# The role of the microenvironment in mediating breast cancer cell responses to xenobiotics

By

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**Abstract**

The estrogen receptor (ER) is implicated in breast cancer progression and is a target of environmental chemicals and breast cancer therapies. Previous studies have shown that ER is regulated by multiple components of the mammary microenvironment, but most *in vitro* models only include the breast cancer cell. I hypothesized that the microenvironment regulates responses to chemicals that target the ER signaling pathway, and that chemical responses are missed when evaluated in traditional *in vitro* platforms. I developed the adverse outcome pathway (AOP) for ER+ breast cancer to identify readouts and culture conditions important to modeling ER-driven responses in breast cancer cells. Using the AOP as my guide, I optimized a microfluidic organotypic model of the breast consisting of a ductal structure lined with ER+ breast cancer cells surrounded by stromal cells in a collagen matrix. I used the organotypic model to show that mammary stromal cells modulate ER protein, and speed estradiol-induced hyperplasia by reducing apoptosis. I also demonstrated that mammary stromal cells indirectly regulate ER-driven responses by producing aromatase, the enzyme responsible for estrogen production. To assess if the organotypic model was more predictive than a traditional co-culture platform, I evaluated the aromatase inhibitor anastrazole when breast cancer cells were co-cultured with mammary adipose stromal cells derived from lean or obese women in a 2D co-culture or the organotypic co-culture platform. Breast cancer cells co-cultured with obese stromal cells in the organotypic model exhibited increased anastrazole resistance. In contrast, the 2D co-culture platform did not segregate the anastrazole responses of lean and obese patients. Next, I hypothesized that environmental chemicals differently affect breast cancer cells when cultured as a 2D monoculture, compared to when co-cultured with adipose stromal cells in 2D or in the organotypic model. MCF7 cells differed in response to tributyltin chloride when cultured alone compared to when co-cultured with adipose stromal cells. Altogether, these studies add to existing information that implicate the mammary

microenvironment as a regulator of ER driven responses and suggest that *in vitro* platforms that consider only the breast cancer cell provide an incomplete picture of chemical responses.

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## Abbreviations

| | |
|---|---|
| 2D | two-dimensional |
| 3D | three-dimensional |
| ADH | atypical ductal hyperplasia |
| AOP | adverse outcome pathway |
| ATP | adenosine triphosphate |
| BLS | basal-like subtype |
| BMI | body mass index |
| CAF | carcinoma associated fibroblast |
| CASP7 | caspase 7 |
| CSRA | chemotherapy sensitivity and resistance assays |
| CYP | cytochrome P450 |
| DCIS | ductal carcinoma in situ |
| DES | diethylstilbestrol |
| DEX | Dexamethasone |
| DHT | 5α-dihydrotestosterone |
| ECM | extracellular matrix |
| ER | estrogen receptor |
| ERE | estrogen response element |
| E1 | Estrone |
| E2 | 17β-estradiol |
| GA | Glutaraldehyde |
| GDF-15 | growth differentiation factor 15 |
| HMF | human mammary fibroblasts |

| | |
|---|---|
| ICI | Fulvestrant |
| IDC | invasive ductal carcinoma |
| MLS | mesenchymal-like subtype |
| MTT | 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide |
| PDMS | polydimethylsiloxane |
| PEI | 2% poly(ethyleneimine) |
| PPARy | peroxisome proliferator-activated receptor gamma |
| OECD | Occupational Economic Cooperation Development |
| OHT | 4-hydroxytamoxifen |
| RXR | retinoid X receptor |
| SERD | selective ER downregulators |
| SERM | selective ER modulators |
| T | Testosterone |
| TBT | tributyltin chloride |
| TOP | therapeutic outcome pathway |

## Chapter 1: Introduction

Accumulating evidence suggests that our ability to predict the effects of chemicals on breast cancer is limited by a lack of physiologically relevant *in vitro* model systems; the typical *in vitro* platform used to study breast cancer includes only the cancer cell, and completely excludes the mammary microenvironment. As understanding of the effects that the microenvironment has on cancer cell behavior and response to chemicals increases, researchers have called for the integration of the microenvironment in *in vitro* chemical testing systems. However, given the complexity of the microenvironment and the wide range of platforms to choose from, identifying the most important parameters to include in a chemical testing platform is a challenging. This chapter reviews the need for more complex *in vitro* breast cancer models and describes the normal and cancerous mammary microenvironment. The different approaches used to model breast cancer *in vitro* are outlined, specific examples of the microenvironment modulating breast cancer cell responses to xenobiotics are provided, and strategies to help pinpoint what components should be included in a model are discussed. The estrogen receptor (ER) is introduced as a case study due to its importance in breast cancer and regulation by the microenvironment. Estrogen signaling and production are discussed in detail, as are the challenges in studying environmental and pharmaceutical chemicals that target ER signaling.

## 1.1 Defining the need for improved systems to test chemical effects on breast cancer

A large fraction of breast cancer research is devoted to identifying and understanding chemicals that reduce or increase, cancerous behaviors in breast epithelial cells. These studies have identified multiple pathways that are effective targets of breast cancer therapies[2; 3], as well as several chemicals present in the environment and consumer products that increase breast cancer risk[4; 5; 6]. However, breast cancer remains the most common non-cutaneous cancer in women and the second leading cause of cancer-related deaths among women worldwide[7]. Clearly, there is still much to be learned about the effect of chemical exposures on breast cancer risk and progression.

The existing framework used to evaluate the effect of chemicals on breast cancer relies on model systems that lie on two ends of a wide spectrum: simple, inexpensive *in vitro* models and resource intensive, physiologically complex mouse models. The simplicity of *in vitro* models is ideal for uncovering the innerworkings of signaling pathways or screening for the molecular targets of chemicals, such as the affinity for a specific receptor. These kinds of experiments are difficult to perform *in vivo*, as animal models are too complex to easily pinpoint molecular interactions. Unfortunately, *in vitro* culture platforms lack the predictability to make reliable decisions about the toxic or therapeutic potential of a chemical. Mouse models are valuable as they incorporate the systematic and local effects of chemicals and enable the assessment of chronic chemical exposures and the importance of specific life stages. However, there are key biological differences between humans and mice that create problems when extrapolating data from mice to humans, such as in the development of breast cancer[8; 9; 10], the physiology of the mammary gland[11; 12; 13], and chemical metabolism[14; 15]. Both established *in vitro* and *in vivo* models are indispensable to the breast cancer field, but each harbor unique shortcomings that limit their utility.

To increase the efficiency of chemical testing, leaders in the pharmacology and toxicology fields have called for the development and implementation of *in vitro* model systems that better recapitulate

human physiology[16; 17; 18]. These new model systems would help bridge the gap between *in vitro* and *in vivo* systems, and consequently improve the ability to predict the effects of chemicals on breast cancer risk and progression. While the approaches differ, the prevailing hypothesis is that inclusion of the mammary microenvironment is critical to increasing the predictability of *in vitro* models. However, when integrating the microenvironment into *in vitro* models, it is important to find a balance between the simplicity and complexity of an *in vitro* model. If a platform becomes too complex, the advantages of its being *in vitro* are lost: chemical mechanisms become difficult to decipher and costs and variability increase. Therefore, to maintain a level of simplicity, it is critical that only the components needed to recapitulate *in vivo* responses are included in a platform. As the mammary microenvironment is complex and contains many different cell types and proteins, a major challenge is identifying which components are needed to predict chemical responses.

This chapter describes the normal and cancerous mammary microenvironment, and the various tactics used to model breast cancer *in vitro*. Scenarios in which the microenvironment regulates chemical responses in breast cancer cells are provided, and strategies to develop and validate *in vitro* models are discussed. Then, a case study of estrogen receptor signaling in breast cancer introduced and discussed in detail.

## 1.2 The normal and cancerous mammary gland

The human mammary gland is composed of a series of ducts that collect into the nipple[19]. Mammary ducts are bilayered: the milk secreting luminal epithelial cells line the ductal lumens while the basal myoepithelial cells face the laminin-rich basement membrane[20]. The basement membrane, a specialized form of extracellular matrix that is rich in laminin and collagen IV, provides mechanical support and separates the ducts from the surrounding stroma[21]. The stroma accounts for approximately 80% of the breast volume[22], and is composed of an extracellular matrix rich in collagen, glycoproteins, and proteoglycans as well as different stromal cell types including fibroblasts, adipose stromal cells,

adipocytes, immune cells, and endothelial cells[21]. The crosstalk between the epithelium and stroma tightly regulates the development and maintenance of the mammary gland[23; 24].

The development of mammary adenocarcinoma is characterized by the abnormal proliferation of epithelial cells into the mammary duct, also referred to as hyperplasia. Ductal carcinoma *in situ* is considered the earliest form of breast cancer, and it occurs when breast cancer cells have proliferated to the extent that the breast ducts are filled solid with cells[25; 26]. When lumens exhibit this high-grade hyperplasia, the intraductal microenvironment becomes hypoxic and deprived of nutrients[27; 28]. The duct diameter increases in size: ducts of DCIS breast tissue exhibit a mean diameter of 360 microns compared to normal ducts with a mean diameter of 90 microns[29]. The transition from ductal carcinoma *in situ* to invasive ductal carcinoma is not well understood, however, the loss of the basement membrane and myoepithelial layer are defining features[30; 31; 32]. The disruption of normal duct architecture enables the escape of cancer cells into the surrounding stroma, where they can enter the vasculature and travel to distant organs.

As cancer progresses, fibroblasts[33; 34; 35], immune cells[35; 36; 37], adipocytes[35; 38; 39], endothelial cells[35; 40; 41], and the extracellular matrix[42; 43] undergo dramatic changes. The extracellular matrix is stiffened due to increased deposition of extracellular matrix proteins and increased collagen crosslinking[44; 45] In contrast to the normal mammary gland in which the collagen fibers are curly and randomly organized, the collagen fibers of breast cancer tissue become linear and thick. These aligned collagen fibers facilitate the invasion of cancer cells into the vasculature[46; 47]. Fibroblasts acquire an activated phenotype, upregulating expression of proteins such as smooth muscle alpha actin and fibroblast activation protein[48; 49; 50]. Adipocytes become delipidated, exhibit reduced expression of adipocyte markers, and acquire a fibroblast-like morphology[51; 52]. Blood vessels grow and develop a leaky phenotype[40; 53] and immune cells are recruited to the tumor site[54; 55].

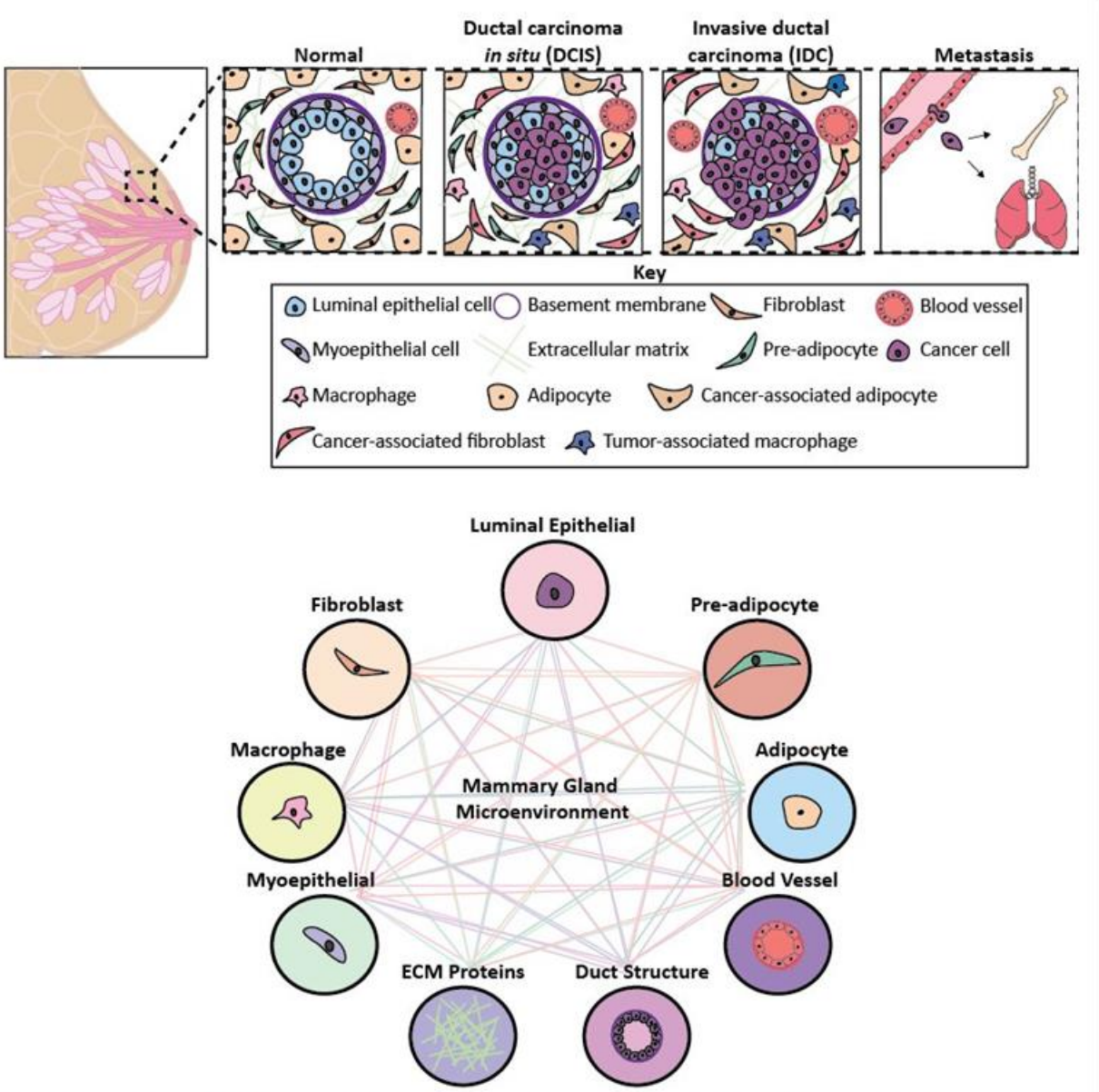


**Figure 1.1. The normal and cancerous mammary microenvironment.** (top panel) The transition from a normal to cancerous mammary gland is characterized by changes in both the epithelium and stroma. The ductal epithelium becomes hyperplastic, the myoepithelial layer is lost, and the basement membrane is degraded. The stromal cell types become activated, immune cells infiltrate to the area, and angiogenesis is increased. (bottom panel) Each component of the microenvironment communicates with the breast cancer cell, as well as with one another.

There is a complex interplay between cancer cells and stromal cells; while cancer cells induce an activated phenotype in stromal cells[56; 57; 58], cancer-associated stromal cells send signals back to cancer cells to fuel their growth and invasion[59; 60]. Components of the microenvironment also reorganize one another; for example, fibroblasts secrete matrix metalloproteinases which degrade the extracellular

matrix, release pro-inflammatory signals that recruit macrophages, and stimulate angiogenesis by secreting angiogenic factors such as VEGF[35; 61]. By the late stages of breast cancer, the microenvironment is entirely remodeled **(Fig. 1.1)**.

### 1.3 Toolbox of *in vitro* culture platforms

A major advantage of *in vitro* platforms over *in vivo* platforms is the ability to precisely control experimental conditions. For this reason, it is easy to understand why most *in vitro* studies use a simple platform consisting of an immortalized cell line cultured on a flat plastic surface: experimental results are easy to interpret, as the variables affecting cell interactions are well defined. However, due to the growing recognition that *in vitro* platforms often fail to predict *in vivo* responses, a variety of strategies have been developed to better recapitulate *in vivo* biology. Here, we discuss the various approaches used to improve *in vitro* breast cancer models. While we focus on the microenvironment, we also discuss the use of primary cells and microfluidic platforms.

**1.3.1 Primary cells** – Most *in vitro* breast cancer studies are conducted using immortalized cell lines because they replicate indefinitely and are easy to culture. However, multiple studies have found that while the genetic profiles of breast cancer cell lines generally reflect the profiles of breast tumors, immortalized cell lines poorly recapitulate the heterogeneity observed within tumors and between different patients[62; 63; 64; 65]. For example, most breast cancer cell lines were derived from metastatic tumors, so cell lines that model early stage breast cancer are limited. In addition, while estrogen receptor (ER) positive breast cancer accounts for approximately 75% of all breast cancer cases, the majority of breast cancer cell lines do not express ER[62]. We expect that immortalized stromal cells suffer from similar issues, as they likely do not recapitulate the heterogeneity of stromal cells seen *in vivo*[66; 67]. While utilizing primary cells would overcome these issues, primary cells are hard to acquire and lose their *in vivo* phenotypes after only a few passages[68; 69; 70]. Consequently, the goal of the experiment should dictate whether to use immortalized cells or primary cells. For instance, immortalized cell lines

may be more useful for hypothesis-generating studies or chemical screens, while primary cells may be more valuable for validation or experiments that study patient-specific responses.

**1.3.2 Tissue architecture** – As breast cancer progresses, the structural environment that houses the cancer cell is remodeled. There is no scenario in which breast epithelial cells reside on a flat, stiff surface as seen *in vitro*. While researchers originally overlooked the impact of the structural environment on cell behavior, landmark studies by Mina Bissell's lab revealed that culturing cells in 3D uncovers striking differences in the phenotypes of normal and cancerous breast epithelial cells. When grown as 2D monolayers, non-tumorigenic and cancerous mammary epithelial cell lines appeared morphologically similar. However, when grown in the reconstituted basement membrane, Matrigel, non-tumorigenic cell lines organized into growth-arrested polarized lumens while cancerous cell lines formed disorganized proliferative colonies[71; 72]. This led to an explosion of publications that reported striking differences in cell behavior when grown in 3D matrices compared to when cultured on plastic[73; 74; 75].

To increase the structural relevance of platforms, researchers have cultured cells on top of matrices (2.5D cultures), embedded cells in matrices (3D cultures), or cultured them independent of matrices (anchorage-independent)[76]. In these platforms, breast epithelial cells self-organize into ductal structures or solid spheres (e.g. spheroids), collectively referred to as organoids, and thus provide a more structurally relevant environment to model preinvasive disease[77]. Multiple groups have generated patient-derived mammary organoids to test potential cancer therapeutics *in vitro,* which has recapitulated the heterogeneity in treatment response often observed *in vivo*[78; 79; 80].

