A Paper-Based Breast Co-Culture Model to Study Direct and Indirect Effects of Endocrine Disrupting Chemicals on Estrogen Signaling

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Table of Contents

Abstract

Three-dimensional (3D) cell culture platforms better recreate the complex microenvironment and cell-cell signaling gradients characteristic of *in vivo* tumors than monolayer cultures on plasticware. While *in vitro* co-culture models of stromal cells and ER(+) carcinoma cells have been developed to study tumor responses, there is a lack of detailed characterization of the expression and activity of aromatase enzyme in mammary fibroblasts in 3D environments. Aromatase converts androgens to estrogen. In this study, I quantify aromatase activity in stromal reductive mammary fibroblasts (RMFs) and estrogen receptor alpha (ERα) transactivation in a breast carcinoma cell line in 2D, 3D, and co-culture platforms. When treated with the aromatase inducers genistein and quercetin, we observed increased E2 synthesis in all culture formats containing RMFs. The 2D monocultures had much higher aromatase activity than 3D monocultures; the greatest increase in activity occurred in the co-cultures. Our results highlight the use of 3D breast cancer models as an improvement over traditional 2D cultures and a useful and efficient alternative to *in vivo* models.

1. Introduction

Breast cancer accounts for nearly 22% of all cancers in women worldwide [1]. Among these cases, approximately 60% of premenopausal and 75% of postmenopausal women have tumors that are estrogen-responsive [2]. The growth and development of these tumors are modulated by estrogen receptor alpha (ERα). The high incidence of estrogen-responsive or ER(+) breast cancers in postmenopausal women is caused, in part, by estrogen that is locally synthesized in neighboring stromal and adipose tissues [3]. Studies have shown that intratumoral estrogen concentrations, specifically the most potent inducer 17-β-estradiol (E2), are approximately 5-9 times those found in the blood plasma of postmenopausal women [4].

Aromatase, a member of the cytochrome P450 family, is the enzyme that catalyzes the final step in estrogen biosynthesis, the aromatization of androgens. Aromatase is expressed at abnormally high levels in stromal tissues of various types of breast cancer [5]. Due to its ability to drive cancer progression through increased estrogen levels in breast tissue, aromatase is one of the primary targets of endocrine therapies [6]. Long-term treatments with aromatase inhibitors such as letrozole, anastrozole, and exemestane have been shown to be more effective than drugs that modulate the expression of or directly inhibit the estrogen receptor, such as tamoxifen [7]. Aromatase inhibitors are also associated with fewer risks of recurrence than alternative treatments.

Mammary tissue is a complex environment composed of different cells types that continuously communicate through signaling molecules, each of which shape the overall tissue microenvironment [8]. Because aromatase is primarily expressed in stromal cells and not in cancer cells [9], the tumor microenvironment is crucial in assessing drug efficacy and the effect of toxins in breast carcinogenesis [10]. While traditional well plate-based screening platforms are amenable to high-throughput screening assays capable of evaluating libraries of hundreds to

thousands of drug molecules or estrogen mimics, they are limited to two-dimensional (2D) surfaces and often exclude stromal cells [11].

Three-dimensional (3D) cultures are increasingly relied upon to study cancer progression and evaluate cellular responses to therapies [12,13]. These culture setups better represent tissue architectures than monolayer cultures, incorporating cell-cell contacts, extracellular matrices, and physiologically relevant gradients. When compared to cells grown on 2D surfaces, cells in 3D culture environments have gene expression patterns and proliferation rates that are more similar to those found *in vivo* [14]. The *in vitro* co-culture of cancer cells with stromal fibroblasts has been shown to induce significant changes in the regulation of hormone signaling. For example, Wang et al. showed that aromatase expression was upregulated in both MCF-7 epithelial breast tumor cells and Hs-578Bst mammary fibroblasts when co-cultured vs. when cultured separately [8]. While a significant amount of research has focused on differences in cancer cells between 2D and 3D cultures, there are few studies that characterize fibroblasts cultured in 2D and 3D formats. Additionally, the inclusion of co-cultures in 3D screening platforms is lacking [10]. Previous examples used microfluidic devices, which are expensive and require engineering expertise.

