposure with a total of 22 significantly altered transcripts. In particular, the expression of epithelial markers *ITGA1* and *GJA1* decreased while epithelial-to-mesenchymal transition (EMT) markers *FNI*, *CLDN1*, and *SNAI2* increased.

These transcriptional changes support other studies, which showed that estrogenic compounds promote EMT in ER+ breast cell lines.^{3,35,41,42} Cells that undergo EMT adopt an invasive phenotype. In scratch assays, MCF7 migration increased upon exposure to E2.³⁵ We found that E2 exposure did not increase the invasiveness of MCF7 cells, highlighting two important points about *in vitro* assays that utilize monolayers. First, changes in EMT markers may not be enough to promote invasion, which requires the expression of proteins needed to remodel the extracellular matrix (ECM). Second, cellular movement along a planar surface may exaggerate the propensity or rate of movement because there is no ECM to remodel.

Paper-Based Platform Can Support More Representative Tissue Models.

Despite the inability of the current setup to better predict the *in vivo* potency of known ER modulators than monolayers of T47D-KBluc cells, the modular nature of the platform allows a number of culture variables to be incorporated and prototyped. There is literature precedence for the cellular microenvironment affecting estrogen signaling, each of which can be tested in the paper platform.

ECM stiffness significantly alters estrogen signaling and enhances prolactin/E2 crosstalk.⁴³ This variable can be altered by changing the density of the collagen the cells are suspended in or through the incorporation of other extracellular components. The stabilization of hypoxia-inducible factor 1 alpha, which occurs in hypoxic environments, regulates ER α protein levels and downstream estrogen signaling.^{44,45} By stacking multiple cell-laden scaffolds together, we have engineered the steepness of oxygen gradient in these cultures.^{46,47} Other studies have shown that environmental chemicals can indirectly influence estrogen signaling by altering the metabolism of testosterone to estradiol in neighboring fibroblasts.⁴⁸ The paracrine signaling between fibroblasts and MCF7 cells directly regulates

ER α protein levels and estrogen-dependent growth.⁴⁹ Cocultures have been prepared with the paper-based cultures^{50,51} and offer the ability to spatially position cells by stacking individual scaffolds containing a particular cell type.

CONCLUSION

The datasets generated here demonstrate the power of the paper culture platform and its ability to interface with assays and tools commonly used in screening applications. While placing ER+ breast cancer cells in a collagen matrix does not better predict the *in vivo* potency of ER modulators than monolayer cultures, the platform offers many distinct advantages which merit its further development. First, is the ability to support complementary assays and probe different cellular responses that are not possible in monolayer formats. These additional assays complement transactivation data of a single set of ER-regulated proteins, which may not be enough to predict ER modulator potency accurately. Second, is the ability to rapidly prototype different culture conditions by simply changing the number of paper scaffolds or the extracellular matrix in which the cells are suspended. Previous examples of paper-based cultures have used different ECMs, introduced extracellular gradients, and incorporated cocultures. We will utilize each of these approaches to refine this screening platform further and generate a more representative breast tissue model capable of identifying potential EDCs as well as predicting their potency.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Photograph of the four primary components of the 96-well holder: (1) an acrylic 96-well insert, (2) a silicone rubber gasket, (3) a wax-patterned paper scaffold with 96 culture zones, and (4) an acrylic base plate. The individual components of the 96-well device are held together with 15 stainless steel screws and washers. (B) Photograph of the assembled device, which has the same footprint as a 96-well plate. The scale bar in both images represents 20 mm.

Figure 2.

(A) Schematic of the checkerboard pattern used to assess chemical crosstalk between the wells of the assembled 96-well device. (B) Fluorescence intensity values obtained from the checkerboard pattern, immediately after plate assembly and after a 24 h incubation in cell culture conditions (37 °C, 5% CO₂). Each bar represents the average and standard deviation of $n = 48$ replicate values.

Figure 3.

(A) Fluorescence image of an assembled 96-well device obtained with a Typhoon scanner. Each zone of the paper scaffold contained 40 000 MCF7-eGFP cells suspended in 1 μ L of collagen. (B) Fluorescence intensities of the MCF7-eGFP cells in each well plotted as a function of column number (left) or row letter (right). Each bar represents the average and standard deviation for an individual column or row. (C) Heat map of luminescence intensities for 40 000 T47D-KBluc cells after a 24 h incubation in either 1 nM E2 or 1 μ M ICI. The plate was dosed in a checkerboard pattern. (D) Luciferase fold-induction for E2-treated wells in the 96-zone or single-zone formats. The bars represent the average and standard deviation for $n = 30$ replicate values.