Later findings revealed that the composition of the hydrogel influences the behavior of cells grown in 3D. When non-tumorigenic mammary epithelial cells are grown in Matrigel they form polarized, hollow lumens. However, when the same cells are grown in a matrix composed entirely of collagen, they exhibit reverse polarity or no polarity[81]. In addition, using the same kind of matrix but changing the stiffness, pH, or geometry of the matrix can change cell behavior[82; 83; 84; 85]. One platform

that compared hydrogels of varying stiffnesses found that plasticity influences the proliferation and invasion of breast cancer cells[86]. This has been related to *in vivo* findings that have found a correlation between increased stromal collagen and tumor formation and metastasis in mice[87; 88]. The stiffness of the matrix has also been found to change the gene expression profiles of breast cancer cells[89]. Therefore, researchers should carefully consider the type and composition of the matrix used their *in vitro* breast cancer model.

**1.3.3 Stromal cells** – As described above, the multiple stromal cell types of the mammary microenvironment have been shown to actively stimulate cancer progression. Moreover, they can modulate cancer cell function by multiple mechanisms, including secreted proteins and direct contact. Several strategies have been developed to study the effect of stromal cells on cancer cell behavior, largely through the use of conditioned media, compartmentalized co-culture systems, and direct co-culture systems[90; 91]. As there are trade-offs with each type of co-culture method, the preferred strategy varies based on the experimental question.

Researchers sometimes include matrix proteins into co-culture platforms, which has revealed dramatic differences in cancer cell behavior when co-cultured with stromal cells in 2D compared to 3D. For example, multiple studies have found that the invasive potential of breast cancer cells is increased when co-cultured with fibroblasts in 2D vs 3D, as fibroblasts in 3D rearrange the extracellular matrix and secrete higher levels of cytokines[92; 93]. Stromal cells have also been shown to direct the organization of mammary epithelial cells grown in 3D. As described above, benign breast epithelial cell lines are polarized when grown in Matrigel but fail to polarize when grown in a 3D collagen matrix. As stromal cell types are important to mammary gland homeostasis, Gudjonsson et al. hypothesized that myoepithelial cells would be sufficient to induce a normal phenotype in collagen organoids. When myoepithelial cells were included in the collagen matrix that housed the luminal epithelial cells, the epithelium organized into a polarized structure that appeared morphologically similar to epithelial cells grown in Matrigel[81]. A

later paper compared the organization of mixed luminal:myoepithelial cultures when grown in a 3D collagen gel, compared to when grown in Matrigel. Myoepithelial and luminal epithelial cells grown in a 3D collagen gel organized into a bilayered structure, where myoepithelial cells laid against the basement membrane and luminal epithelial cells lined the inner part of the lumen. However, the two cell types did not self-organize when grown in Matrigel; instead, luminal and epithelial cells were randomly organized throughout the organoid[77]. Several other groups have found that stromal cell types regulate the tissue morphogenesis of mammary epithelial cells grown in 3D; in many cases the structures developed in co-culture are more complex than structures developed in mono-culture[94; 95; 96; 97; 98; 99; 100].

**1.3.4 Microfluidics** – One challenge with increasing the complexity of *in vitro* models is that standard culture platforms (e.g. well plates) offer limited control over the culture environment. For example, the majority of 3D breast cancer models rely on cancer cells to self-assemble into luminal structures, which can be inefficient as in some scenarios only a small percentage of samples will undergo structural morphogenesis and/or the structures vary dramatically in shape or size[101; 102]. In addition, when studying stromal:epithelial interactions, cells are typically either mixed together or layered on top of one another, while in the *in vivo* mammary gland the spatial organization of cells is tightly regulated. Consequently, microfluidic platforms, also referred to as to as microphysiological platforms or organ-on-chips, are increasing in popularity due to enhanced control over the culture environment[103; 104].

Microfluidic platforms are highly modular as they are fabricated using cheap and easy to modify materials; microfluidic platforms can easily incorporate multiple culture areas, diffusion channels, gradients, and/or structures[105; 106]. A wide range of microfluidic platforms have been developed to study breast cancer, such as simple compartmentalized co-culture models[93; 107] and complex microphysiological systems[108; 109; 110; 111]. Kamm and colleagues have developed several microphysiological models that culture metastatic microenvironments adjacent to a biomimetic vasculature; cancer cells can be added to the vasculature and monitored for extravasation into the bone

microenvironment[112; 113; 114]. Our lab has published multiple studies using a microfluidic platform that cultures a biomimetic mammary duct surrounded by a collagen matrix containing stromal cells, which is similar to the organization of a normal or premalignant mammary gland[115; 116]. In addition to their increased spatial control, microfluidic platforms require small amounts of cells and reagents, which can be especially advantageous when working with primary cells or expensive matrix proteins. The high surface area to volume ratio in microfluidic platforms also increases the sensitivity of paracrine signaling: there's less medium volume per cell, which results in a higher concentration of secreted factors[117; 118; 119]. While there are clear advantages to utilizing microfluidic platforms, most of these platforms are in their infancy and future studies are needed to confirm if they recapitulate human responses more faithfully than traditional culture platforms. In addition, microfluidic devices can be technically challenging to learn, and are usually constructed by academic labs so are not commercially available[120]. Commercializing platforms to decrease variability and increase usability, will be essential for widespread adoption of microscale platforms.

**1.4 Examples of chemical interactions that are regulated by the microenvironment** – Due to the myriad effects that the microenvironment has on cancer cell behavior, the responses of cancer cells to chemicals can differ dramatically depending on the surrounding microenvironment. In addition, some chemicals that target the microenvironment directly, such as immunotherapies or aromatase inhibitors, cannot be evaluated when the cancer cell is grown alone. To illustrate this point, we provide examples of chemical interactions in the mammary gland that are regulated by the microenvironment.

**1.4.1 Stromal cell modulation of estrogen receptor expression in breast cancer cells** – In breast cancer, a number of studies have found that stromal cells regulate the expression of the estrogen receptor (ER) [116; 121; 122; 123; 124], a nuclear receptor protein that controls breast cancer cell proliferation in approximately 75% of breast cancer cases[125]. One study found that stromal cells that lack the cell surface marker CD146 suppress ER expression and induce ER-independent growth in breast cancer cells

via activation of the EGFR, HER2, and IGF1R pathways. By inducing ER-independent growth, CD146- cells confer resistance to tamoxifen, an ER antagonist commonly used to treat breast cancer. Interestingly, the authors showed that an epithelial gene signature indicative of a high percentage of CD146- fibroblasts is predictive of decreased recurrence-free survival in breast cancer patients treated with tamoxifen[126].

**1.4.2 Stromal cell modulation of apoptosis in breast cancer cells** - Triple negative breast cancer (TNBC) accounts for approximately 15% of all breast cancers and can be organized into different subtypes. Two subtypes of TNBC, the mesenchymal like-subtype (MLS) and the basal-like subtype (BLS), differ in chemotherapeutic resistance: the mesenchymal-like subtype of TNBC exhibits higher resistance to chemotherapies in comparison to the basal-like subtype of TNBC[127; 128]. Interestingly, when MLS and BLS cell lines were grown as a monoculture and exposed to breast cancer chemotherapeutics, they did not segregate in chemotherapeutic resistance. However, comparison of drug sensitivity when the cancer cell subtypes were co-cultured with fibroblasts revealed differences in drug sensitivity similar to what is observed clinically. While the mechanism is not entirely understood, the authors found that the fibroblasts differently altered mitochondrial priming depending on the TNBC subtype[129].

**1.4.3 Chemotherapeutic-induced activation of the stroma** - Recently researchers have found that chemotherapies exert direct effects on fibroblasts: exposure to the chemotherapeutics doxorubicin, 4-hydroxy-cyclophosphamide, and paclitaxel induced an activated phenotype in human mammary fibroblasts, as indicated by a dramatic induction of the ELR+ chemokines associated with aggressive tumors. When co-cultured with chemotherapy-treated fibroblasts, breast cancer cells exhibited more proliferative and invasive behavior compared to those co-cultured with vehicle-treated fibroblasts[130]. This finding has been observed by other breast cancer researchers[131] as well as in other types of cancer[132; 133; 134]. Importantly, adjusting the dosing paradigm was sufficient to prevent fibroblast activation: treatment of fibroblasts with a low dose of chemotherapies for ten days rather than a high

dose for one day prevented fibroblast activation[130]. This data has prompted some authors to hypothesize that therapeutics that prevent fibroblast activation would decrease the incidence of chemotherapeutic resistance[61].

**1.4.4 Hepcidin expression differs in 3D and 2D** - Hepcidin is a hormone that plays an important role in breast cancer progression due to its regulation of iron metabolism[135; 136]. A recent study found that the expression of hepcidin differs dramatically depending on the culture condition. Compared to when grown in 2D, breast cancer cells grown as spheroids exhibited remarkedly higher levels of hepcidin mRNA and protein, as well as increased iron retention. Through a series of mechanistic studies, the authors concluded that the BMP signaling pathway was activated when breast cancer cells were grown as spheroids, which caused hepcidin to be regulated by growth differentiation factor 15 (GDF-15). The expression of hepcidin and GDF-15 were correlated in human breast cancer tissue, while no relationship was observed between hepcidin and GDF-15 when breast cancer cells were cultured in 2D. The authors also found that breast cancer cells grown as spheroids exhibited an even higher expression of hepcidin when co-cultured with fibroblasts, as fibroblasts secreted IL-6 which induced hepcidin expression in breast cancer cells. Interestingly, IL-6 had no effect on hepcidin expression when breast cancer cells were cultured in 2D[137]. This study provides a clear example of a signaling pathway that is differentially activated when breast cancer cells are cultured in 3D as compared to 2D. Future studies that evaluate the therapeutic value of hepcidin inhibitors should carefully consider their choice of *in vitro* culture model.

**1.4.5 Environmental obesogens target adipocytes** – Some environmental chemicals have been found to alter the differentiation and/or proliferation of adipocytes, primarily by interacting with the master regulators of lipogenesis: peroxisome proliferator-activated receptor (PPARy) and retinoid X receptor (RXR)[138; 139; 140]. Named after their ability to increase body weight, obesogens are an emerging area of research in toxicology[141; 142]. Obesogens are relevant to the breast cancer field because obesity is

associated with an increased risk of developing breast cancer[143; 144]. While the mechanisms are not understood, signals from the adipose microenvironment are thought to be primarily responsible: adipocytes secrete hormones and cytokines, coined adipokines, which are upregulated in obese individuals and are mitogenic to breast cancer cells[143; 145; 146; 147]. Consequently, some authors have hypothesized that obesogens can indirectly increase breast cancer risk or progression by altering the adipose stroma[144].

**1.4.6 Modulation of aromatase activity in adipose stromal cells** –The primary source of estrogen in the mammary gland of postmenopausal women is mammary adipose stromal cells. Mammary adipose stromal cells produce the enzyme aromatase, which metabolizes androgens to estrogens[148; 149]. Like many potential targets in the cancer microenvironment, aromatase is a target of both breast cancer therapies and environmental chemicals: aromatase inhibitors letrozole and anastrazole are the first line treatment for post-menopausal women with ER+ breast cancer[150; 151] and several chemicals present in pesticides and plasticizers have been found to modulate aromatase expression[152; 153]. Interestingly, some environmental chemicals that modulate estrogen signaling also target aromatase expression[152; 154; 155]; since aromatase controls estrogen production, these chemicals could lead to synergistic or neutralizing effects on ER signaling. While aromatase studies have mostly been limited to human studies or clinical studies, inclusion of adipose stromal cells in *in vitro* breast cancer models would provide a more tractable approach. Van den berg and colleagues have co-cultured mammary adipose fibroblasts with human breast cancer cells to study the aromatase modulating effects of chemicals[156; 157].

Another factor to consider when evaluating aromatase modulators is inflammatory cells. Under chronic states of inflammation such as obesity, the pro-inflammatory cytokines secreted by immune cells are hypothesized to enhance aromatase expression in individual breast stromal cells, leading to aromatase inhibitor resistance[158; 159; 160]. In support of this hypothesis, *in vitro* studies have found that the conditioned media from macrophages increases aromatase expression in adipose stromal cells[159; 161].

Another study found that the frequency of crown-like structures formed by macrophages correlates with aromatase expression[162; 163]. Macrophages have also been found to produce aromatase themselves[164; 165]. Therefore, studies that evaluate aromatase inhibitor resistance *in vitro* should likely incorporate breast cancer cells, adipose stromal cells, and macrophages.

### 1.5 Considerations on the design of *in vitro* models

While we argue that the microenvironment is critical to the improvement of *in vitro* chemical testing systems, there is a delicate balance between the complexity and simplicity of a model system. As additional components are integrated into a model system, the model becomes more resource intensive and responses become difficult to interpret. The ideal model would only incorporate the components needed to recapitulate an *in vivo* response, but there are many strategies to building an *in vitro* model and the ideal approach is usually unclear. Consider a situation in which a researcher wants to develop an *in vitro* co-culture model and is deciding between a platform that contains no matrix proteins, collagen, or Matrigel, as well as a monoculture or co-culture; in this scenario there are six different combinations to choose from. If the researcher also chooses between a macroscale or microscale platform, there are now twelve options. When the variety of cell lines, cancer subtypes, cell types, and life stages are included in the decision, there is an infinite number of different possibilities. Pinpointing what components to include in a model and what responses are predictive of a human, is an ongoing challenge.

The significance of the microenvironment can differ dramatically depending on the question being asked. Breast cancer is a heterogenous disease encompassing multiple subtypes that differ in signaling and progression, and chemicals vary in what biological pathways they target. Therefore, the importance of the microenvironment likely depends on the context – such as the chemical being evaluated, and the cancer subtype or stage – and should therefore be evaluated case by case. To inform the design of future model systems and help define the scenarios where the microenvironment is

needed, we must increase understanding of the influence that the microenvironment has on breast cancer development and xenobiotic responses.

To help identify what kind of model systems are required to predict *in vivo* responses, studies need to assess if more complex *in vitro* models respond to chemicals differently than traditional *in vitro* models, and whether the response is predictive of what is seen in humans. As failure to compare to standard culture platforms stunts the ability to determine if the microenvironment is needed in platforms, researchers that evaluate chemicals in a complex *in vitro* model should concurrently evaluate chemicals in a traditional *in vitro* model. *In vitro* responses must also be compared directly to human data to validate if the responses observed *in vitro* are reflective of *in vivo* responses. Models for pharmaceutical research can be validated by conducting *in vitro* studies alongside or retrospectively with clinical trials. For example, Hans Clever's group generated organoids from the needle biopsies of five breast cancer patients who varied in response to tamoxifen. When they exposed the organoids to tamoxifen, they found that the organoids responded to tamoxifen similar to their respective donors. While the sample size was small, these results are promising for the personalized medicine field[78]. One challenge is that while multiple drug combinations can be evaluated from a single patient's cells *in vitro*, patients can only receive one drug treatment. Therefore, only one *in vitro* drug condition can be compared per patient. Validation of models is even more difficult when evaluating chemicals for their ability to increase cancer risk: we cannot intentionally expose humans to possible toxicants. While results can be compared to animal studies, there are critical differences between the physiology of animals and humans that present uncertainties when extrapolating data from *in vivo* studies. Consequently, validating *in vitro* data regarding chemical risk factors is limited to studying accidental or occupational exposures, which can be challenging to analyze as the dose, length of exposure, and other factors are usually unclear. Comparing data about unknown chemicals to well characterized chemicals, such as pharmaceuticals, is one strategy to validate chemical hits.

## 1.6 The estrogen receptor as a target of environmental chemicals and pharmaceuticals

As discussed in section 1.4, the microenvironment regulates estrogen receptor signaling in breast cancer cells. For example, several different kinds of stromal cells regulate ER protein in breast cancer cells[122], and mammary adipose stromal cells produce the estrogen producing enzyme aromatase[148; 149]. In addition, studies have found that the extracellular matrix can change the expression of ER-driven genes[89]. Consequently, the estrogen receptor is an ideal case study for investigating the effect of the microenvironment on chemical responses, and for characterizing the need for more complex *in vitro* models for chemical testing. Here, estrogen signaling and production are introduced, and the challenges with studying environmental and pharmaceutical chemicals that target ER signaling are described.

**1.6.1 Estrogen signaling in the normal and cancerous breast –** The estrogen receptor belongs to the nuclear receptor family of transcription factors[166]. When ER is not activated, it resides in the nucleus sequestered by heat shock proteins[167; 168]. Once bound by a ligand, ER undergoes a conformational change, disassociates from heat shock proteins, dimerizes, then binds to DNA indirectly or directly[169]. ER directly binds to sequences of DNA referred to as estrogen response elements (EREs), which are located primarily in the promotors of genes[170; 171]. ER can indirectly regulate gene transcription by binding to other transcription factors such as AP-1[172], SP-1[173], or NF-kB[174]. Following DNA binding, co-regulators are recruited to the promotor site, where they interact with the estrogen receptor to regulate gene transcription[175; 176]. The effects of ER activation on gene transcription differs depending on the ligand, the tissue type, and cell type[70; 177].

The estrogen receptor is expressed in both normal mammary epithelial cells and breast cancer cells, although its function differs between the two cell types[178]. In the normal mammary epithelium, ER is expressed in approximately 10-20% of cells, and the ER+ population is primarily non-proliferative[179]. In preinvasive stages of breast cancer, the percent expression of $ER^+$ cells lining the duct increases

dramatically to 95% of the population, and ER+ cells gain the ability to proliferate in response to estrogen[179; 180]. In addition to proliferation, ER activation in breast cancer cells induces other pro-cancerous effects in breast cancer cells, such as modulation of mitochondrial dynamics[181; 182], and a reduction in apoptosis[183; 184]. While mouse studies have shown that ER is essential for the postnatal development of the mammary gland[185], the exact role of ER in the normal mammary gland is largely unknown; ER is notoriously difficult to retain in mammary epithelial cells *in vitro*[186], and consequently, the only ER+ breast cell lines are of cancerous origin. Recently, Ronnoev Jessen and colleagues found that ER can be maintained in normal mammary epithelial cells when grown in the presence of TGFB inhibitors[187]. The authors later compared the ER+ normal mammary epithelial cell line to the ER+ MCF7 breast cancer cell line which revealed differences in estrogen-regulated genes, Ki67/ER localization, and spheroid formation[70]. Future studies are needed to elucidate the function of ER in the normal mammary epithelium.