In this study, I adapted the paper-based culture platform being developed in our laboratory to study the regulation of aromatase expression in co-cultures containing ER(+) breast carcinoma cells and reductive mammary fibroblasts. The paper-based scaffolds provide a 3D culture environment using equipment that is readily available to any laboratory conducting tissue culture. The aims of my thesis project are two-fold: 1) characterize aromatase expression and activity in 2D and 3D cultures of fibroblasts, and 2) study the impact of stromal fibroblasts on estrogen signaling in an ER(+) breast cancer cell line. We found that aromatase activity is increased in across all platforms when treated with inducers and is highest when RMFs are co-

cultured in 2D with T47D-Kbluc cells in 3D. Consequently, ERα transactivation is higher in co-cultures when treated with 30 µM genistein.

2. Experimental Detail

2.1 Chemical reagents

All reagents were purchased from Sigma-Aldrich and used as received, unless otherwise noted. Stock solutions (1000-fold of the highest working concentration) of 17β-estradiol (E2) and testosterone were dissolved in ethanol (Fisher Scientific). Stock solutions of the aromatase inhibitor letrozole, and aromatase inducers quercetin, genistein, and dexamethasone were reconstituted and diluted to protocol concentrations with DMSO (Fisher Scientific). CellTiter-Glo, ONE-Glo and 5x Reporter Lysis Buffer were purchased from Promega and used according to the manufacturer's protocol. Cell culture medium and supplements were purchased from Gibco, except for fetal bovine serum (FBS, VWR International) and collagen I (rat tail, Corning).

2.2 Cell lines and cell culture

The T47D-KBluc cell line, which was stably transfected with the luciferase reporter gene downstream of three estrogen-responsive elements, was kindly provided by Dr. Vicky Wilson at the EPA. These cells were cultured in DMEM base medium supplemented with D-glucose (4.5 g/L), L-glutamine (4 mM), HEPES (25 mM), FBS (10%), sodium pyruvate (1 mM), Geneticin (0.5 mg/mL), gentamicin (0.05 mg/mL), and nonessential amino acids (0.1 mM). The T47D-KBluc cells were grown to 100% confluency in a humid atmosphere of 5% CO_2 at 37°C, and passed at either 1:10 or 1:5 dilution, depending on the experiment. Culture medium was exchanged every 48-72 h. Two days prior to use, the T47D-KBluc cells were placed in withdrawal medium consisting of DMEM base medium supplemented with D-glucose (4.5 g/L), L-glutamine (4 mM), HEPES (25 mM), gentamicin (0.05 mg/mL), nonessential amino acids (0.1 mM), and 10% charcoal-stripped FBS.

Reduction mammoplasty fibroblasts (RMFs) were kindly provided by Dr. Melissa Troester at the Gillings School of Public Health. These cells were maintained in DMEM medium supplemented with FBS (10%), HEPES (25 mM), and penicillin-streptomycin (1%). The RMFs were grown to 85-95% confluency in in a humid atmosphere of 5% CO_2 at 37°C, and passed at either 1:10 or 1:5 dilution, depending on the experiment. Culture medium was exchanged every 48-72 h.

2.3 **Preparation of the paper scaffolds**

The single-zone paper scaffolds used in this work fit directly into commercially available 96-well plates. Each scaffold, which was surrounded by a wax-patterned border, was prepared as previously described [15]. Briefly, sheets of Whatman 105 lens paper were wax patterned with a ColorQube 8650 printer. Each single-zone was sterilized under UV light for 24 h prior to use.

2.4 **Culture formats**

In the 2D monoculture formats, 40,000 RMF cells were seeded directly onto the bottom of a 96-well plate. These cells were maintained in 100 μL of fresh medium. In the 3D monocultures, either RMF or T47D-KBluc cells suspended in a neutralized solution of collagen I (pH 7.4, 1.2 mg/mL), were seeded into the paper scaffolds at a density of 40,000 cells/0.5 μL collagen. The seeded single-zones were incubated in fresh medium for 24 h prior to transferring to a 96-well plate. These cells maintained in 100 μL of fresh medium.

In the indirect co-cultures, 40,000 RMFs were seeded as a monolayer at the bottom of 96-well plate and 40,000 T47D cells were seeded in the paper scaffolds. Both cell lines were supplemented with withdrawal medium two days prior to use.

2.5 **Cell viability and ERα transactivation assays**

Cell viability and ERα transactivation were assessed with the CellTiter-Glo and One-Glo reporter kits, respectively. Luminescence was recorded on a SpectraMax M5 plate reader with an

integration time of 500 ms. Values are reported as fold-induction relative to untreated or vehicle-treated cultures grown in hormone-stripped medium.