Figure 4.

(A) Dose–response curves of 40 000 T47D-KBluc cells exposed to E2 in 2D and 3D culture formats. Luciferase activity was measured with the ONE-Glo assay after a 24 h incubation. Data points represent the average and SEM of 10 replicates collected from two different cell passages. (B) ERα levels of T47D-KBluc cells dosed with vehicle or 10 nM E2 for 24 h, determined with ELISA. (C–F) Growth curves for T47DKBluc or MCF7-eGFP cells in 2D (C, E) or 3D (D, F) culture formats. Both cell lines were dosed with vehicle or 10 nM E2. Cell viability was determined with the CellTiter-Glo assay. Each point represents the average and SEM of four replicates from one passage of cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 5.

Representative dose-response curves from single-zone cultures of 40 000 T47D-KBluc cells suspended in collagen, dosed with (A) increasing concentrations of ERα agonists E2, genistein, or BPA or (B) 100 pM E2 and increasing concentrations of ERα antagonists ICI 182 780 or 4-hydroxytamoxifen. (C) E2 dose-response curve comparison for single- and 96-zone cultures. (D) Representative dose-response curves for DES, genistein, and cycloheximide generated in the 96-zone device. Each concentration, along with vehicle and 100 pM E2 controls, was included in triplicate on the plate. In each setup, luciferase activity

was measured with the ONE-Glo assay after a 24 h incubation. Each point represents the average and SEM for a minimum of six replicates collected from two different cell passages (A, B) or a minimum of three replicates collected from one cell passage (C, D).

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Table 1.

Library of ER Transcriptional Activation Reference Substances Screened

chemical	ER TA ^a	chemical class
E2	+++	steroid
DES	+++	cyclic hydrocarbon
4-nonylphenol	++	phenol
BPA	+	phenol
methoxychlor	+	halogenated hydrocarbon
HPTE	++	halogenated hydrocarbon
genistein	+	flavonoid
4-hydroxytamoxifen	-/+	cyclic hydrocarbon
ICI 182 780	-	steroid
ammonium perchlorate	n.d.	amine
cycloheximide	n.d.	heterocyclic compound
sodium azide	n.d.	azide, salt

^a +++ = strong agonist, ++ = moderate agonist, + = weak agonist, -/+ = ambiguous, - = antagonist, and n.d. = activity not detected.

Table 2.Observed EC₅₀ Values and the Corresponding Estradiol Equivalency Factors

chemical	3D			2D		
	EC ₅₀ (95% CI)	EEF ^a	fold induction	EC ₅₀	ref	
E2	5.47×10^{-12} (4.26×10^{-12} – 7.18×10^{-12})	1.00	102.2 ± 6.0	2.68×10^{-12}	15	
DES	1.88×10^{-12} (9.72×10^{-13} – 4.54×10^{-12})	2.90	93.3 ± 4.8	1.40×10^{-11}	14	
4-nonylphenol	2.39×10^{-8} (1.22×10^{-8} – 5.55×10^{-8})	2.29×10^{-4}	107.0 ± 5.0	2.60×10^{-7}	52	
HPTE	2.86×10^{-8} (2.16×10^{-8} – 3.71×10^{-8})	1.91×10^{-4}	102.2 ± 6.0	not reported		
genistein	3.15×10^{-8} (1.81×10^{-8} – 5.67×10^{-8})	1.74×10^{-4}	101.2 ± 5.4	1.00×10^{-7}	52	
BPA	2.07×10^{-7} (1.36×10^{-7} – 6.52×10^{-7})	2.64×10^{-5}	93.2 ± 8.1	1.27×10^{-7}	15	
Methoxychlor	5.82×10^{-6} (3.46×10^{-6} – 1.49×10^{-5})	9.40×10^{-7}	93.7 ± 10.4	1.47×10^{-6}	15	

^aEEF = Estradiol Equivalency Factor (EC50E2/EC50chemical)