**1.6.2 Estrogen production -** 17β-estradiol, the most potent endogenous ligand of the estrogen receptor[188], is produced by the cytochrome p450 enzyme aromatase. Aromatase is located in the endoplasmic reticulum where it metabolizes the androgens testosterone or androstenedione to 17β-estradiol or estrone, respectively[148; 149]. The granulosa cells of the ovaries produce the majority of aromatase in pre-menopausal women[148]. After menopause, the ovaries stop producing estrogen, and consequently, the level of circulating estrogens is dramatically lower[189] and ER expression in breast epithelial cells is increased[190]. In the postmenopausal mammary gland, mammary adipose stromal cells[191] are primarily responsible for aromatase production. The expression of aromatase is increased in breast cancer tissue, relative to normal breast tissue[192; 193; 194]. Breast cancer cells have been found to produce aromatase[195; 196], although breast cancer cell lines[197] generally lack aromatase.

**1.6.3 Environmental chemicals that target estrogen signaling** – Breast cancer is a multifactorial disease that is influenced by several factors, such as genetics[198], reproductive history[199; 200], alcohol

use[201], obesity[202], and age[203]. In addition, multiple studies have found that exposure to high levels of estrogens increases breast cancer risk[204; 205]. For example, elevated levels of circulating estrogens[206; 207; 208; 209] and current use of hormone-replacement therapy[210; 211] are associated with an increased breast cancer risk in post-menopausal women. Epidemiological studies have found that women exposed to high levels of xenoestrogens also have an increased breast cancer risk[212; 213; 214; 215]. In addition, there is evidence that exposure to estrogens during development increases breast cancer risk later in life[216; 217]. For example, women who were exposed to diethylstilbestrol *in utero* have an increased risk of developing breast cancer after the age of 40[218; 219]. Consequently, identifying and understanding chemicals that modulate estrogen signaling is one focus of preventative breast cancer research.

While it is accepted that breast cancer risk is influenced by environmental chemicals, most registered chemicals in the United States have not been tested for toxic effects. However, increased pressure from the government and scientific community have sparked interest in evaluating the effects of chemicals on the risk of diseases, such as breast cancer[220; 221]. Due to the high costs and ethical considerations associated with *in vivo* models, initial screens will focus on the evaluation of chemicals *in vitro*. As described above, *in vitro* chemical screens mostly rely on breast cancer cells cultured on a flat plastic surface and exclude the microenvironment. However, some chemicals directly target the stromal cell types of the microenvironment, which can have indirect effects on ER signaling. As described in section 1.4.6, some environmental chemicals have been found to target aromatase[152; 153], which is present in adipose stromal cells but not breast cancer cell lines. In addition, PPARγ[222; 223] and RXR[223] inhibit aromatase expression, which could reduce estrogen signaling in breast cancer cells. Therefore, it the effects of some chemicals are most likely missed when evaluated in simple, monoculture platforms.

**1.6.4 Breast cancer therapies that target estrogen signaling –** Women diagnosed with ER+ breast cancer are typically treated with hormone therapies such as selective ER modulators (SERMs), selective ER downregulators (SERDs), or aromatase inhibitors[224; 225]. SERMs such as tamoxifen or raloxifene act as

ER antagonists in some tissues but weak agonists in others[226]. For example, tamoxifen acts as an ER antagonist in the breast and an agonist in the bone, uterus, as well as other tissues[227; 228]. The mechanism of tamoxifen inhibition of ER-driven gene expression in the breast is known; when tamoxifen binds ER, the binding pocket does not seal which prevents the binding of co-activators[229]. Consequently, the AF-2 transactivation domain is blocked, which is primarily responsible for hormone-driven gene transcription[230]. The SERD fulvestrant is a pure ER antagonist that disrupts ER signaling by competitively binding to and degrading ER[231]. While three generations of aromatase inhibitors have been developed, only the third-generation aromatase inhibitors are used today; the third generation aromatase inhibitors cause less severe side effects and are more effective at reducing aromatase than the previous two generations. The previous two generations also target other types of metabolic enzymes, while the third-generation aromatase inhibitors have higher specificity to aromatase[226; 232]. The third-generation aromatase inhibitors include steroidal and nonsteroidal aromatase inhibitors. Anastrazole and letrozole are nonsteroidal aromatase inhibitors that reversibly bind to the heme iron of aromatase, and consequently prevent androgens from binding to aromatase[233; 234]. The steroid aromatase inhibitor exemestane irreversibly binds to aromatase similar to the androgen androstenedione, and permanently inactivates aromatase[235].

Identifying what treatment to use for a given breast cancer patient is an ongoing challenge. There are several factors that dictate a patient's treatment, such as genetic factors, age, and cancer stage[150; 236; 237]. Postmenopausal women are usually treated with either tamoxifen or aromatase inhibitors, however clinical data suggests that tamoxifen is less effective than aromatase inhibitors at reducing mortality rates and recurrence[150; 151; 158; 238; 239]. In either case, some patients fail to respond to breast cancer therapies initially (*de novo* resistance) or develop resistance over time (acquired resistance)[238; 240]. Women can develop resistance to breast cancer therapies through various mechanisms such as loss of ER[241; 242], ER-independent growth[243; 244], or mutations in ER[245; 246]. The

microenvironment is thought to also contribute to hormone therapy resistance. Clinical trials have shown that aromatase inhibitors are less effective in obese patients than lean patients[247; 248; 249]. While the mechanism is not fully understood, one hypothesis is that estrogen signaling is not sufficiently inhibited in obese women because obese women have higher aromatase expression in the stroma of their breast adipose tissue[159; 162; 250; 251]. Studies have shown that after treatment with aromatase inhibitors, patients with a higher body mass index (BMI) have higher levels of 17β-estradiol and estrone compared to patients with a lower BMI[247; 252; 253]. An alternative hypothesis is that the increased secretion of adipocytokines by the adipose stroma of obese individuals stimulates breast cancer proliferation and survival through an ER-independent mechanism [254]. Multiple studies show that obese stromal cells increase the proliferation of breast cancer cells more than lean stromal cells[250; 255; 256], and that stromal cells can induce ER-independent growth in breast cancer cells *in vitro* and *in vivo*[122; 257]. Future studies are needed to elucidate the role of the microenvironment in mediating aromatase inhibitor resistance in breast cancer cells.

### 1.7 Rationale and Summary

Accumulating evidence suggests that the *in vitro* tools used to study breast cancer are too simple to recapitulate *in vivo* responses, thereby hindering our ability to predict the effects of chemicals on breast cancer risk and progression. While advances in tissue engineering and cancer biology have enabled researchers to integrate components of the microenvironment into *in vitro* platforms, a major challenge is identifying which components to include in *in vitro* models to predict *in vivo* responses. Future studies are needed to determine the effect of the microenvironment on xenobiotic responses, and to define the scenarios in which complex *in vitro* models are needed to better understand chemical actions.

As outlined above, ER is a target of environmental chemicals and breast cancer therapies and multiple components of the microenvironment have been shown to regulate ER-driven responses in

breast cancer cells. With this in mind, I hypothesized that the microenvironment regulates responses to chemicals that target the ER signaling pathway, and that consequently, that chemical responses are missed when evaluated in traditional *in vitro* platforms. In Chapter 2, I describe how the adverse outcome pathway framework can be used to guide the development of *in vitro* chemical testing systems. I develop the adverse outcome pathway for ER mediated breast cancer and use it to identify cell types and readouts that are important to modeling ER+ breast cancer progression. In Chapter 3, I optimize an ER+ organotypic breast model to study how stromal cells affect ER-driven responses in breast cancer cells. I show that immortalized mammary fibroblasts reduce ER protein in breast cancer cells and speed estradiol-induced hyperplasia by reducing apoptosis. In Chapter 4, I incorporate primary adipose stromal cells into the organotypic breast model and use it to study hormone metabolism, as adipose stromal cells produce aromatase. To test if the organotypic breast model is more predictive than a traditional 2D co-culture platform, I evaluate the response of both systems to the aromatase inhibitor anastrazole when breast cancer cells are co-cultured with primary stromal cells derived from lean or obese women. I show that breast cancer cells co-cultured with obese stromal cells in the organotypic model exhibit increased resistance to anastrazole, while the traditional co-culture platform does not segregate the anastrazole responses of lean and obese patients. Next, in Chapter 5, I hypothesize that environmental chemicals differently affect breast cancer cells when cultured as a 2D monoculture, compared to when cultured as a 2D co-culture or organotypic co-culture. I found that the organotin compound, tributyltin chloride, differently affected ER transactivation in monoculture compared to coculture because tributyltin chloride induced aromatase activity in adipose stromal cells. Finally, Chapter 6 discusses the overarching conclusions of the dissertation and a description of possible future directions that will further enhance understanding of how the microenvironment regulates ER-driven responses in breast cancer cells, as well as the need of the microenvironment when testing chemicals *in vitro.* Altogether, this work highlights the gap that exists between *in vitro* and *in vivo*

models and demonstrates that the microenvironment can be incorporated into *in vitro* models to better recapitulate chemical effects.

## Chapter 2: Personalized *in vitro* Cancer Models to Predict Therapeutic Response: Challenges and a Framework for Improvement

Personalized cancer therapy focuses on characterizing the relevant phenotypes of the patient, as well as the patient's tumor, to predict the most effective cancer therapy. Historically, these methods have not proven predictive in regards to predicting therapeutic response. Emerging culture platforms are designed to better recapitulate the *in vivo* environment, thus, there is renewed interest in integrating patient samples into *in vitro* cancer models to assess therapeutic response. Successful examples of translating *in vitro* response to clinical relevance are limited due to issues with patient sample acquisition, variability and culture. We will review traditional and emerging *in vitro* models for personalized medicine, focusing on the technologies, microenvironmental components, and readouts utilized. We will then offer our perspective on how to apply a framework derived from toxicology and ecology towards designing improved personalized *in vitro* models of cancer. The framework serves as a tool for identifying optimal readouts and culture conditions, thus maximizing the information gained from each patient sample.

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## 2.1 Introduction: Need for personalized medicine, current approaches and *in vitro* models

Classically, patients have relied on systemic treatments and invasive procedures such as chemotherapy, radiation therapy, and surgery to combat cancer. Choosing the right cancer treatment is difficult because limited tools, money and time are available to guide this decision. The therapeutic index for most cancer treatments is extremely narrow, requiring a balance between drug efficacy and toxicity tolerance. In a given population, drug efficacy and tolerance can differ greatly between individuals and even across an individual tumor [258; 259]. The mechanisms underlying cancer development and progression vary drastically from patient to patient and a cure for one patient can be ineffective or harmful to another. Researchers have shifted from trying to identify silver bullet "cures" for a given cancer to attempting to find solutions to combat patient specific cancer subtypes or in other words personalized medicine [260; 261].

Personalized medicine encompasses both tumor and non-tumor systemic patient phenotypes that contribute to the effectiveness of a treatment/therapeutic [262; 263]. Human populations can exhibit profound systemic differences in drug disposition, which can also contribute to therapeutic response. Identifying polymorphisms in genes that encode for drug metabolizing enzymes can help determine if a patient will experience adverse effects or even no effects in response to a drug [264; 265]. For instance, individuals that carry the uridine diphosphate glucuronosyltransferase (UGT) 1A1*28 gene variant produce less of the UGT1A1 enzyme and are at high risk for irinotecan associated morbidity and mortality [266; 267].

Assessment of tumor cells themselves provide a lens into susceptibilities of a tumor to targeted therapies. One method to evaluate tumor cells is through profiling the tumor's DNA, RNA, or protein, to identify molecular biomarkers that are predictive of patient response. A second method is to integrate tumor cells into chemosensitivity and resistance assays (CSRAs), a term used to describe an *in vitro* functional assay that measures response to a drug *ex vivo*, which will be discussed in detail later.

Investigating the molecular profile of cancer cells to identify biomarkers can help predict drug resistance, and have identified gene expression profiles that correlate with cancer recurrence after specific drug treatments [268; 269; 270]. Small molecule kinase inhibitors such as gefitinib or erlotinib are the suggested drug treatment for lung cancer patients whose tumors have mutations in epidermal growth factor receptor. However, if those patients also have mutations in KRAS they are at high risk for gefitinib and erlotinib resistance [271]. Gene expression combined with protein secretion profiles of cancer cells also identify biomarkers for targeted therapies [272; 273; 274]. One study of patients with advanced cancer including over 15 different tumor types found that patients who underwent targeted therapy had a significant increase in overall response rate, time to treatment failure, and survival duration [275]. While molecular profiling of the tumor is a powerful approach, the focus of this review is to discuss the technological advancement of CSRAs and methods to improve their clinical use.

Beyond tumor cells themselves, some cancer therapeutics do not target the tumor alone but also the associated microenvironment. In breast, colorectal and prostate carcinomas, stromal components actively participate in cancer development and are continuously modified as the disease progresses. It has been demonstrated that tumor-associated stroma undergoes extensive changes in gene expression and that a stromal transcript signature correlates with histological tumor grade [268; 276; 277]. Accordingly, targeting stromal changes is gaining acceptance as an alternative option for treating cancer. For example, vascular endothelial growth factor (VEGF) inhibitors such as bevacizumab combat cancer by targeting tumor angiogenesis [278]. These examples of tumor and tumor microenvironment contributions to response underscore the complexity of individual tumors and highlight the need for an expanded view when considering therapeutic response of individuals to cancer therapies.

While patient profiling can improve patient outcome, cancer mortality remains high and it is clear that we need additional approaches [279]. In response, there is once again increasing interest in integrating patient samples into *in vitro* models to predict *in vivo* response. These models, most

commonly referred to as chemosensitivity and resistance assays (CSRAs), have the potential to provide a rapid, high throughput and inexpensive approach to predicting therapy for individuals, but have thus far faced challenges that have hindered their success [263]. Poor *in vitro* culture conditions [263; 280], the limited information offered by traditional *in vitro* readouts [281; 282], and tumor tissue heterogeneity [259; 263; 280; 282] have been invoked to explain discordance between *in vivo* and *in vitro* therapeutic response.

In 2004 and 2011 the American Society of Clinical Oncology reviewed personalized *in vitro* models or what they referred to as "chemotherapy sensitivity and resistance assays" (CSRAs). In both instances, they recommended not to use these models to identify appropriate therapeutic agents outside of clinical trials but stressed their potential importance [282; 283]. Advances in molecular biology, toxicology, biomedical/tissue engineering and other fields offer advances that may help move CSRAs into clinical use.

In an effort to improve CSRAs, we propose a guiding framework coined a therapeutic outcome pathway (TOP), to select culture conditions, cellular readouts and key components to include in a CSRA. The TOP concept is based on the adverse outcome pathway (AOP) framework used in toxicology to map the molecular, cellular and tissue level targets of a toxin or in this case a therapeutic. We will begin our discussion by addressing challenges with *in vitro* personalized models and the emerging solutions. This will familiarize the reader with the advantages of personalized *in vitro* models and the obstacles that hamper their success. We will then introduce the TOP framework and describe how it might be used to overcome these obstacles and maximize the information retrieved from a patient sample. The TOP framework will facilitate the development of personalized *in vitro* models because it will highlight what should be incorporated into models and what readouts will be most predictive of response. To illustrate the usefulness of the framework, we will provide an example of estrogen receptor (ER) positive breast cancer and tamoxifen treatment.

## 2.2 The nature of patient derived samples: Heterogeneity, sample acquisition, and challenges with primary cell culture

Intertumor heterogeneity (heterogeneity between tumors of different patients) has driven the need for personalized medicine [284]. Even within the same cancer subtype, each patient's tumor can have distinctly different gene expression profiles, tumor microenvironments and behaviors [285]. Early attempts to predict *in vivo* response made use of immortalized cell lines *in vitro.* However, immortalized cell lines are highly selected subpopulations and do not adequately reflect the heterogeneous function and behavior of tumors [286; 287]. Consequently, there is increased interest in using primary cells in personalized *in vitro* models for evaluating drug response [259; 288; 289].

While ideal, patient derived primary cells present their own set of challenges in areas such as sample acquisition, variability and culture. First, they are difficult to obtain in the standard diagnostic workflow. Traditionally, standard of care dictates that tissue removed during biopsy be immediately sent for pathological examination where it is assessed for morphologic and molecular markers that are indicative of disease stage and cancer subtype [290]. Pathological review is well established and is a reliable method to gain valuable information about a patient's cancer, but it offers limited predicted power [291; 292; 293]. Integrating patient samples into *in vitro* models requires that some part of the sample, which would ordinarily go to pathology, be relinquished for research purposes. With early detection and small tumor samples, obtaining a sample for research prior to or concurrent with pathological review is often not possible. Second, there are logistical complexities in transferring sample from operating room, to surgical pathology, and ultimately to researchers while maintaining cellular viability. Institutional tissue biobank can be useful in this regard [294; 295]. Once researchers have received primary samples, those tumor pieces are further processed, introducing additional variability.

Intratumor heterogeneity (heterogeneity within the same person's tumor) underlies many of the failures of CSRAs to date. The source of intratumoral heterogeneity stems from natural variations in

tissue architecture, tumor evolution, selection of cells during processing, as well as changes induced when tumor cells are removed from the *in vivo* environment. This heterogeneity is thought to partly explain the high variability and response when utilizing primary cells [296]. Perhaps this shouldn't be surprising since treatment induced regression *in vivo* can also sometimes be seen in some areas of an individual tumor but not others, indicating that a tumor sample may not be representative of overall tumor behavior [259].

Finally, primary cells become senescent after only a few passages and in many cases only a small number of patient cells are available for testing [297; 298]. The limited availability and life span of primary cells makes it difficult to develop, optimize and validate new assay platforms. It is important, therefore, to consider if the information gained by personalized *in vitro* models outweighs what is gained from evaluation of standard clinical parameters [286]. In summary, patient sample acquisition, variability and culture has hindered progress in personalized *in vitro* models.

## 2.3 The current state of primary cell based *in vitro* models: What has been done and how we can improve

The idea of personalized *in vitro* models is not new; researchers have been attempting to develop *in vitro* methods to predict therapeutic response for several decades [299; 300]. Despite these efforts, CSRAs are not recommended outside of a clinical trial setting [282]. While these assays have had some success with predicting drug resistance, they generally fail to predict drug sensitivity [301]. Overall, current personalized *in vitro* models do not offer significant clinical benefit and must be improved [301; 302; 303]. Several reviews evaluate the limitations of predictive *in vitro* models and their use in clinical practice [280; 302; 304].

Several potential contributing factors likely underlie the historical failures of CSRAs, but one significant problem is that *in vitro* models are too simplistic to accurately model and predict *in vivo* response to therapeutics [263; 282]. In hope of developing more robust cancer models, some groups have

begun to incorporate additional microenvironment components factors into *in vitro* cancer models [71; 91; 305; 306]. In addition to creating more biologically relevant culture conditions, we speculate that analyzing several orthogonal readouts per sample will improve model predictability. Here, we will summarize the technical evolution of CSRA platforms. We will first discuss what these models have traditionally entailed and then discuss if incorporating additional microenvironmental factors and readouts into these platforms is needed to predict patient response.