2.6 **Aromatase activity**

To quantify the aromatase activity of 40,000 RMF cells in the 2D and 3D culture formats, the concentration of E2 released into the culture medium was determined with an Estradiol EIA Kit (Cayman Chemical), according to the manufacturer's protocol. In these experiments, RMFs were dosed with vehicle (Veh, DMSO), dexamethasone (100 nM), quercetin (30 μM), genistein (30 μM), or letrozole (100 nM) in the presence or absence 10 nM testosterone. The cells were incubated for 48 h before culture medium was collected for analysis. Samples were incubated for 1.5 h before taking absorbance measurements at 412 nm on a SpectraMax M5 plate reader. Each sample was assayed in duplicate. Estradiol calibration curves were generated for each experiment.

2.7 **Statistics**

Graphpad Prism v7.0b was used for all statistical analyses. Unless otherwise stated, two groups were compared using an unpaired student's t-test with Welch's correction. A one-way ANOVA test was used to determine significance among multiple comparisons. A p-value of < 0.05 was considered significant.

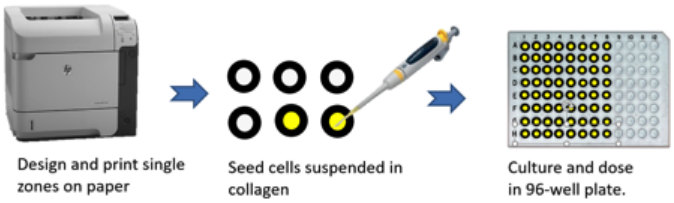
3. Results

This work focused on two cell lines, RMFs and the ER(+) T47D cell line. The T47D cell line was engineered with a luciferase reporter gene located downstream of three estrogen-responsive elements. This construct allowed us to quantify estrogen signaling, through the activation of ERα. In these studies, the 2D cultures were prepared as monolayers in cell culture-treated plates. The 3D cultures were generated by pipetting cell-collagen suspensions into wax-patterned paper scaffolds. Indirect co-cultures, which contained both 2D and 3D cell cultures, were prepared by seeding RMFs at the bottom of 96-well plates and seeding T47D-KBluc cells in the paper scaffolds. The indirect co-culture format was chosen over the direct method (both cell lines seeded within the same layer) because aromatase activity was higher in RMFs in 2D than in 3D. Indirectly co-culturing the cells lines allowed for sufficient E2 synthesis for ERα transactivation while still providing a 3D microenvironment for the T47-KBluc breast cancer cells. Schematics of the paper-based culture workflow (A) and the co-culture format (B) are shown in **Fig. 1.**

3.1 **Aromatase activity is higher in 2D than 3D culture formats**

To assess aromatase activity and expression, 2D and 3D monocultures containing 40,000 RMF cells were treated for 48 h in the presence or absence of substrate (testosterone) and inducer (dexamethasone). We determined the optimal cell density (40,000 cells per well) and inducer concentration (100 nM) in previous experiments (data not shown) and used these parameters for each experiment presented here. Estradiol concentrations were measured with ELISA and compared to standard curves.

(A)



(B)

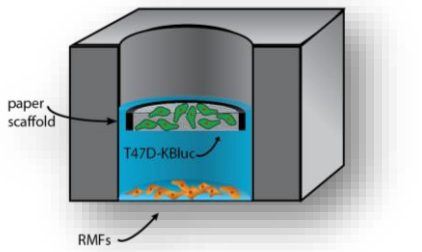


Figure 1. (A) Schematic of the paper-based culture workflow. Single-zones, which fit directly into a commercial 96-well plate, are seeded with a cell-containing, collagen suspension. (B) Schematic of the indirect co-culture setup containing RMFs and T47D-KBluc cells. The RMFs were seeded as a monolayer at the bottom of 96-well plate. The T47D-KBluc cells were suspended in collagen I and seeded in the paper scaffolds. Both cell lines were seeded at a density of 40,000 cells per well.

Basal levels of aromatase activity were low, yet above the limit of detection of the assay, in both the 2D and 3D culture setups. Treatment with dexamethasone significantly increased the conversion of testosterone to E2 in both formats: from 0.57 nM to 4.14 nM E2 in the 2D format (**Fig 2A**), and from 0.015 nM to 0.078 nM E2 in the 3D format (**Fig 2B**). Our findings that dexamethasone induces aromatase activity in mammary fibroblasts are consistent with previous reports [16]. A comparison across culture formats reveals that aromatase is much more highly expressed in 2D than in 3D cultures: 38-fold higher levels in the untreated samples and 52-fold higher levels in the dexamethasone-treated samples. We observed similar decreases in both the expression and transcriptional activity of ERα when comparing monocultures of T47D-KBluc cells in 2D and 3D cultures in a previous study, which is currently under review [17].