### 2.3.1 Traditional CSRA platforms

In traditional *in vitro* CSRAs, researchers isolate cancer cells from the patient tumor, culture them on a two-dimensional (2D; flat plastic or glass surface) well plate and expose them to an array of drugs to assess response. These platforms typically assess response using one readout per sample, most often cell survival/death [280]. For example, the adenosine triphosphate (ATP), 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT), extreme drug resistance (EDR), and ChemoFX assay have been widely used as the sole endpoint in personalized *in vitro* models [307]. Several groups have attempted to use the ATP assay to predict drug effectiveness of patient derived cancer cells. However, the assay has been found to be sensitive but not specific, and could therefore predict drug sensitivity but not resistance [308]. One study reported increased overall survival in the ATP assay directed group [309] while another group reported that survival was not improved [310]. The MTT assay was also used to evaluate epirubicin and docetaxel sensitivity in primary human breast cancer cells and correctly predicted changes in cancer cell proliferation but not overall tumor size [311]. The MTT assay was used to predict drug effectiveness in 353 gastric cancer patients but no difference in clinical outcome was found between the control group and the MTT sensitive group [303]. EDR assays have also had mixed success, where some groups found that the test could predict patient survival where others could not [312; 313; 314]. The ChemoFX assay is promising but may not be generalizable. Several groups have reported that the ChemoFX assay is predictive of patient response [315; 316] and that ChemoFX directed drug treatment

improves overall survival [317]. However, one group found that ChemoFX did not correlate with patient outcome and did not improve overall survival [318]. In the latter, the authors assessed tumors from patients with a variety of cancers, where ChemoFX has been mostly used to predict therapeutic response in ovarian cancer patients [319]. Thus, there has been mixed success with the one readout, one cell type on a 2D architecture approach.

These models do not incorporate key features of the *in vivo* environment such as tissue structure, matrix composition, an immune component or stromal cells despite evidence suggesting the pivotal role each play in cancer progression [320; 321; 322]. Mounting evidence supports that the absence of these components result in a decreased drug resistance, suggesting incorporation of at least some of these factors is necessary to accurately predict drug response [37; 323; 324; 325; 326; 327].

Cells cultured in 2D environments undergo changes in morphology, deposition of ECM proteins, migration, cell growth, invasion and apoptosis [324; 328; 329; 330; 331]. Primary cells also lose critical cell functions under these conditions [332; 333]. It has been shown that cells cultured in 2D conditions experience a reduction or complete loss of some receptors and signaling molecules known to be critical to tumor progression in many different cancers, such as androgen receptor, human epidermal growth factor receptor, and estrogen receptor [334; 335; 336].

### 2.3.2 Emerging CSRA platforms with ECM and microenvironmental components

Researchers have attempted to improve predictability of *in vitro* models and have begun to develop more complex CSRAs that promise to be more robust by incorporating key features of the *in vivo* environment [91]. We distinguish examples of predictive *in vitro* cultures that have incorporated primary cancer cells embedded in extracellular matrix proteins, from those with spheroid structure, organoid structure and/or incorporated stromal cell types into different categories **(Fig. 2.1)**. These platforms can be well-plate based or constructed using microfluidic technologies. Microscale *in vitro* models can provide unique functionality and controllability (e.g., enhanced spatial and temporal

controls) and are emerging as practical tools to investigate tumor-stroma interactions in various three dimensional (3D) conditions more efficiently. The fundamentals and applications of microfluidic *in vitro* models have been extensively reviewed [106; 337; 338; 339]. One well-known advantage of microfluidic systems is the reduced number of cells required for each endpoint, which makes the microfluidic system especially attractive when working with patient samples [298].

3D cell culture models allow cells to be cultured in natural hydrogels, such as collagen or Matrigel, or synthetic biomaterials, such as polyethylene glycol (PEG) gels. The incorporation of extracellular matrix (ECM) components into cultures has shown striking differences between 3D and 2D cultures, including differences in morphology, adhesion, polarity, differentiation and gene expression [92; 328; 340; 341]. For instance, in some cases culturing primary cells in 3D conditions can prevent the prototypical loss of receptor expression seen when primary cells are placed in 2D culture conditions [335; 342]. Morphological differences have also been reported in primary cells when cultured in 2D vs. 3D, where a more *in vivo* like morphology was observed in 3D [324]. It is speculated that these differences are because materials used in 3D cultures provide structural and chemical components that are similar to ECM found *in* vivo [343]. It is also important to note that chemical and mechanical properties of various ECM materials influence cell behaviors in 3D *in vitro* cultures considerably [71; 323]. Various synthetic and natural materials that have been used in designing 3D *in vitro* system have been previously reviewed [305; 344; 345]. The collagen gel droplet embedded culture drug test (CD-DST) is one example of a 3D CSRA. Similar to 2D predictive assays, the predictive value of the CD-DST is mixed because in some cases it has high sensitivity but low specificity [346; 347].

Co-culture of cancer cells with other cellular components of the microenvironment has been shown to influence the growth rate, protein secretion and drug sensitivity of cancer cells [348]. Several groups incorporated patient stromal cells into a variety of personalized *in vitro* platform architectures [298; 324; 349; 350; 351]. In four cases that assessed the influence of stromal cells on drug sensitivity, the authors

found differences in the effective dose of primary cancer cells cultured in a monoculture compared to co-culture, highlighting the importance of integrating supportive cell types into predictive models [298; 324; 350; 351]. The majority of these platforms assessed cytotoxicity as their readout [324; 352], however, a microscale platform developed by Young et. al allowed a functional analysis of patient samples in addition to evaluation of cell viability, where NF-kb and STAT3 nuclear translocation were evaluated following drug treatment [298].

Spheroid culture models, where cells self-arrange or proliferate into spherical balls of cells, sometimes with a hollow lumen, are another popular tool to study cancer in a more physiologically relevant system [353]. There is evidence that organization of cells in spheroids, rather than a monolayer, better reflects the *in vivo* behavior of tumors [354; 355; 356]. While there are several techniques to generate spheroids, most commonly cells are placed in a 2D or 3D environment that encourages cell-cell adhesion rather than matrix adhesion [357]. With growing recognition of the importance of 3D microenvironment, spheroids also can be generated in various biomaterials to achieve spheroid-ECM interactions [323; 358; 359]. Several groups have compared response when primary tumor cells are cultured in traditional 2D conditions and tumor spheroids. One group cultured primary oral cancer cavity cells in microscale 2D, 3D or spheroid platform and found that the culture method influenced drug sensitivity, metabolic activity and proliferation [352]. Kim et al. found that patient derived tumor spheroids composed of mesothelioma cells, macrophages and collagen demonstrated apoptotic resistance to chemotherapeutics, whereas mesothelioma cell monolayers did not [349]. Other groups have used spheroids to evaluate sensitivity to chemotherapeutics, where the authors suggested that the *in vitro* model could be used to determine the dose most appropriate for a given patient. However, this claim was not validated and purely speculative; these authors did not (to our knowledge) use doses identified in the *in vitro* assay to guide the treatment of patients in the clinic [360].

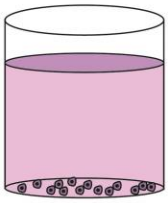
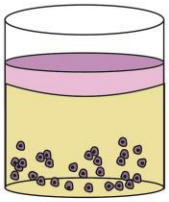
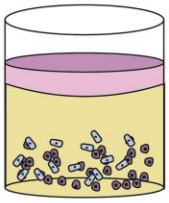
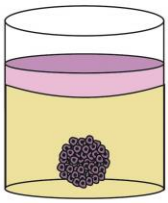
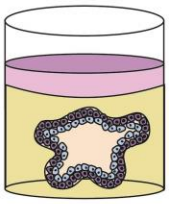
| Culture Method | Schematic | Architecture | Example |
|---|---|---|---|
| Traditional (2D) |  | Monolayer of cancer cells on a flat plastic or glass surface | Sevin et al., 1998; Singer et al., 2013; Wu et al., 2003 Mehta et al., 2001 Matsuo et al., 2010 Hsieh et al., 2015 Kim et al., 2015 Pak et al 2015 Edmond et al, 2012 |
| 3D |  | Cancer cells embedded in matrices such as collagen I or Matrigel | Xu et al., 2013 Higashiyama et al., 2010 Takebayashi et al., 2013 Kim et al., 2005 Hsieh et al., 2015 |
| Coculture |  | Cancer cell type along with another cell type of the microenvironment (fibroblast, non cancerous epithelial cell, etc.) | Xu et al., 2013 Janine et al., 2015 Edmond et al., 2012 Kim et al., 2005, Pak et al., 2015 |
| Spheroid |  | Cell aggregates in a 3D or 2D environment that prevents cell-matrix adhesion and stimulates cell-cell adhesion | Kim et al., 2005 Konodo et al., 2015 Hsieh et al., 2015 Xu et al., 2013 Janine et al., 2015 |
| Organoid |  | Organ-like structure typically derived from a single stem cell | Gao et al., 2014 van de Wetering et al., 2015 Huang et al., 2015 |

**Figure 2.1. Examples of traditional and emerging personalized *in vitro* platforms.** While personalized *in vitro* platforms have traditionally evaluated cancer by studying one cell type on a plastic or glass surface, researchers have begun to increase physiological relevance by incorporate extracellular matrix proteins, additional cell types and/or structure.

Finally, the emergence of patient-derived organoids has garnered a great deal of interest in the personalized medicine field. Organoids are derived from stem cells and are considered more complex/differentiated than spheroids because they recapitulate some aspects of organ structure and function including differentiation into multiple cell types (i.e. basal and luminal epithelial cells) [361; 362; 363].

Perhaps the most discussed example is the generation of crypt-villus structures from a single intestinal stem cell [364]. Patient-derived organoids are advantageous because they can be cultured long term, are amenable to drug screening and retain histological, immunohistochemical and genetic features of the tissue they were derived from [365; 366; 367; 368]. One study sequenced the genomes of organoids derived from castration resistant prostate cancer patients to identify molecular signatures of drug resistance. They also evaluated the influence of three cancer drugs on the growth rate of the organoids and demonstrated that analyzing both drug sensitivity and genomic data can offer a more complete insight into patient response. [365]. Another study generated organoids from 20 colorectal carcinoma patients and evaluated 80 different compounds on organoid viability. The authors coupled genomic data with *in vitro* drug response to gain a more comprehensive understanding of how each patient may respond to a given drug. As expected, these studies revealed heterogeneity in patient response, for instance, organoids with KRAS mutations were more resistant to anti-EGFR inhibitors. Interestingly, these studies also identified patients who had high sensitivity to compounds with no known genetic biomarker. [367].

### 2.3.3 Choosing the right platform complexity, culture conditions and assay readouts

We argue that using more physiologically relevant cultures may improve the likelihood that an *in vitro* model can predict response, and should be further explored. Ideally, a model would incorporate all components of the tumor and microenvironment but there is a trade-off between complex and physiological relevance with reproducibility, ease of use, and cost. It is essential that we recapitulate the *in vivo* environment well enough so we can accurately model drug response, while also maintaining the simplicity necessary for evaluating drugs in a manner that is succinct and cost efficient [323]. In section 4.0, we will offer our perspective on how to guide this process.

Some authors have argued that the simplicity of a “one readout approach” is beneficial for evaluating clinical response [302]. While this approach may allow a more straightforward assessment of *in vitro* response, cancer is a complex disease that occurs as a result of multiple hits or changes to several

critical components (e.g. changes in gene expression, stromal changes, angiogenesis etc.) [277; 369]. Moreover, some subtypes of cancers are tightly regulated by a biological pathway, such as the androgen receptor pathway in prostate cancer [370] or the estrogen receptor pathway in breast cancer [371]. These pathways are complex and in order to determine if a drug can act on and produce a therapeutic response to combat a subtype of cancer, it is often necessary to run multiple assays both *in vitro* and *in vivo*. Using one endpoint per *in vitro* assay has been sufficient for drug development because it is possible to run several separate experiments on immortalized cell lines and use *in vivo* models to evaluate tissue level changes [372]. However, this is problematic when working with patient samples because only a small number of cells are available for testing and this approach is costly and time consuming.

We hypothesize that one of the major challenges that has hindered the success of personalized *in vitro* models is the limited information elicited from each sample. To increase confidence that personalized *in vitro* models can predict drug response, it is important to analyze multiple readouts in a single sample at different levels of organ complexity. While we were previously limited by the available technologies, there has been a great deal of improvement in assay development, analytical equipment (e.g. microscopes, plate readers), sequencing and culture platform architectures [46; 261; 281; 373]. Genomic characterization of tumors enables the identification of mutations, gene deletions, etc. that are indicative of drug sensitivity/resistance [374]. Additionally, reagents that readout diverse physiologies and are compatible with live cell assays have been developed, enabling researchers to multiplex several readouts in one model [375; 376]. The emergence of 3D, spheroid, and other organotypic cultures allows the analysis of tissue level changes [1; 377; 378]. The term “3D model” has been used interchangeably with “organotypic model”. While similar, the organotypic model refers more specifically to 3D models that faithfully recapitulate *in vivo* like structures *in vitro*. In particular, the integration of microfluidics with organotypic models enables the generation of tissue structures, such as a circular shaped lumen system,

with enhanced controls and throughput [377]. Organotypic models enable researchers to evaluate changes that were previously difficult to quantify *in vitro,* including key events in cancer progression, such as invasion, angiogenesis, intravasation, and extravasation [1; 114; 378; 379]. Evaluating responses from functional assays in addition to the molecular signatures of a given tumor would offer valuable insight into optimal drug options for the individual patient.

The development of models that analyze multiple endpoints at a molecular, cellular and tissue level may increase the likelihood that evaluating a patient sample will provide predictive information for identifying successful treatments. However, with the countless assays available and many changes that occur during cancer, it is often difficult to determine which endpoints would provide the most valuable information for evaluating drug response.

### 2.4 The therapeutic outcome pathway: A conceptual framework to aide in the development and application of personalized CSRAs

We have so far discussed how increasing the physiological relevance of our cultures and integrating readouts at a molecular, cellular and tissue level should improve the predictive value of personalized *in vitro* models. While these advantages may be intuitive, it is less apparent what specific elements of the *in vivo* environment should be incorporated into models to best evaluate response for a given therapeutic. Cancer development and progression can vary drastically depending on cancer subtype [380]. Even therapeutics that target the same cancer subtype can work through different mechanisms and have different risk factors for resistance [381; 382]. Consequently, factors that are important when evaluating drug response may vary depending on the cancer subtype and therapeutic being tested. To determine what factors are most important when assessing therapeutic response, we must ask several questions. For instance, 1) are there any microenvironmental components that are important in disease progression or are targeted by the therapeutic? 2) What events at a cellular, molecular and tissue level are most important when evaluating response to a given cancer drug? 3) Are there any genetic

signatures that might predispose a patient to therapeutic resistance or toxicity? To better organize this information, we propose the use of the therapeutic outcome pathway (TOP), a variation of the adverse outcome pathway (AOP), a framework commonly used to model mechanisms of drug/chemical induced toxicities [383]. In context of personalized medicine, the purpose of the TOP is to identify what cellular and microenvironmental factors are essential and what endpoints are critical for evaluating response to a given therapeutic, in order to maximize the likelihood that a personalized in vitro model is able to predict therapeutic response.

### 2.4.1 Introduction to Outcome Pathways

It is our perspective that a generalizable framework that graphically describes the essential components and molecular, cellular and tissue level events that occur during cancer progression and targeted therapy is key to advancing personalized CSRAs. The AOP framework, utilized in designing toxicity screening platforms, is an existing framework that has already been used to predictively model disease and screen for compounds that speed cancer progression [383; 384]. The visual representation of key events in disease progression provides a clear understanding of how a chemical exposure can result in an adverse outcome. AOPs employ a hierarchical approach of increasing biological complexity from the earliest molecular event (molecular initiating event) to the advanced tissue level adverse outcome (tumor growth) and beyond to death **(Fig. 2.2)** [383]. Some examples include estrogen receptor driven breast cancer, androgen receptor driven prostate cancer or NFkB activation driven multiple myeloma [332; 385; 386]. The intermediate steps or key events that directly link the molecular initiating event and adverse outcome are mapped using the basic science literature and noting the weight of evidence that connects these events. Key events include responses that are both driven by the molecular initiating event but occur independently of each other perhaps even in different cell types or tissues, referred to as orthogonal events. Previous reports have discussed strategies to construct AOPs, including a formal document published by the Organisation for Economic Co-operation and Development (OECD) [383; 387; 388].

While AOPs are constructed for the purpose of identifying early molecular or cellular endpoints to incorporate into chemical screening platforms, TOPs go beyond this utility to incorporate molecular, cellular and tissue level effects.

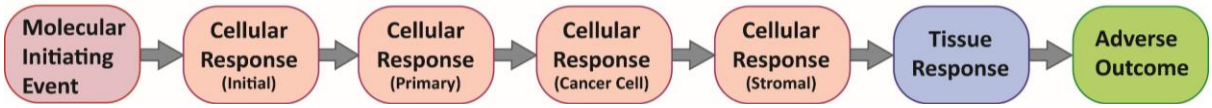



**Figure 2.2. The AOP framework.** A typical layout of an AOP, a framework that defines the key events between a molecular initiating event and an adverse outcome.

### 2.4.2 Identifying key components and readouts for CSRAs

We propose that the outcome pathway framework could be used to improve predictive medicine by guiding the development and implementation of personalized CSRA platforms. In this regard, the AOP would be used in the identification of key components of a system that are needed to recapitulate the mechanism by which a given compound causes toxicity (the adverse outcome, cancer progression and death). The identification of events at different levels of biological organization is especially useful when developing organotypic models.

In addition to identifying microenvironmental components that are needed to recapitulate a therapeutic effect, we have found that an extension of the AOP, what we have coined the therapeutic outcome pathway (TOP), can help identify how proposed therapeutics target key events of an AOP (e.g. which event(s) of the AOP does the therapeutic target). The TOP will help pinpoint key events that may be useful as readouts to determine therapeutic effectiveness. In contrast to the traditional AOP framework, a TOP should also include key effectors that alter the metabolism and excretion of the therapeutic as well as measures of therapeutic resistance, as these are all critical in therapeutic response and effectiveness.

As an example, we created an AOP for a specific cancer subtype, estrogen receptor driven breast cancer, which is shown below in section 4.3. We then developed a TOP in section 4.4 by assessing tamoxifen in context of the AOP.

### 2.4.3 Utilizing the AOP of estrogen receptor activation and breast cancer progression to guide the development of CSRAs

Identifying the key events that occur during ER+ breast cancer progression can help determine what microenvironmental components and readouts (readouts are discussed in the next section) may be most predictive when modeling breast cancer response. We have developed the ER mediated breast cancer AOP to frame this information and help make decisions regarding model components.

Major sources of estrogen receptor (ER) agonists include estrogen synthesis in the ovaries and local synthesis through conversion of androgens to estrogens via aromatase [148; 389; 390]. Estrogens and other ER agonists bind to ER and stimulate ER activation, the molecular initiating event of the AOP [391]. Initial cellular changes include ER-induced genomic and non-genomic effects. In the genomic pathway, estrogen binding to ER results in a series of events (posttranslational modification, dimerization, conformational change). This leads to ER binding either directly (the classical pathway) [392; 393; 394] or indirectly (the non-classical pathway) [169; 391; 395; 396] to DNA and the initiation of transcription. The rapid, non-genomic effects of ER are less characterized but are thought to include stimulation of signaling pathways through mechanisms such as an increase in production of secondary messengers [397; 398].

Following the initial cellular responses, there are primary cellular responses such as changes in gene transcription and protein production [392; 393; 397; 399]. Well-characterized ER induced cellular responses of breast cancer epithelial cells include changes in mitochondrial dynamics [181; 400; 401], oxidative stress and DNA damage [390; 402; 403]. These changes are thought to influence proliferation [397; 404; 405; 406; 407] and apoptosis [183; 408; 409]. For instance, ER induced changes in mitochondrial dynamics, oxidative stress, and DNA damage have been shown to increase proliferation of ER+ breast cancer epithelial cells [402; 410; 411]. Modulation of mitochondrial dynamics, proliferation and oxidative stress increase DNA damage, which can lead to pro-cancerous mutations [390; 400; 402]. Estradiol exposure can also result in DNA damage

through estrogen metabolism, although this mechanism is independent of ER activation [403; 412]. While more controversial, some authors have observed an ER induced increase in motility [413; 414; 415] where others have reported a decrease [416; 417; 418].

Cells from the microenvironment can also be incorporated in this framework. Endothelial cells express ER and are stimulated by ER to proliferate and migrate [407; 419; 420]. Macrophages, fibroblasts and adipocytes undergo changes and become 'cancer associated' during breast cancer [35; 36; 38; 421]. While these cells are influenced by and influence cancer progression, it is unclear if there is a direct relationship between estrogen receptor activation and cancer association, although some evidence supports a direct relationship between ER activation and macrophage function [422; 423]. M2 macrophages, coined tumor-associated macrophages (TAMs) secrete cytokines and other factors that stimulate angiogenesis, suppress immune response and encourage tumor cell proliferation and invasion [165; 424]. Cancer associated fibroblasts (CAFs) increase proliferation and invasion of cancerous epithelial cells and recruit inflammatory and endothelial cells to tumor sites [425; 426]. Cancer associated adipocytes (CAAs) become delipidated, obtain a fibroblast-like morphology and encourage cancer cell proliferation and invasion [35; 38]. CAFs, CAA and TAMs secrete proteins that degrade and remodel the extracellular matrix, such as matrix metalloproteinases [36; 51; 427]. They also produce unusually high levels of aromatase that greatly contribute to estrogen rich environment found in ER+ breast cancer tissue [38; 165; 428; 429; 430].

The next level examines tissue-level responses. Decreased apoptosis and proliferation cause ductal filling (tumor growth) [75] and changes in endothelial cell migration and proliferation result in increased angiogenesis, which supports tumor growth [407; 419; 431; 432]. The extracellular matrix is remodeled due to stromal cell induced alterations of matrix stiffness, collagen I orientation (the most abundant protein in the breast), and the composition of other extracellular matrix proteins [46; 87; 433; 434; 435]. While important in disease progression, it is unclear if ECM changes are directly related to ER. Finally, these changes collectively encourage invasion out of the mammary duct and colonization of

secondary sites, which is ultimately what leads to the adverse outcome, breast cancer related death [46; 436]. Whether ER plays a direct role in motility and invasion processes has not been established, in part due to lack of good models of ER+ metastasis. Nevertheless, the existence of ER+ CTCs and metastatic tumors provide evidence that ER+ cancer cells are subject to the same pressures at the tissue level [437].

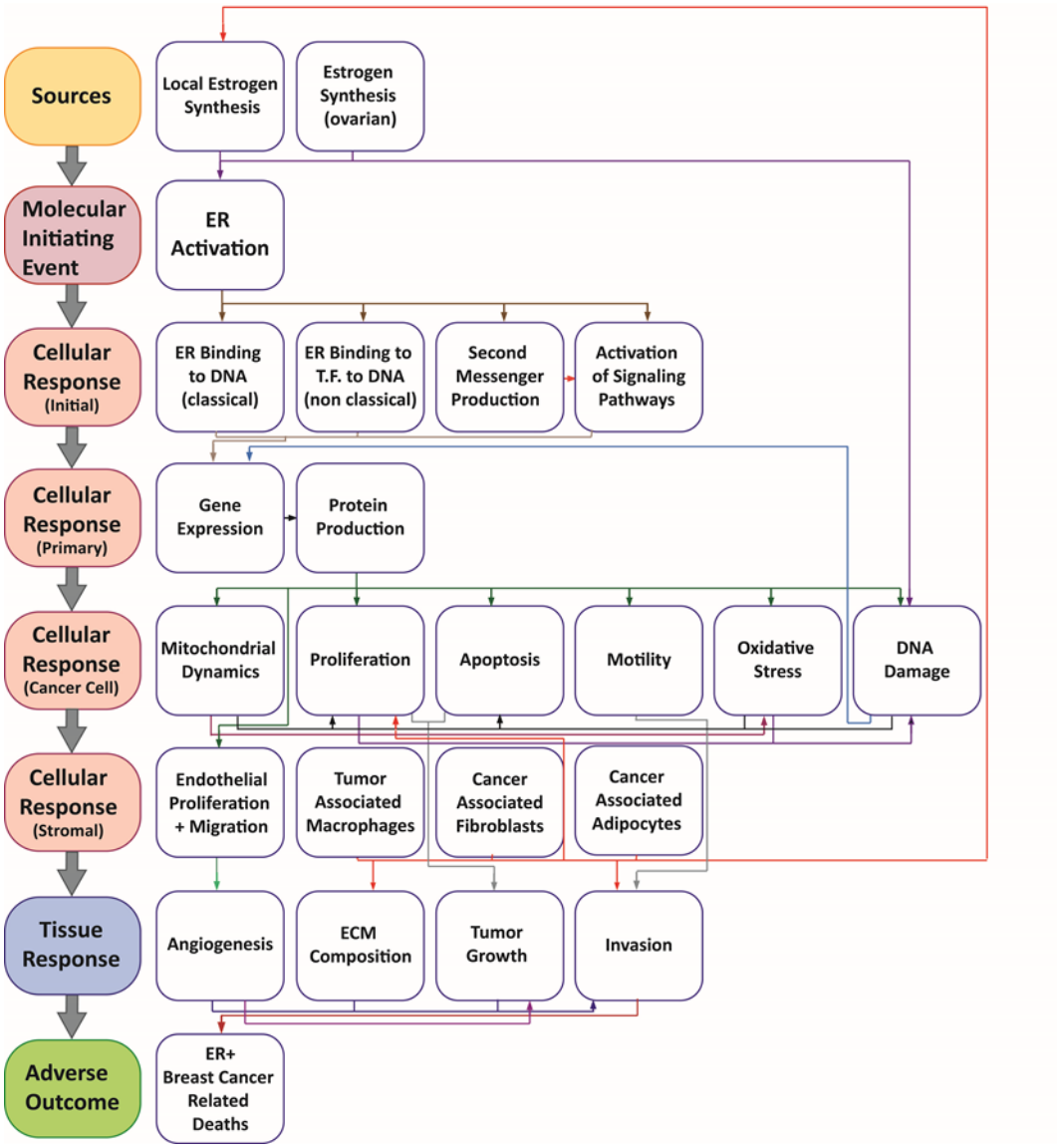



**Figure 2.3. The AOP of ER mediated breast cancer progression.** We have identified the key events that occur between the molecular initiating event, estrogen receptor activation and the adverse outcome, breast cancer related death. This framework highlights what microenvironmental factors may be important when evaluating response.

| *Event* | *Weight of Evidence* |
|---|---|
| ***Molecular Initiating Event*** | |
| Estrogen receptor activation | Very Strong |
| ***Cellular Effects (Initial)*** | |
| ER binding to DNA (classical) | Very Strong |
| ER binding to T.F. to DNA (non classical) | Very Strong |
| Second messenger production | Strong |
| Activation of signaling pathways | Strong |
| ***Cellular Effects (Primary)*** | |
| Gene expression | Very Strong |
| Protein production | Very Strong |
| ***Cellular Effects (Secondary)*** | |
| Proliferation | Very Strong |
| Apoptosis | Very Strong |
| Oxidative Stress | Very Strong |
| DNA Damage | Very Strong |
| Mitochondrial Activity | Very Strong |
| Motility | Moderate |
| ***Cellular Effects (Stromal)*** | |
| Endothelial Proliferation + Migration | Very Strong |
| Cancer association of fibroblasts | Weak |
| Cancer association of adipocytes | Weak |
| Cancer association of macrophages | Moderate |

| ***Tissue Effects*** | |
|---|---|
| Angiogenesis | Very Strong |
| ECM Composition | Moderate |
| Tumor Growth | Very Strong |
| Invasion | Moderate |

**Table 2.1.** A weight of evidence assessment was done for each key event according to the Bradford Hill Criteria.

To ensure reliability and robustness of our AOP, we have used the Bradford Hill Criteria outlined by the OECD to assess weight of evidence for each key event (Table 1) [438]. Here, we have identified several key events that link the molecular initiating event (ER activation) and the adverse outcome (breast cancer related deaths), and several microenvironmental components that play a role in breast cancer progression. While it is not feasible to include all of these factors into a CSRA, we can use the framework depicted in **Figure 2.3** as a guide to determine what components are most important. The cancerous epithelial cells incorporated in the model should be ER+, as ER activation is thought to drive the AOP. ER status is commonly lost when primary cells are placed in 2D conditions. However, Novaro et. al reported that ER expression is better maintained in murine cells when cultured on an extracellular matrix [335]. Therefore, ECM proteins such as laminin or collagen IV could be incorporated into the model to help maintain ER function. In addition to epithelial cells, endothelial cells may be of importance when modeling disease progression. Endothelial cells are directly influenced by ER activation and angiogenesis supports tumor growth and epithelial cell invasion. Fibroblasts could also be important to include into the CSRA. As discussed in section 3.2, fibroblasts influence drug sensitivity. In context of the AOP, fibroblasts play a role in extracellular matrix reorganization, epithelial proliferation and provide routes of invasion.

### 2.4.4 Utilizing the TOP of tamoxifen and ER mediated breast cancer progression to help identify predictive readouts

Tamoxifen is a selective estrogen receptor modulator that is commonly used to combat ER+ breast cancer progression [439]. Unfortunately, therapeutic response to tamoxifen can vary and it is often unclear if a patient will benefit from the drug [396; 439; 440]. The application of tamoxifen towards the ER mediated breast cancer progression AOP can help frame what readouts are most important for predicting tamoxifen effectiveness. This will generate the tamoxifen TOP. Given the limited success of CSRAs to date, we will outline considerations relevant to inclusion of patients within a study, as well as factors that influence choice of endpoints to monitor.

It is important to first discern what patient factors may result in a CSRA failing to predict response including ADME and resistance mechanisms. Tamoxifen is a prodrug and is metabolized to more active forms, such as 4-hydroxytamoxifen and endoxifen. These compounds are much more potent at modulating ER driven genes than tamoxifen because they have a higher affinity for ER. Therefore, the effectiveness of tamoxifen as a therapeutic agent can be strongly influenced by polymorphisms of genes that encode for tamoxifen metabolizing enzymes. Several cytochromes P450 (CYP) are responsible for tamoxifen metabolism including CYP2D6, CYP3A4, CYP3A5, CYP2B6 and CYP2C19 [441]. Individuals who carry alleles for these enzymes that exhibit reduced enzymatic activity, “poor metabolizers”, can less efficiently generate more active compound and thus, experience less therapeutic benefits of the drug [442]. CYP2D6 is primarily responsible for metabolizing tamoxifen to endoxifen. Poor metabolizers of CYP2D6 gene have lower concentrations of endoxifen, and a worse clinical outcome [236; 237; 443]. Enzymes that are responsible for the elimination of tamoxifen, such as sulfotransferase 1A1 and UDP-glucuronosyltransferases 2B7 and 1A8 may also play a role [444; 445; 446]. Similarly, genetic polymorphisms that encode for drug transporters may also influence clinical efficacy. P-glycoprotein (ABCB1) is involved in the transport of tamoxifen metabolites and differences in ABCB1 levels are thought to influence serum concentrations of these metabolites [447; 448; 449].

Combining tumor genome profiles with CSRAs could enhance their predictive power. Tumors can express predictive therapeutic resistance markers within their gene expression profile [371; 450]. For instance, breast cancer cells that express high levels of the ER co-activator, amplified in breast cancer 1 (AIB1) and nuclear receptor HER2, are resistant to tamoxifen inhibition and instead stimulated by tamoxifen. For individuals with this tumor profile, tamoxifen is not recommended as therapy because they are very prone to resistance [451]. Therefore, incorporating the genomic and pathological data could help stratify patient samples and refine the design and analysis of the CSRA.

Given that the patient is a good candidate for a therapeutic, it is helpful to outline the key portions of the AOP targeted by the therapy to determine the best readouts for predicting therapeutic response (TOP depicted in **Fig. 2.4**, where targeted key events are highlighted in pink). In the case of tamoxifen and its metabolites, they compete with estrogen for ER binding and modulate ER induced genomic effects through the classical pathway. Consequently, tamoxifen influences ER driven gene transcription and protein production [442; 452]. Through modulating the expression of ER-targeted genes, tamoxifen decreases proliferation and increases apoptosis [406; 453]. There are conflicting reports on the ability of tamoxifen to modulate cell motility and invasion both *in vitro* and *in vivo*, with some reports claiming a protective effect while others claim an invasive effect [454; 455; 456]. However, clinical trials have shown that tamoxifen decreases the risk of invasive breast cancer and reduces breast cancer related deaths due to its ability to reduce metastasis [457; 458]. Tamoxifen decreases endothelial cell proliferation and migration, thereby decreasing angiogenesis [459]. While it is clear that tamoxifen has anti-angiogenic effects, there is speculation whether this is independent of ER [460].

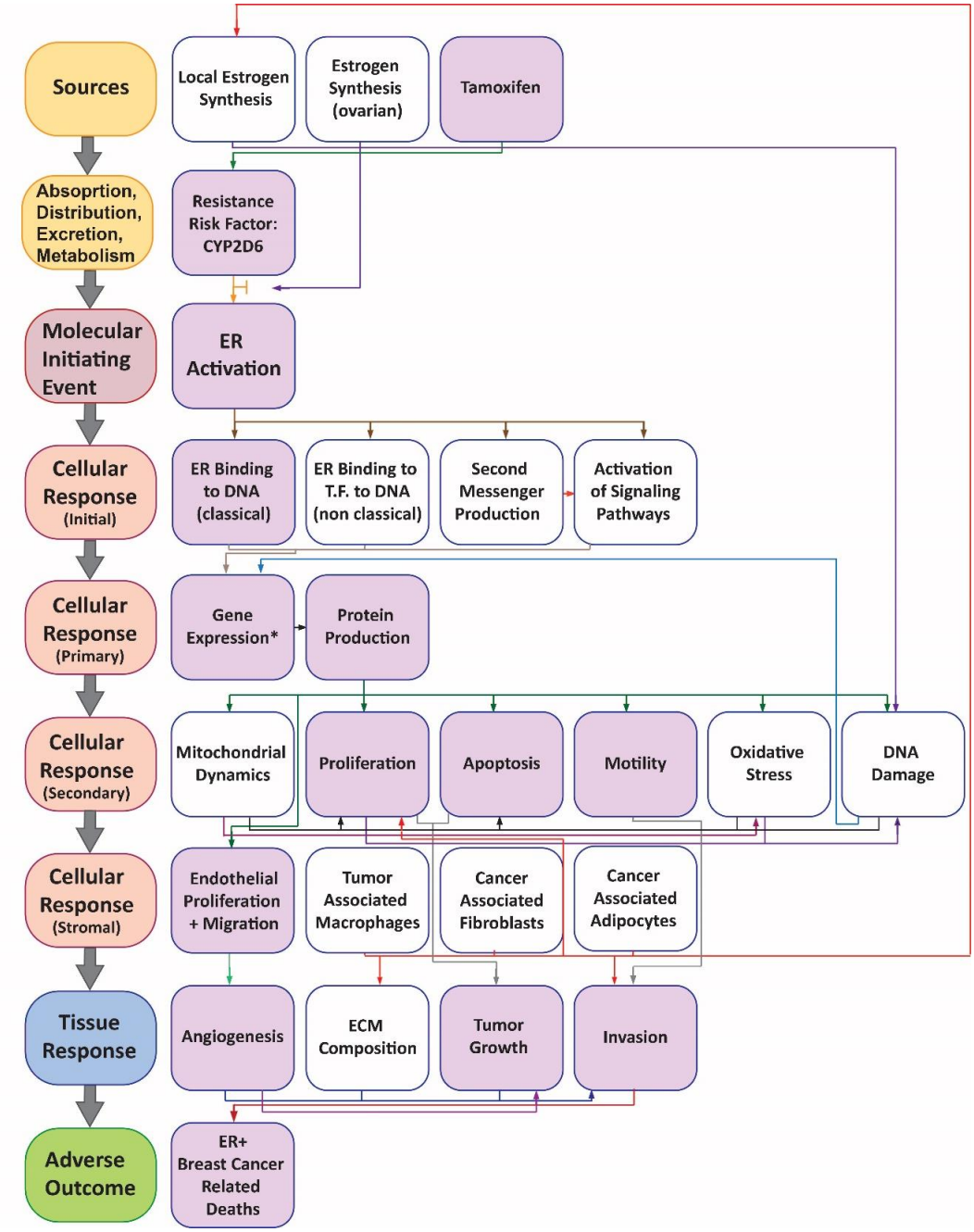



**Figure 2.4. The TOP of tamoxifen and ER mediated breast cancer progression.** We have identified the key events of the AOP that tamoxifen targets in pink. The TOP emphasizes what events are important when evaluating tamoxifen effectiveness. This map can help determine what readouts should be incorporated into CSRAs. *Evaluation of gene expression should include known markers of tamoxifen resistance, in addition to tamoxifen-targeted genes.