To determine whether the E2 concentrations generated by the RMFs were enough to modulate ERα transcriptional activity in breast cancer cells, we dosed T47D-KBluc cell cultures with spent medium collected from monolayer cultures of RMFs treated in the presence and absence of both testosterone and dexamethasone. ERα transactivation was assessed by quantifying luciferase activity in the T47D cells with the ONE-Glo assay. As expected, medium from RMF cultures treated with both the inducer and the substrate resulted in elevated luciferase activity: a 15-fold increase in the 2D format (**Fig 2C**) and a 4-fold increase in the 3D format (**Fig 2D**), compared to the respective formats treated only with vehicle. These results demonstrate that

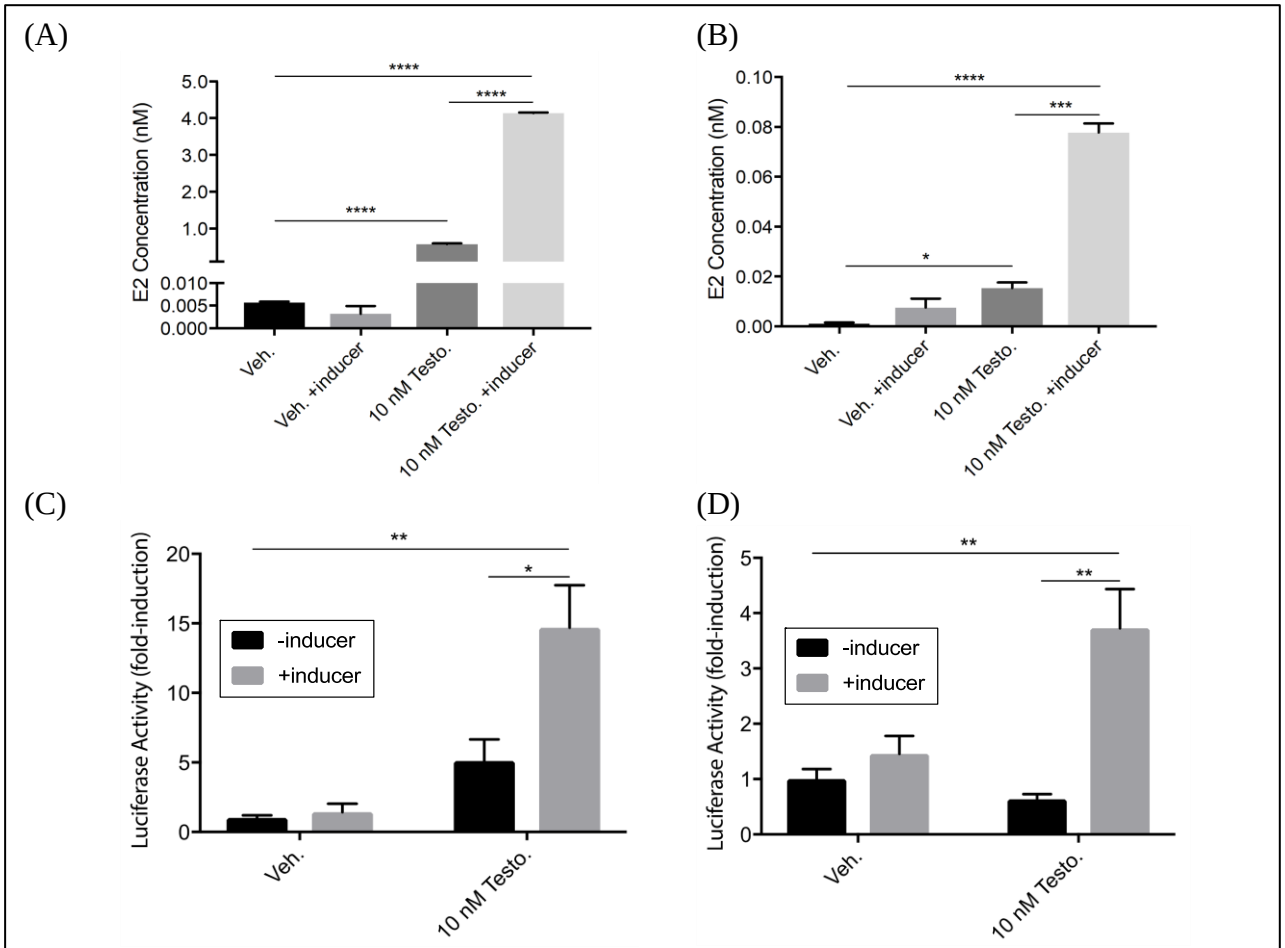


Figure 2. Comparison of estradiol concentration in 2D (A) and 3D (B) cultures 40,000 RMF cells with or without the substrate (10 nM testosterone) and inducer (100 nM dexamethasone). ERα transactivation was assessed with a luciferase-based readout. T47D-KBluc cells were incubated in spent medium collected from 2D (C) and 3D (D) RMF cultures treated with and without the substrate and inducer. Each data point represents the average ± SEM for n = 3 separate experiments, prepared on separate days with different cell passage numbers. *p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001; **** p ≤ 0.0001.

aromatase is upregulated in 2D when compared to 3D and indicate that stromal-derived estrogens are able to activate the ERα pathway. These findings were essential in deciding the optimal co-culture format, namely the indirect format where RMF's were cultured as monolayers in 2D and T47D's were seeded in the paper scaffolds.

3.2 **Genistein and quercetin upregulate aromatase expression in 2D and in co-cultures.**