TOPs can help us identify what readouts on a molecular, cellular and tissue level will be most useful for the CSRA. While it is not possible to measure all key events that tamoxifen targets, the TOP at least highlights which may be considered. In this case, evaluating the ability of tamoxifen to influence gene and protein expression, invasion and motility through ECM and in the context of multiple cell types, and determination of tamoxifen metabolism, provides a more comprehensive analysis that adds information to proliferation and apoptosis that could better inform patient response to tamoxifen and provide insight into complementary strategies.

## 2.5 Concluding Remarks

The application of AOP/TOPs towards personalized medicine has great potential because it helps organize information about a given therapeutics mechanism of action within its cellular context. While the AOP has been criticized for simplifying a complex process (e.g. breast cancer development) [383], providing a clear representation of how a therapy impacts outcome could reveal key events or properties of the system that improve response prediction and hence, aide in the decision-making process. The example shown above helps illustrate how the AOP/TOP framework can be used to choose components and identify readouts when building CSRAs using a well-characterized anti-cancer agent. The general AOP framework highlights what role cell types and other microenvironmental components play in progression. Understanding these components can help determine what needs to be incorporated into *in vitro* models to reflect *in vivo* response. The TOP framework can help identify what readouts are most important when predicting drug effectiveness. We believe that in addition to aiding in the development of CSRAs, the TOP can also be used to improve the predictive potential of other types of CSRA platforms, such as whole mount tissue slices or tissue scaffolds. We are currently applying these concepts to observe therapeutic responses to neoadjuvant chemohormonal therapy in a clinical trial for advanced prostate cancer patients. We will utilize the TOP-based approach to identify readouts and the microenvironmental compartments that are most important to examine when evaluating therapeutic

response in PSMA-PET guided responding and resistant tumor foci *ex vivo*. While CSRAs are not widely used, we believe that the TOP/AOP can help overcome some of the problems hindering their success.

**2.6 Acknowledgements.** This review was supported by EPA grant number 83573701, University of Wisconsin Carbone Cancer Center, Grant P30 CA014520, NIH T32 CA157322, NIH R01CA186134 and Prostate Cancer Foundation Challenge Award.

## Chapter 3. Mammary fibroblasts reduce apoptosis and speed estrogen-induced hyperplasia in an organotypic MCF7-derived duct model

The estrogen receptor (ER) regulates the survival and growth of breast cancer cells, but it is less clear how components of the tissue microenvironment affect ER-mediated responses. We set out to test how human mammary fibroblasts (HMFs) modulate ER signaling and downstream cellular responses. We exposed an organotypic mammary model consisting of a collagen-embedded duct structure lined with MCF7 cells to 17-β estradiol (E2), with and without HMFs in the surrounding matrix. MCF7 cells grown as ductal structures were polarized and proliferated at rates comparable to *in vivo* breast tissue. In both culture platforms, exposure to E2 increased ER transactivation, increased proliferation, decreased apoptosis, and induced ductal hyperplasia. When the surrounding matrix contained HMFs, the onset and severity of E2-induced ductal hyperplasia was increased due to decreased apoptosis. The reduced apoptosis may be due to fibroblasts modulating ER signaling in MCF7 cells, as suggested by the increased ER transactivation and reduced ER protein in MCF7 cells grown in co-culture. These findings demonstrate the utility of organotypic platforms when studying stromal:epithelial interactions, and add to existing literature that implicate the mammary microenvironment in ER+ breast cancer progression.

This chapter has been adapted from the manuscript published in *Scientific Reports* in 2018: "Mammary fibroblasts reduce apoptosis and speed estrogen-induced hyperplasia in an organotypic MCF7-derived duct model." The manuscript was authored by Molly M. Morgan, Megan K. Livingston, Jay W. Warrick, Eli M. Stanek, Elaine T. Alarid, David J. Beebe, Brian P. Johnson.

## 3.1 Introduction

Breast cancer is the most common non-cutaneous cancer in the United States, responsible for approximately 15% of all cancer cases and 7% of all cancer related deaths[7]. Estrogen receptor (ER) signaling regulates the survival and growth of mammary epithelial cells[439] and is altered in premalignant breast lesions such as atypical ductal hyperplasia (ADH) and ductal carcinoma in situ (DCIS), as well as in invasive ductal cell carcinoma (IDC)[461; 462]. Consequently, studying ER signaling is a major focus of breast cancer research; ER is a common target for breast cancer therapies[439], and exposure to ER agonists has been found to increase breast cancer risk[463].

Classically, *in vivo* models have been used to characterize the effects of ER-targeted cancer therapeutics and toxicants, but are expensive, time consuming, ethically challenging and often lack human relevance[12]. In addition, the complexity of *in vivo* models poses challenges when trying to decipher the molecular and cellular effects of chemicals, as specific interactions are difficult to isolate. Breast cancer models constructed *in vitro* are appealing tools for chemical testing because they are dramatically less resource intensive, more biologically tractable, and could be more relevant to the human condition than *in vivo* animal models. Unfortunately*, in vitro* models often fail to predict *in vivo* response[464; 465], a problem that some have attributed to the oversimplified culture conditions of *in vitro* models[466; 467]. Overall, our understanding of how therapeutics and toxicants affect ER+ breast cancer is hindered by the lack of available models that are both predictive and resource efficient.

Researchers have speculated that a more physiologically relevant *in vitro* culture environment would push cells to behave more similarly to cells grown *in vivo*, which would increase the predictability of *in vitro* models[467]. The traditional cell culture model evaluates cells grown on a flat plastic surface and lacks components of the mammary microenvironment such as extracellular matrix proteins, tissue structure, or cellular communication; the absence of these components may contribute to the low predictability of *in vitro* models as they could influence ER-driven responses. Mounting studies have

found that tissue structure[468; 469; 470], extracellular matrix proteins (ECM)[72; 98], and supportive cell types[30; 379] regulate the behavior of cancer cells, such as invasion, cytokine secretion, and gene expression. There is also evidence that the breast microenvironment regulates ER signaling and function of breast epithelial cells[335; 469]. Of particular importance, there is some evidence that mammary fibroblasts regulate ER dynamics in breast cancer cells. For example, mouse mammary fibroblasts have been found to regulate how mouse epithelial cells respond to hormones[123]. While informative, these studies must be cautiously evaluated due to species differences; murine mammary fibroblasts express ERα, where human mammary fibroblasts do not[471; 472]. *In vitro* studies using human breast cancer cells have also reported that fibroblasts regulate ER dynamics, where fibroblasts were found to regulate ERα expression and tamoxifen resistance[122; 126]. While these studies made important advances, they were confined to two-dimensional (2D) platforms that did not incorporate tissue structure or ECM proteins. Accordingly, pathological endpoints such as ductal hyperplasia, which is a key step in premalignant breast cancer progression, could not be evaluated in these models. In summary, there is a need for physiologically relevant *in vitro* breast cancer platforms that can be used to investigate how components of the mammary microenvironment (such as fibroblasts) affect ER signaling in early stage breast cancer.

Here, we utilized an organotypic mammary model that incorporates a MCF7 cell-derived duct surrounded by a collagen matrix to test the hypothesis that incorporating fibroblasts into the matrix surrounding the duct would lead to differences in estrogen induced cellular responses. We found that fibroblasts modulated epithelial ER protein levels, resulting in increased ER transactivation. Additionally, the incorporation of fibroblasts increased the severity and sped the onset of estrogen-induced hyperplasia. We determined the augmented hyperplasia was due to reduced apoptosis in the co-culture model. Overall, this work presents a structurally relevant platform to evaluate the impact of the mammary microenvironment on ER signaling and suggests that fibroblasts can regulate ER protein as well as apoptosis in breast cancer cells.

## 3.2 Materials and Methods

**3.2.1 Cell culture.** We used a variant of the immortalized ER+ human mammary epithelial cell line MCF7, MVLN (referred to as MCF7s) that has previously been stably transfected with an ERE-luciferase construct[394]. Immortalized human mammary fibroblasts (referred to as HMFs) derived from a reduction mammoplasty[9] were gifted to us from Dr. Lisa Arndt's lab (University of Wisconsin, Madison). MCF7s and HMFs were grown in high glucose DMEM (4.5 mg/ml, Gibco, Gaithersburg, MD, USA; #11965092) supplemented with 10% fetal bovine serum (FBS, VWR #97068-085) and 1% penicillin/streptomycin (Thermo Fisher, Waltham, MA, USA; #15140-122) and maintained in a 5% $CO_2$ 37°C incubator.

**3.2.2 Device fabrication.** Fabrication of the LumeNEXT microfluidic devices has been described previously[1]. Briefly, standard photolithography methods were employed to create the SU-8 masters that were used as molds for the polydimethylsiloxane (PDMS) (Dow Corning, Auburn, MI, USA; Sylgard 184 Silicone Elastomer Kit #3097358-1004) device. After the two layers of the devices were bonded, 340-µm diameter PDMS rods were placed into the device chamber. The device was oxygen-plasma-treated and bonded to a 48x65 mm Gold Seal cover slip (Thermo Fisher, 48X65-1-002) that was taped to an omnitray (Thermo Fisher #242811). Once the device was bonded, the middle chamber of the device was closed to the air and facing the glass, while the device ports were open (fabrication shown in **Fig. 3.1A**). A single 86 x 128 mm omnitray can hold up to 36 lumen devices (example of a 24 lumen array shown in **Fig. 3.1D**). Devices were UV sterilized for 15-20 minutes then transferred to a biosafety hood.

**3.2.3 Organotypic culture preparation.** To minimize evaporation, sterile Kimwipes soaked in PBS were placed along the corners of the Omnitray that held the devices. To aide collagen attachment to the device, a 2% poly(ethyleneimine) (PEI) (Sigma-Aldirch, St. Louis, MO, USA; #03880) solution diluted in deionized (DI) water was loaded into the side ports and incubated at room temperature for 10 minutes. The PEI solution was removed from the channel and a 0.4% glutaraldehyde (GA) (Sigma #G6257) solution diluted in DI water was loaded into the side ports and incubated at room temperature for 30

minutes. While the GA incubated, a collagen solution was prepared on ice. High-density rat-tail collagen type 1 (Corning, Corning, New York, USA; #354249, will refer to as collagen for the remainder of the paper) was diluted with 10X PBS and neutralized with 0.5 M NaOH to a final concentration of 6 mg/ml collagen, a final concentration of 1X PBS, and a pH of 7.2. After the GA incubation, the devices were washed three times with DI water. Directly prior to collagen loading, the 6 mg/ml collagen solution was diluted 3 to 1 in media (for the monoculture) or in a 5,000 cell/µl fibroblast cell solution (for the co-culture). The final 4.5 mg/ml collagen solution was loaded into the side ports and incubated at room temperature until the collagen turned white, then transferred to the incubator for one hour. A small drop of media was placed into the input port and the PDMS rod was pulled out through the output port, leaving a hollow lumen structure that connected the input and output port. 1.5 µl of a MCF7 solution (~50,000 cells/µl) was pipetted into the input port and into the lumen. Devices were placed in the incubator and flipped from bottom to top every 20 minutes for 100 minutes. The output port was aspirated and filled with media then devices were cultured upside down overnight. The next morning, media was flowed through the input port then aspirated out of the output port to clear the lumen of dead or unattached cells. The media was changed every day. Device design and culture conditions are illustrated in **Figure 3.1.**

**3.2.4 Hormone treatments.** Prior to all estrogen experiments, cells were grown in estrogen-deprived media containing phenol red-free DMEM (Thermo Fisher #31053-028) supplemented with 10% charcoal dextran stripped FBS, 1% penicillin/streptomycin and 1% L-Glutamine for 48 hours. Where hormone treatment is indicated, a vehicle (0.1% ethanol) control or hormone treatment (estradiol (Sigma-Aldrich, #E2758) or diethylstilbestrol (Sigma-Aldrich #D4628)) at the indicated concentrations were added directly to the input port of the lumens and replenished daily.

**3.2.5 Immunofluorescence staining.** Cells were fixed with 4% paraformaldehyde (PFA) (Alfa Aesar, Tewksbury, MA, USA; #43368) then washed with PBS three times. To visualize filamentous actin (F-

actin), cells were stained with Texas red phalloidin (1:200, Thermo Fisher #T7471). For all staining experiments, cells were stained with Hoechst (1:500, Thermo Fisher #H3570) to visualize nuclei. For intracellular stains, cells were permeabilized with 0.2% Triton for 20 minutes then blocked with 3% BSA overnight. To evaluate ERα expression in 2D, cells were stained for ERα (1:200, anti-mouse, Thermo Fisher #MA5-13304) overnight, washed with 0.1% TWEEN three times, and incubated with Alexa flour 488 (1:200, anti-mouse; Abcam, Cambridge, MA, USA) for 2 hours. Cultures were washed 3 times with 0.1% TWEEN 20 diluted in PBS then imaged. The following antibody stains were performed in our organotypic cultures and have different dilutions/staining times than cells plated in 2D. To evaluate apical-basal polarity, cells were stained for basal marker laminin-5 (1:50, anti-mouse, Abcam #ab78286) and apical marker GM130 (1:50, anti-rabbit, Abcam #ab52649). To evaluate proliferation, cells were stained for proliferation marker Ki67 (1:50, anti-rabbit, Thermo Fisher #RM9106-S). To evaluate ER expression, we used the same ER antibody as in our 2D ER staining protocol. After cells were incubated with primary antibodies for 48 hours, cells were washed 5 times over a 24-hour period with 0.1% TWEEN 20 diluted in PBS. Next, cells were incubated with Alexa Flour 488 (1:50, anti-mouse, Abcam #ab150113) and/or Alexa Flour 647 (1:50, anti-rabbit, Thermo Fisher #a-21244) for 2 days. The samples were then washed 5 times over a 24-hour period then imaged.

**3.2.6 RT-qPCR.** Prior to mRNA isolation, MCF7s and HMFs were grown on a 96 well plate and exposed to a vehicle control or 10 nM E2 for 24 hours. mRNA isolation was performed with Dynabeads mRNA direct purification kit (Thermo Fisher #61011) and reverse transcription was done with the high capacity RNA to cDNA kit (Thermo Fisher #4387406). RT-qPCR was conducted using Taqman Probes (Thermo Fisher). The ΔΔCT method was used to evaluate relative gene expression, where expression of ER-driven genes ESR1 (Hs00174860_m1), TFF1 (Hs00907239_m1), PGR (hs01556702_m1), and JAG1 (Hs01070032_m1) were normalized to housekeeping genes GAPDH (Hs99999905_m1) and HPRT (11501003267_m1). A student's t test was used to evaluate significant differences between vehicle and

E2 treated cultures (defined as p>0.05). Graphs displayed are representative of data normalized to the GAPDH housekeeping gene, although the same findings were observed when the data was normalized to HPRT.

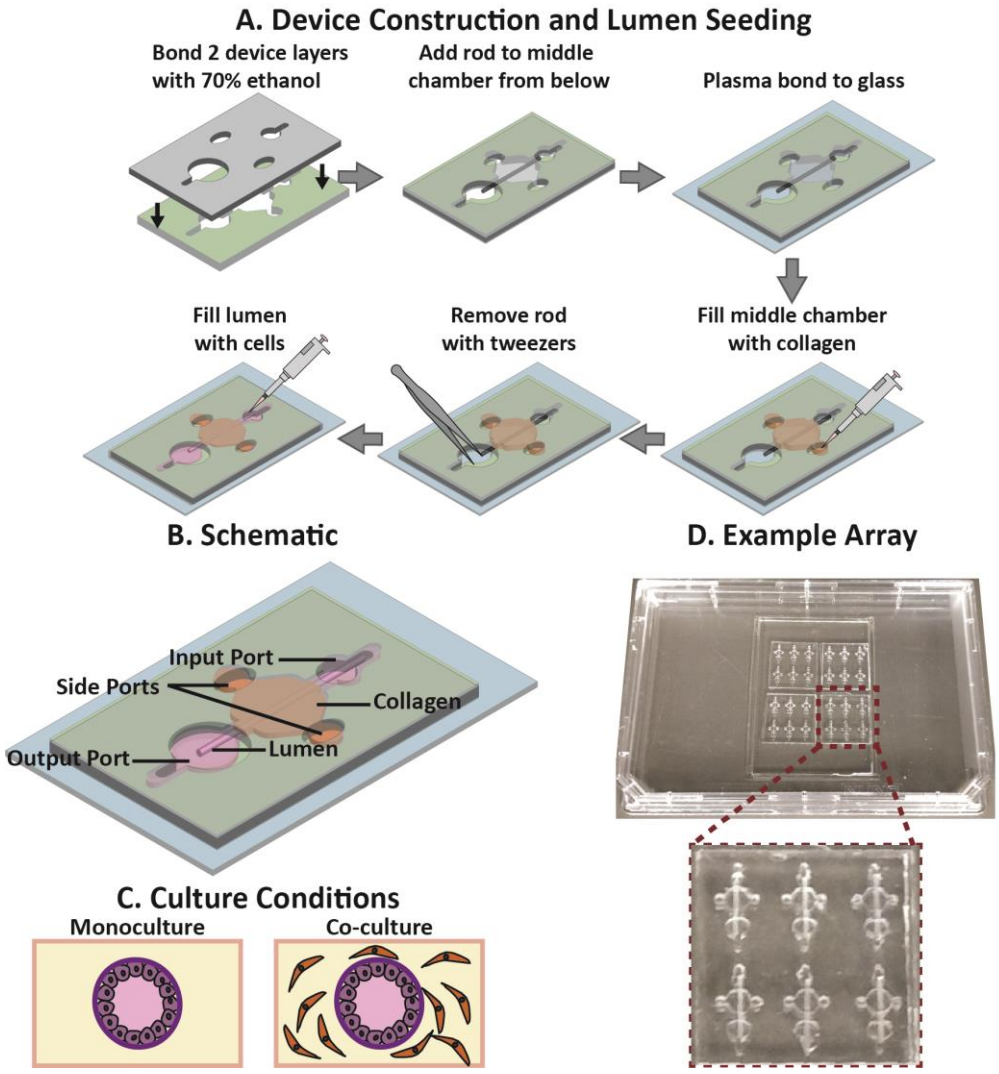



**Fig. 3.1. Device illustration and culture conditions. (A)** The LumeNEXT method[1] was used to create the two-layered microfluidic devices. **(A)** depicts device construction and use, and **(B)** shows a detailed device schematic. **(C)** The platform allows the investigation of stromal cells on duct function and estrogenic response, as the duct can be cultured in a collagen matrix, as well as cultured in a collagen matrix that has stromal cells embedded throughout. An example of a device array is shown in **(D)**, where 24 devices are plasma bonded to a cover slip attached to an omni tray.

**3.2.7 Live/dead assay.** MCF7-derived ducts were washed with serum-free phenol red-free DMEM then replaced with a solution containing calcein AM (2:500, Thermo Fisher #c3100mp), ethidium

homodimer-1 (2:500, Thermo Fisher #e1169), and Hoescht (1:500) diluted in serum-free phenol red-free DMEM for 30 minutes at 37°C.

**3.2.8 Apoptosis assay.** CellEvent Caspase 3/7 Green Detection Reagent (1:1000, Thermo Fisher #C10423) and Hoescht (1:500) were diluted in estrogen-free media and incubated with the cells at 37°C for 30 minutes. MCF7s do not express caspase-3[473] so apoptosis identified by this assay is indicative of caspase-7 activity only.

**3.2.9 Evaluation of cell loss.** Starting 24 hours after the first dosing period, media was collected from lumens daily prior to cell feeding and continued until the end of the 72-hour dose period. Media was pipetted from the large port then transferred to a 96 well plate containing the live/dead staining solution described above. Number and viability of collected cells was quantified at each day of collection. For each technical replicate, the number of collected cells was summed to estimate the total cell number that left the lumen over the course of the 3 days. Likewise, for each technical replicate, the viability of collected cells was averaged to estimate the viability of cells that left the lumen over the course of the 3 days.

**3.2.10 Lumen cross sectioning and quantification.** After the lumens were fixed and stained, the lumen was aspirated and filled with 3% low melting point agarose (IBI Scientific, Peosta, Iowa, USA; #IB70056) diluted in PBS to prevent the lumen from collapsing during cross-sectioning. Once the agarose solidified, tweezers were used to remove the top layer of the PDMS device then the sample was completely embedded in agarose. The sample was glued to a mounting block, with the lumen faced orthogonal to the mounting block, then sliced with a VT-300 Compresstome (Precisionary Instruments, Greenville, North Carolina, USA). The resulting 100-µm thick cross-sections were immediately imaged. For each cross-section, hyperplasia was quantified by counting the number of nuclei lining the duct (referred to as epithelial thickness) at eight evenly spaced points then averaging the epithelial thickness.

**3.2.11 Image acquisition**. All images were taken with fluorescent microscope Nikon Eclipse Ti. Unless otherwise indicated, images were taken of the bottom plane of the lumen.

**3.2.12 Fluorescent image quantification.** The automated image-processing program, JeXperiment[474] was used for image quantification. First, we conducted a rolling ball background subtraction. Region of interests (ROIs) were drawn over the lumens in one Z-plane to exclude fibroblasts and minimize noise. The ROI dimension and background subtraction was kept constant throughout a dataset.

To count total nuclei, CASP7+ nuclei, as well as Ki67+ nuclei, we counted all maxima within the ROI defined by a set threshold. To evaluate the percentage of Ki67+ cells and the percentage of CASP7+ cells, the number of Ki67+ or CASP7+ nuclei was divided by the total number of nuclei for each sample. The percentage of viable cells was determined by dividing the number of viable cells by the total number of nuclei.

ER protein was quantified as described previously[122]. Briefly, single cell analysis was performed on samples stained for nuclei and ERα protein, and was used to quantify ERα protein levels per individual cell. First, masks outlining the nuclear stain Hoescht were generated. The nuclear masks were overlayed on top of the ERα images and the ER staining intensity within each nuclear mask was quantified. We presented the data in two ways. For each culture condition, we graphed the means of ER expression to gain an understanding of how the intensity of ERα expression changes with the addition of estrogen as well as fibroblasts. Second, we created a density plot in the statistical program R to describe the distribution of ERα protein expression within each population.

**3.2.13 Luciferase transactivation assay.** Prior to conducting the luciferase assay, samples were incubated at room temperature for 10 minutes. 6 ul of 1 mM beetle luciferin (Promega,

Fitchburg, Wisconsin, USA; #E1601) solution diluted in estrogen-deprived media was added into the input port and luminescence was quantified with a Biorad Chemidoc imager. Luminescence is indicative of ER transactivation, as characterized previously and described above[394].

**3.2.14 Statistics.** Graphpad Prism was used for statistical analysis. Any lumens that were not viable the day following cell seeding were excluded from analysis. A two-way ANOVA followed by a multiple comparisons test was used to determine significance (as defined by $p<0.05$) for experiments evaluating how estrogen exposure in addition to stromal cells influenced an endpoint. We used nonlinear regression (equation: Y=Bottom + (Top-Bottom)/(1+10^((LogEC50-X)*HillSlope))) to determine EC50s for dose response curves. Data shown is representative of multiple experiments, as indicated in the graphs. Error bars represent standard deviation in all graphs except for the dose response graphs, where standard error was used.

## 3.3 Results

### 3.3.1 MCF7s grown in a mammary duct structure form confluent polarized epithelial layers

Due to previous studies that report the importance of tissue structure on cell behavior[475; 476], the LumeNEXT method (device construction, use and schematic shown in **Fig. 3.1A** and **B** and described in methods) was used to create a lumen structure within a collagen matrix for the base model. To model an early stage breast cancer, the lumen was lined with cancerous mammary epithelial cells and was surrounded by a collagen matrix with or without embedded normal human mammary fibroblasts (HMFs) (shown in **Fig. 3.1C**, device example shown in **Fig. 3.1D**). Primary mammary epithelial cells rapidly lose ER when grown *in vitro* and consequently are challenging to use for ER-related studies[186]. Therefore, we lined the ductal structures with MCF7s, an immortalized human mammary epithelial cancerous cell line, due to their high expression of ER, their ability to recapitulate hormonal responses

similar to ER+ breast tumors *in vivo*, and the wealth of literature on MCF7s that can be used to interpret our results[477].

We first characterized the model in the absence of embedded HMFs. To characterize the morphology of the biomimetic duct, MCF7s were grown as ductal structures for 48 hours in normal growth media then were fixed and stained for nuclei and F-Actin. Fluorescent microscopy was used to image the bottom plane of the lumen, which revealed that MCF7s formed confluent epithelial layers within the lumen **(Fig. 3.2A)**. To investigate cellular pathology, the ducts were embedded in agarose and cross-sectioned which revealed that the cells grow around the periphery of the lumen **(Fig. 3.2B)**. To evaluate apical-basal polarity, samples were stained for basal marker laminin-5 and apical marker GM130. Laminin-5 was localized to the basal side of the lumen, where GM130 faced the inside of the lumen **(Fig. 3.2C)**.

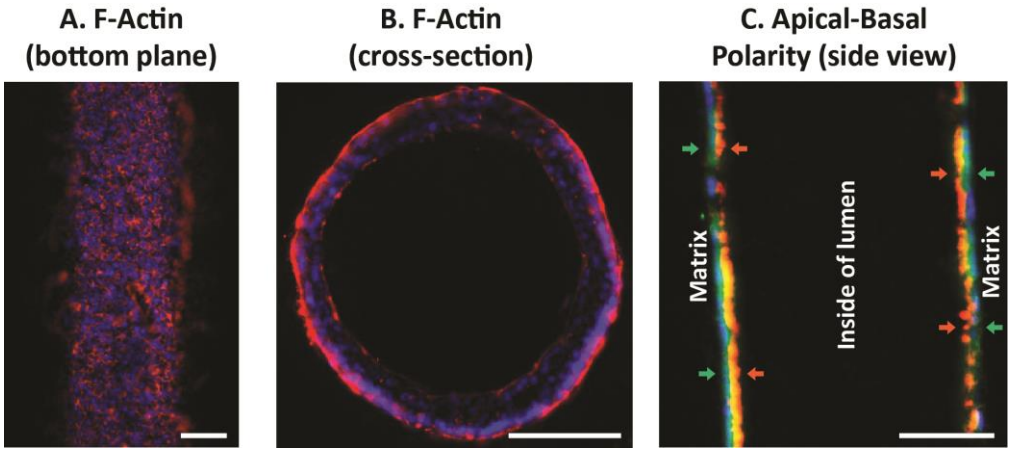



**Fig. 3.2. MCF7s form confluent polarized ductal structures.** The **(A)** bottom plane and **(B)** cross-section of MCF7-derived lumens stained with F-Actin (red) and nuclei (blue) were imaged. **(C)** To evaluate apical-basal polarity, samples were stained for nuclei (blue), laminin-5 (green; basal), and golgi (red; apical). Scale bars represent 100 µm.

Before integration of fibroblasts into the matrix surrounding the mammary duct, the ER status of the HMFs was evaluated. qRT-PCR was performed to evaluate the expression of ERα- and ERβ- driven genes after exposure to 10 nM E2 for 24 hours **(Fig. 3.3A)**. E2 exposure did not induce expression of ER-

driven genes in the HMFs. *ESR2* mRNA was also evaluated after treatment with a vehicle control or 10 nM E2, but *ESR2* mRNA was not detected in HMFs of either condition [data not shown]. Immunocytochemistry was used to stain for ERα. In contrast to MCF7s, HMFs did not stain positively for ERα **(Fig. 3.3B)**. We were unable to evaluate ERβ protein because we do not know of a reliable ERβ antibody, which is a known challenge in the field [478].

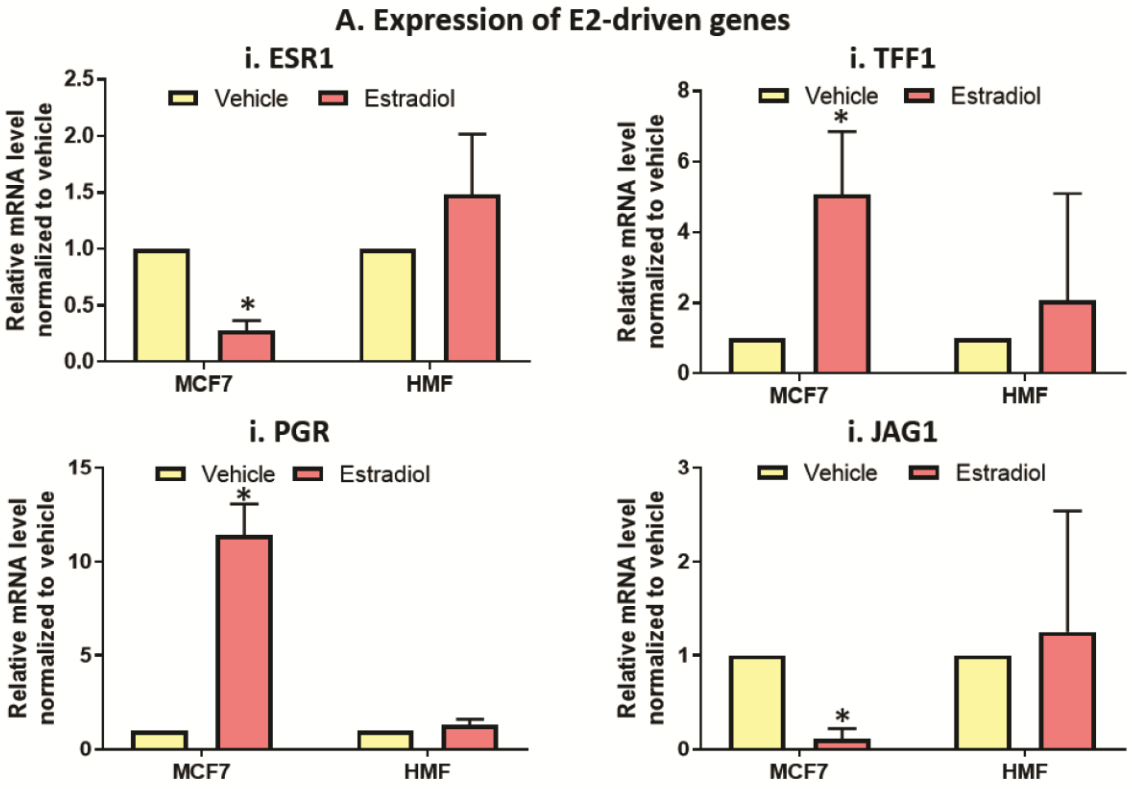



**Fig. 3.3. ERα- and ERβ- driven genes are not induced in HMFs after E2 exposure, and HMFs do not stain positively for for ERα protein. (A)** After MCF7 cells and HMF cells were exposed to E2 for 24 hours, qRT-PCR was used to evaluate expression of ERα-driven genes *ESR1*, *TFF1*, and *PGR* and ERβ-driven gene *JAG1*. Each sample was normalized to the expression of the housekeeping gene *GAPDH*. A student's t test was used to evaluate significance. * = vs. respective vehicle ($p < 0.05$).

### 3.3.2 Fibroblasts reduce ER protein and increase ER transactivation in MCF7s

As previous studies using 2D culture platforms found changes in ER protein when breast cancer cells were cultured near stromal cells[122; 126], we first evaluated the impact of HMFs on ER protein of the MCF7-derived ducts. Immunocytochemistry was used to evaluate ERα protein after the MCF7-derived

duct model was cultured alone or when fibroblasts were cultured in the surrounding matrix, after exposure to a vehicle control or to a saturating concentration (100 nM) of 17β-estradiol (E2) for 24 hours **(Fig. 3.4A)**. Quantification of ER expression in the bulk cell population **(Fig. 3.4B)** as well as in individual MCF7s **(Fig. 3.4C)** revealed that vehicle-treated MCF7-derived ducts had decreased ER protein when grown in a mixed fibroblast:collagen matrix, compared to when grown in a collagen only matrix. In both culture conditions, E2 significantly reduced ER protein, which is consistent with previous studies that found ER downregulates its own expression upon activation[479]. Additionally, the estrogen-treated monocultures showed similar levels of ER protein as the estrogen-treated co-cultures. We suspect that the estrogen-treated cultures displayed similar ER protein expression because ER protein is at maximal downregulation in both systems, as the cultures were exposed to a saturating concentration of E2.

To determine if the differences in ER protein correlated with changes in ER function, MCF7-derived ducts were exposed to five doses of E2 when cultured alone and in co-culture then evaluated for ER transactivation after 24 hours. Upon activation, ER dimerizes and binds to regulatory regions of ER-driven genes called estrogen response elements (EREs), initiating gene transcription. The MCF7s used in the model stably express an ERE-luciferase reporter plasmid; therefore, ER transactivation can be measured by luminescent signal, as luminescence is linear to ERE activity[394]. Both the monoculture and co-culture model responded to E2 in a dose-dependent manner, however, maximal response was slightly increased in the presence of fibroblasts **(Fig. 3.5A).** Next, the model was exposed to xenoestrogen diethylstilbestrol (DES) which revealed a similar trend **(Fig. 3.5B)**. To further investigate the finding that maximal response was increased in co-culture, MCF7-derived ducts were grown in monoculture and co-culture in the presence of a vehicle control or 100 nM E2 for 48 hours. Similar to at the 24-hour time point, the luminescent signal was higher in the co-cultures. Vehicle-treated MCF7-derived ducts co-cultured with surrounding fibroblasts had 4-fold higher ERE activity compared to when

cultured alone. There was significantly higher ER transactivation in the E2-treated co-culture compared to the E2-treated monoculture **(Fig. 3.5C)**.

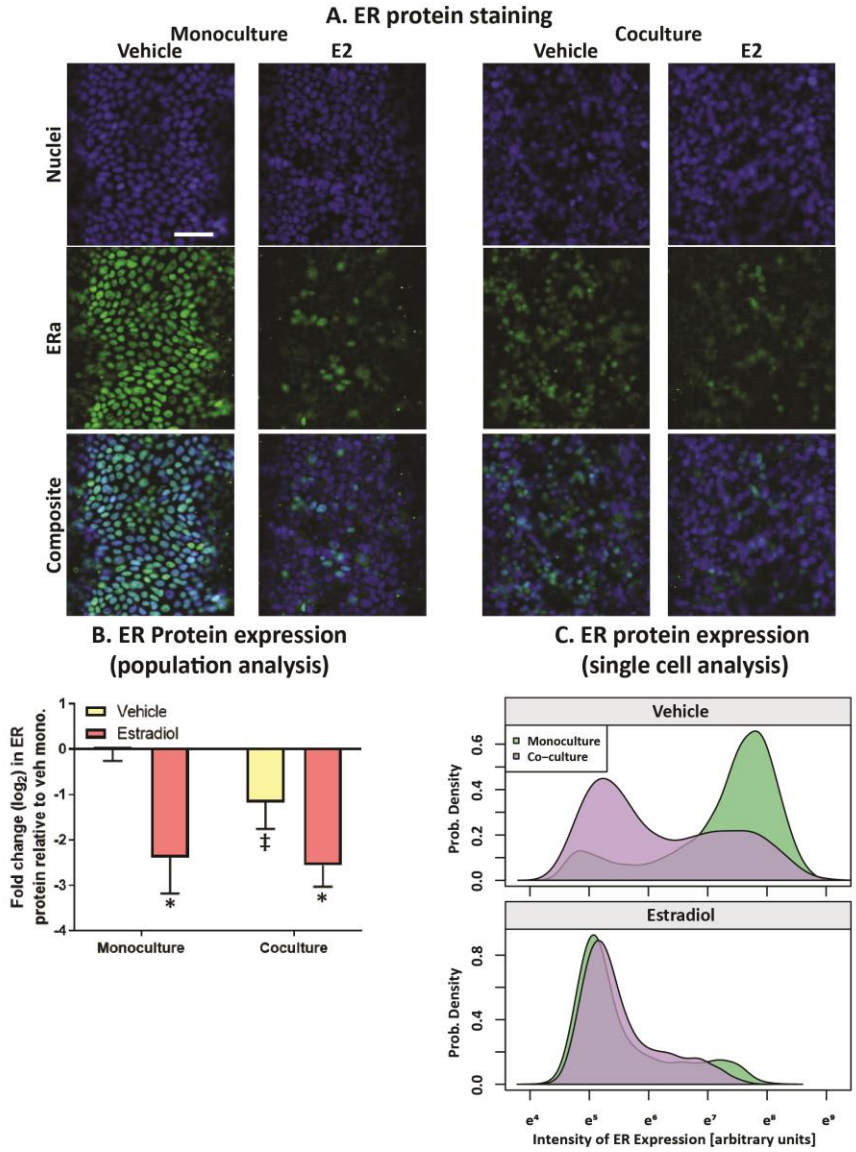



**Fig. 3.4. MCF7-derived ducts grown in a mixed collagen:fibroblast matrix express lower levels of ER protein.** MCF7-derived ducts were cultured alone or in co-culture with HMFs, treated with a vehicle control or 100 nM E2 for 24 hours. **(A)** Cultures were fixed and stained to visualize nuclei and ERα protein. Scale bar represents 100 µm. To quantify ER protein, the Hoescht stain was used to generate nuclear masks then ER expression was quantified within each nuclear mask. **(B)** Mean ER expression per condition was graphed to visualize ER protein changes within the entire population. A two-way ANOVA was run to evaluate interactions between conditions, followed by a Tukey's multiple comparison test to identify significant differences. **(C)** A density plot was created to show the frequency of individual cells (x-axis) that expressed various levels of ER protein (y-axis). * = vs. respective vehicle ($p < 0.05$) and ‡ = vs. respective monoculture ($p < 0.05$). Data is representative of three independent experiments.