To evaluate the effects of known environmental endocrine disruptors on aromatase expression, we measured the concentrations of E2 released into the culture medium after a 48 h incubation testosterone, in the presence or absence of either 30 µM genistein or 30 µM quercetin. We chose these inducer concentrations because they have been previously shown to induce the expression of aromatase without decreasing cell viability [18]. Similar to the results we observed with dexamethasone treatment, E2 production increased in both culture formats in the presence of the inducer. The 2D cultures had a much higher conversion rate (22.4 pg/mL for quercetin and 48 pg/mL for genistein) than the 3D formats (3.5 pg/mL for quercetin and 23.9 pg/mL for genistein). The results for each inducer are summarized in **Fig 3A**.

Treatment with genistein in 2D and co-cultures resulted in a 5-fold and a 10-fold increase in E2 compared to testosterone-only treated cells, respectively. Treatment with quercetin in 2D and co-cultures resulted in a 2-fold and an 8-fold increase compared to testosterone-only treated cells, respectively (**Fig 3C**). Although not as high, these induction patterns have been previously reported [18]. To determine if the aromatase enzyme could be inhibited in each format, we added both dexamethasone and the aromatase inhibitor letrozole (IC_{50} = 20 nM). Inhibition was effective across all platforms and no statistical difference was observed between treated cultures and those that received no dexamethasone.

Finally, we quantified the ability of the RMFs to influence estrogen signaling in T47D-KBluc cells. Here, we quantified ERα transactivation with the luciferase assay in monocultures and co-cultures (**Fig 3D**). Quercetin, genistein, and letrozole-treated monocultures showed a small, but significant increase in ERα activity compared to the vehicle-treated cells. Estrogen signaling was upregulated by 2-fold in co-cultures containing genistein, when compared with cultures treated with a saturating concentration of E2 (10 nM).

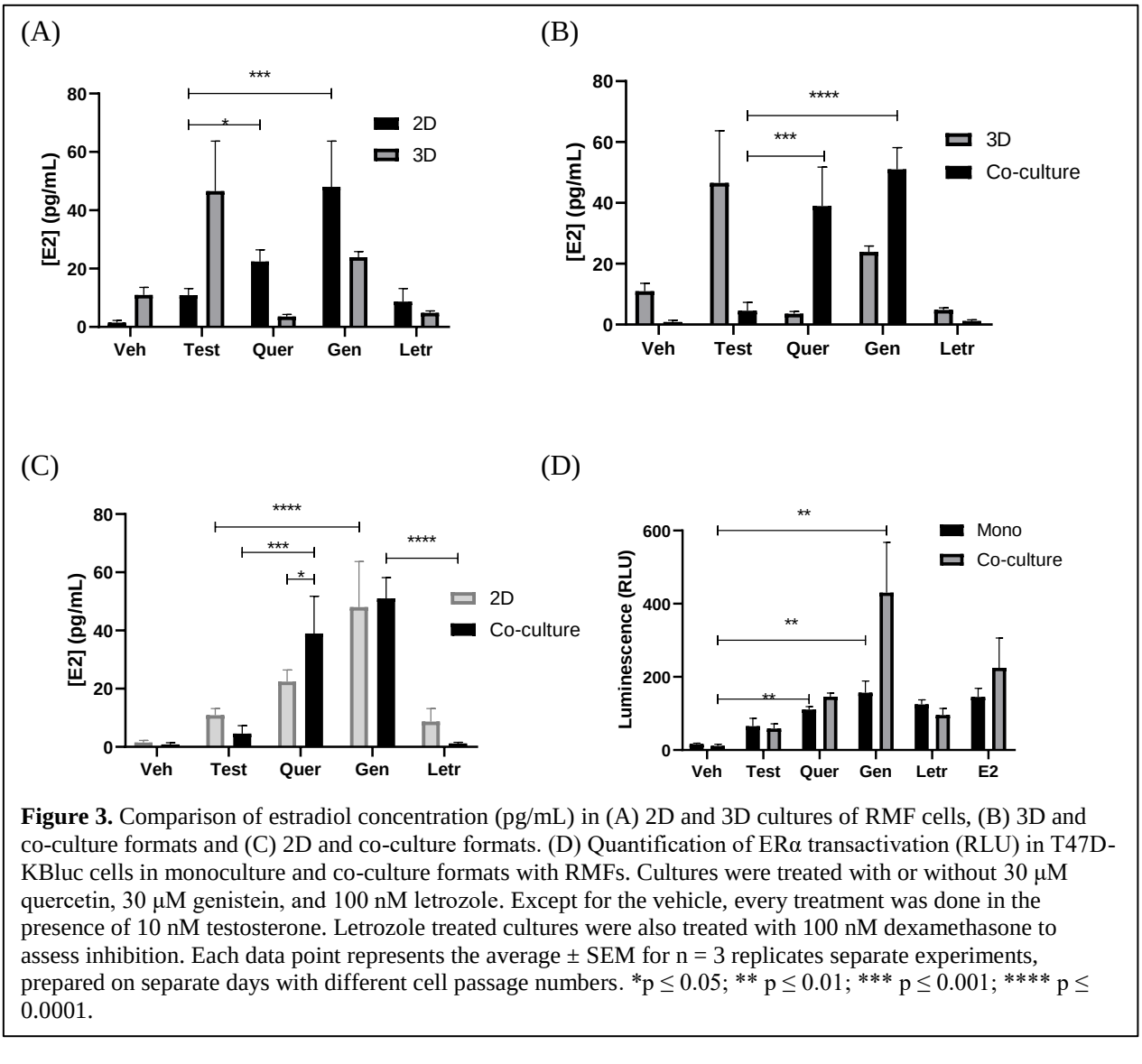


Figure 3. Comparison of estradiol concentration (pg/mL) in (A) 2D and 3D cultures of RMF cells, (B) 3D and co-culture formats and (C) 2D and co-culture formats. (D) Quantification of ERα transactivation (RLU) in T47D-KBluc cells in monoculture and co-culture formats with RMFs. Cultures were treated with or without 30 μM quercetin, 30 μM genistein, and 100 nM letrozole. Except for the vehicle, every treatment was done in the presence of 10 nM testosterone. Letrozole treated cultures were also treated with 100 nM dexamethasone to assess inhibition. Each data point represents the average ± SEM for n = 3 replicates separate experiments, prepared on separate days with different cell passage numbers. *p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001; **** p ≤ 0.0001.

4. Discussion

Breast tissue is a complex, three-dimensional microenvironment in which a variety of cell types communicate via intricately regulated signaling mechanisms [8, 20]. ER(+) breast carcinoma cells rely on estrogens for proliferation, gene regulation, and invasiveness [21]. The stromal and adipose cells in these tissues are the main source of locally produced estrogen. To prepare *in vitro* models that better predict *in vivo* responses of breast tumors, the characterization of these cell types and their influence on the 3D cellular microenvironment is crucial. In these studies, I determined that basal and induced aromatase activity was higher in 2D cultures of RMFs than in 3D cultures where the cells were suspended in collagen. These differences in activity were not caused by differences in total cell number as each format had similar numbers of viable cells. Although we have not encountered a study that quantitatively compares the conversion of testosterone to estrogen in 2D and 3D RMF monocultures, others have noted the presence of feedback mechanisms in 3D cultures that are not observed in 2D [16, 22]. Because cell-cell contact is restored in 3D cultures and there is a greater exchange between the fibroblasts and other ECM components, it is possible that a negative-feedback mechanism regulated by estrogen concentration is established.

The comparisons across the different culture setups shown in **Fig 1** were essential in deciding the optimal co-culture format. While the indirect co-culture format supports both cell types, the long-distance signaling can result in protein regulation that is not representative of the physical contact between stromal and epithelial cells found *in vivo*. However, the very low aromatase activity of the RMF cells in collagen, even when induced with dexamethasone, required they be cultured in a 2D format.

Treatment of 2D and 3D RMF monocultures with dexamethasone, quercetin, and genistein induced aromatase expression and activity. Genistein had the highest aromatase induction of all

the culture formats, resulting in a 2-fold increase in ERα transactivation in the T47D cells, when compared to cultures dosed with the saturating E2 concentration (10 nM). This increase is most easily explained the dual role of genistein, inducing aromatase in the RMF cells and agonizing the estrogen-dependent ERα pathway in the T47D cells [23]. The estrogenic properties of quercetin and genistein may also explain the small but significant increase of ERα activity in the T47D monocultures.

Finally, a significant increase in E2 concentration (~16 pg/mL) was observed in co-cultures compared to the 2D RMF monocultures. Two important paracrine signaling mechanisms involving an estrogenic positive feedback and hepatocyte growth factor (HGF) production are responsible for increased aromatase activity and cell proliferation, respectively. Wang et al. showed that increased fibroblast estrogen synthesis had an inductive effect in aromatase expression in neighboring MCF-7 breast carcinoma cells [8]. Furthermore, co-culturing stromal and breast cancer cells established a positive feedback regulated by estradiol, growth factors, and interleukins that would otherwise not be present in monocultures. This mechanism is shown in **Fig 4.**

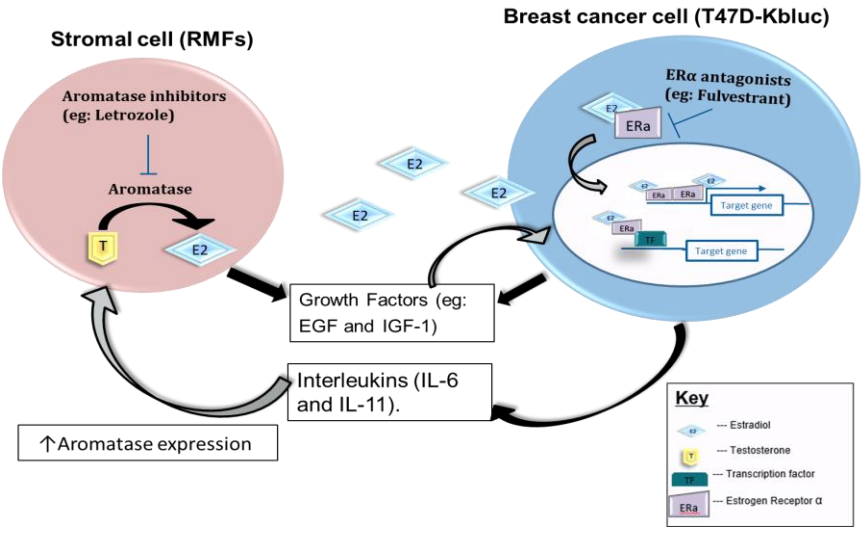


Figure 4. Positive feedback mechanism established between co-cultured RMF and T47D-Kbluc cells. Reproduced with permission from reference [16].

5. Conclusions

Unlike 2D platforms, 3D cell culture formats recapitulate key components of tissue microenvironments. Using the 3D environments generated in the paper-based culture platform, I characterized aromatase expression in stromal cell monocultures and co-cultures with ER(+) breast carcinoma cells. This characterization is necessary for the development of more representative and efficient 3D co-culture models, which include the tissue microenvironment.

While the usefulness and significance of 3D co-cultures have been demonstrated, our work shows key differences in aromatase activity. In the presence of endocrine disruptors, aromatase activity is induced in RMFs and their E2 synthesis increases when co-cultured with T47D-Kbluc breast carcinoma cells. In the future, we will use these models to screen for environmental chemicals that modulate the ERα pathway in estrogen-responsive tumors.

6. Acknowledgements

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