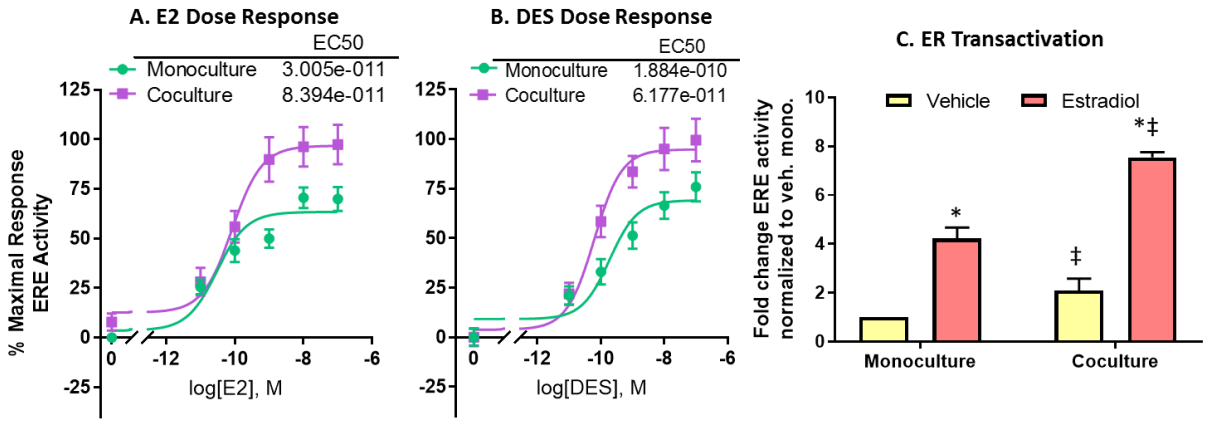



**Fig. 3.5. MCF7-derived ducts respond to ER ligands in a dose-dependent manner, and fibroblasts regulate ER transactivation.** ER transactivation was evaluated after cultures were exposed to 5 doses of **(A)** E2 and **(B)** DES for 24 hours. EC50s were generated using nonlinear regression. **(C)** ER transactivation in monoculture and co-culture with fibroblasts was evaluated after 48 hours of exposure to a vehicle or 100 nM E2. Luminescent signal was normalized to the total number of nuclei to account for differences in cell number. A two-way ANOVA was run to evaluate interactions between conditions, followed by a Tukey's multiple comparisons test to identify significant differences. * = vs. respective vehicle ($p < 0.05$) and ‡ = vs. respective monoculture ($p < 0.05$). Graphs represent data from three independent experiments.

### 3.3.3 MCF7s exhibit increased cell density, reduced apoptosis, and similar levels of E2-induced proliferation in the presence of fibroblasts

The observed changes in ER transactivation and ER protein next led us to look for differences in cell fate when the MCF7-derived ducts were cultured alone or with fibroblasts, after exposure to a vehicle control or 100 nM E2 for 72 hours. E2 treatment as well as co-culture with stromal cells increased the total number of nuclei **(Fig. 3.6A).** To confirm this was not due to differences in adherence during cell seeding, the number of nuclei was quantified the day following seeding which revealed no difference between the monoculture and co-culture (**Fig. 3.6C&D**)**.** This led us to suspect that fibroblasts were decreasing epithelial cell apoptosis or increasing proliferation.

Cell proliferation was characterized by staining for the cell proliferation marker, Ki67. E2 increased the percentage of Ki67+ cells 2-3 fold in both culture conditions. Despite the differences in confluency between the culture conditions, there was no difference in the percentage of Ki67+ cells in the monoculture compared to the co-culture **(Fig. 3.6D).**

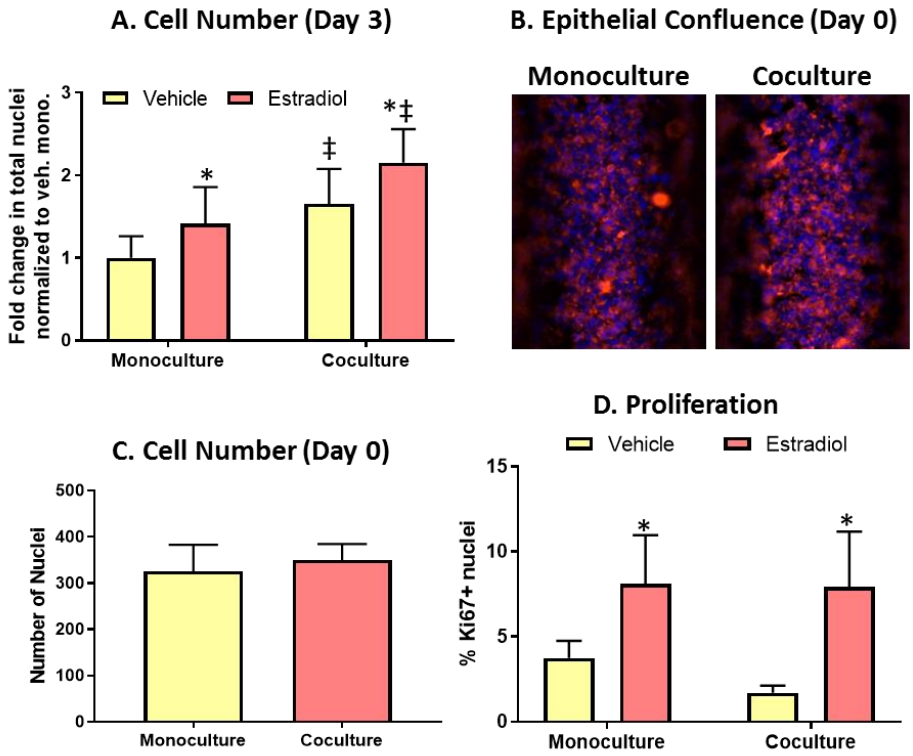



**Fig 3.6 Fibroblasts increase epithelial cell number but have no effect on proliferation. (A)** After cultures were exposed to a vehicle control or 100 nM E2 for 72 hours, the total number of nuclei was measured to assess cell number. The day following seeding, samples were fixed and stained with phalloidin (red) and Hoescht (blue) to visualize F-Actin and nuclei, respectively. **(B)** shows representative images while **(C)** shows quantification of cell number. **(D)** After a 72 hour exposure to a vehicle control or 100 nM E2, proliferation was measured by the percentage of cells positive for proliferation marker Ki67. Graphs A and D represent data from three independent experiments.

To evaluate cytotoxicity, a live/dead stain was performed on the ducts. E2 significantly increased cell viability in the monoculture platform, where 77% of cells were stained as live in the vehicle-treated monocultures compared to 93% in the E2-treated monocultures. Stromal cells also improved cell viability, where similar cell viability was observed in the E2-treated monocultures as the vehicle-treated

co-cultures. There was no difference in viability between the vehicle-treated co-cultures and the E2-treated co-cultures **(Fig. 3.7A).**

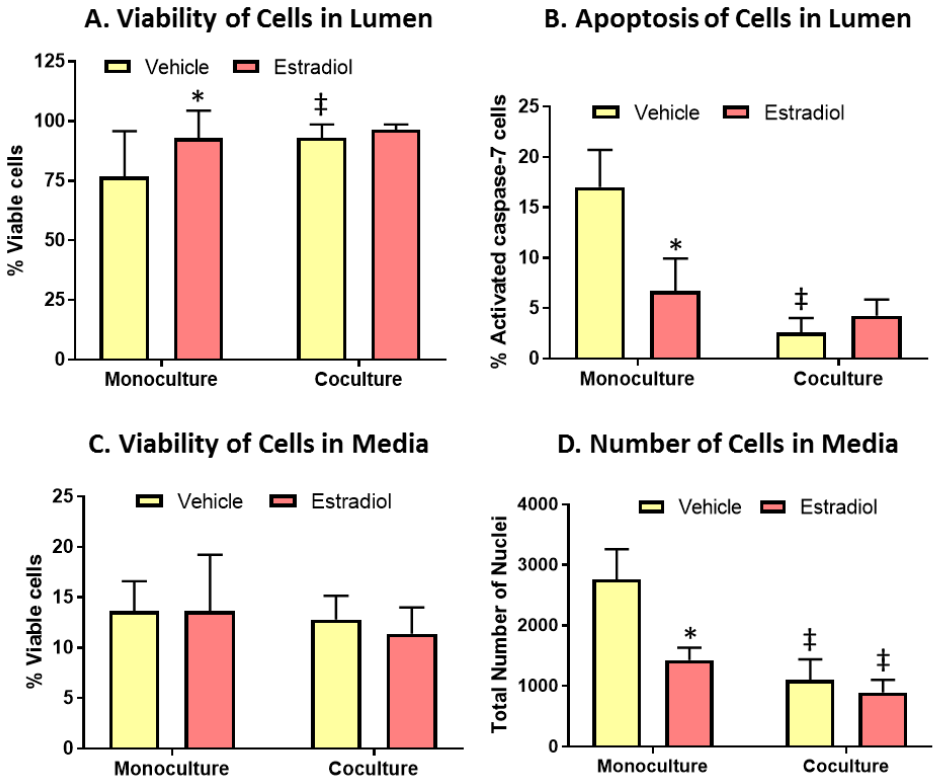



**Fig. 3.7. Fibroblasts increase cell viability and reduce apoptosis**. After cells were exposed to a vehicle control or estradiol for 72 hours, **(A)** cell viability and **(B)** cell apoptosis were assessed. A live/dead assay was used to evaluate cell viability and cell apoptosis was measured by assessing the expression of the apoptosis protein activated caspase-7. Graphs represent data from three independent experiments. Culture media was collected from lumens after 24, 48, and 72 hours of exposure to a vehicle control or estradiol. Cells in the culture were measured for **(C)** cell viability using a live/dead assay, and **(B)** cell number by staining for nuclear marker Hoescht and quantifying the total number of nuclei. The viability and cell number shown is the sum of cells collected during the 24, 48, and 72-hour time points. A two-way ANOVA was run to evaluate interactions between conditions, followed by a Tukey's multiple comparisons test to identify significant differences. * = vs. respective vehicle ($p < 0.05$) and ‡ = vs. respective monoculture ($p < 0.05$).

Next, cell apoptosis was evaluated by staining for the apoptotic marker activated caspase-7 (CASP7). MCF7s have been reported to undergo BCL-2[184] as well as BAX[480] mediated apoptosis in E2 starved conditions, and CASP7 is downstream of BCL-2. E2 exposure decreased the percentage of CASP7+ cells approximately 2-fold in the monoculture platform. Fibroblasts decreased the percentage of CASP7+ cells over 5-fold, where 17% of CASP7+ cells were observed in the vehicle-treated monocultures

compared to 3% in the vehicle-treated co-cultures. There was no significant difference in apoptosis between the vehicle-treated and E2-treated co-cultures, with 3% and 4% CASP7+ cells, respectively. **(Fig. 3.7B)**.

As cells become less adherent when they die, we suspected that a portion of the dead cells fell off the lumen walls and into the culture media. Prior to drug feeding, culture media was collected from the lumen 24, 48, and 72 hours after drug exposure. In all culture conditions, the majority (85-90%) of cells that were in the culture medium were not viable **(Fig. 3.7C**). Hoechst staining and quantification of the cells revealed that significantly more cells left the lumen in the vehicle-treated monocultures, compared to the other culture conditions. In monoculture, E2 reduced the amount of cells that sloughed off the lumen. The presence of fibroblasts in the collagen matrix led to a further reduction of cells washed from the lumen, where the E2-treated co-cultures had significantly less cells leave the lumen compared to the E2-treated monocultures **(Fig. 3.7D)**.

### 3.3.4 Fibroblast co-culture prolongs epithelial confluence and increases E2-induced ductal hyperplasia

As we saw a difference in apoptosis and cell number, we next tested if these cellular-induced changes were great enough to affect tissue morphology. MCF7-derived ducts grown in monoculture were compared to MCF7-derived ducts grown in co-culture, after a 3-day and 10-day exposure to a vehicle control or to 100 nM of E2. At the end of the culture period, ducts were fixed and stained for F-actin and nuclei then the bottom plane of the ducts were imaged. Despite both culture platforms exhibiting a confluent monolayer the day following seeding **(Fig. 3.6C&D),** MCF7-derived ducts grown in monoculture in E2-starved conditions had a visible loss of confluency after 3 days in culture, which worsened by day 10. In contrast, the monolayer was maintained in both the vehicle and E2-treated co-cultures. In both culture conditions that were exposed to estrogen, there were areas with cells piled up on top of one another, suggestive of ductal hyperplasia. (**Fig. 3.8A).**

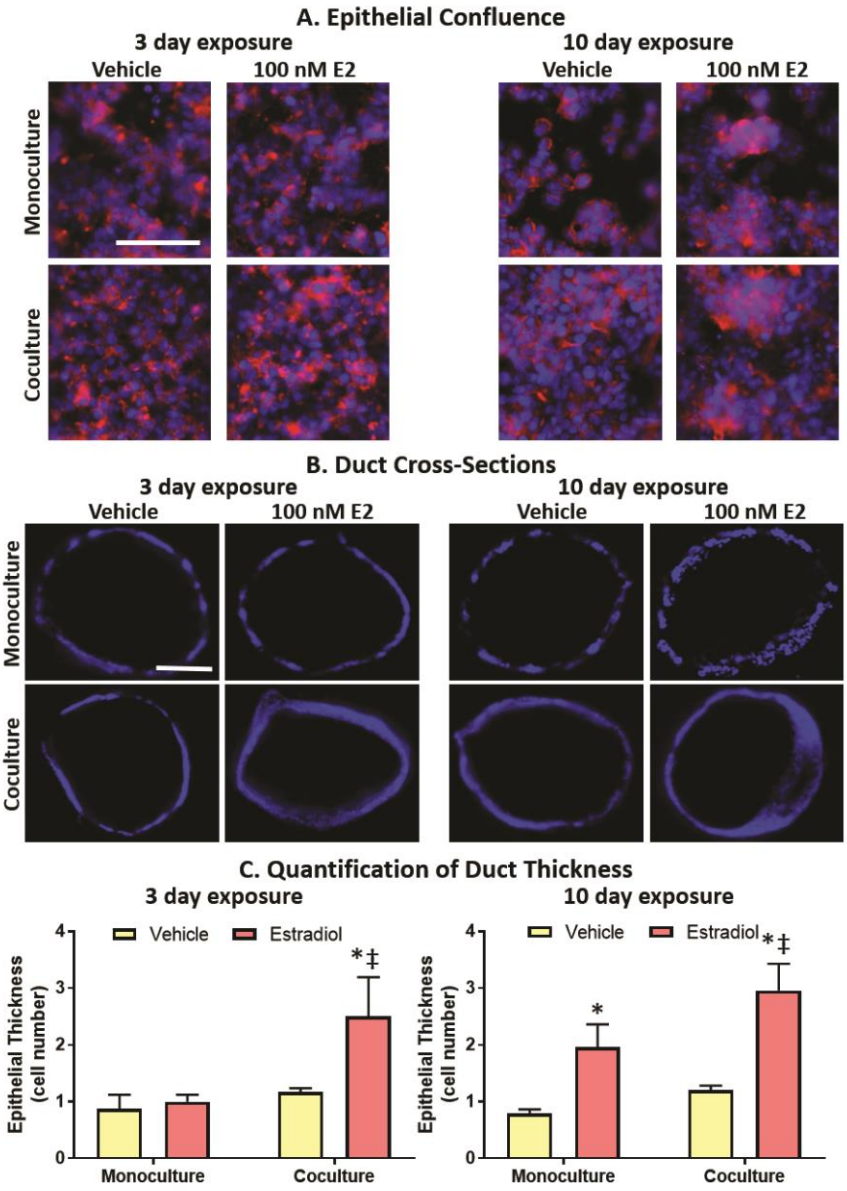



**Fig. 3.8. Fibroblasts prolong epithelial confluence and speed the onset as well as the severity of E2-induced hyperplasia. (A)** Samples were stained for Hoescht (blue) and phalloidin (red) to visualize nuclei and F-actin, respectively. **(B)** To visualize hyperplasia, ducts were cross-sectioned and stained with Hoescht (blue). Scale bars represent 100 µm. **(C)** Quantification of hyperplasia. Each condition represents the cross-sections of three ducts. A two-way ANOVA was run to evaluate interactions between conditions, followed by a Tukey's multiple comparisons test to identify significant differences. * = vs. respective vehicle ($p < 0.05$) and ‡ = vs. respective monoculture ($p < 0.05$). Data is representative of three independent experiments.

To investigate the presence of E2-induced ductal hyperplasia, MCF7-derived ducts were stained with Hoescht, embedded in agarose, cross-sectioned, and imaged. For each lumen, the number of

epithelial layers lining the duct was counted at eight evenly spaced points then averaged **(Fig. 3.8B & C)**. After 3 days of exposure, the ducts of the vehicle-treated monocultures, E2-treated monocultures and vehicle-treated co-cultures were lined with a single layer of cells. In contrast, the E2-treated co-culture ducts averaged approximately 2.5 cell layers. After 10 days of exposure, both the E2-treated monoculture and co-culture ducts had multiple cell layers with around 2 cell layers and 3 cell layers, respectively.

## 3.4 Discussion

Despite the accepted role of ER in breast cancer progression, little is known about how different components of the tissue microenvironment modulate ER signaling or response to ER ligands. With this mind, we developed an organotypic breast model based on the ER-driven breast cancer adverse outcome pathway, which helped frame which readouts (e.g. ER transactivation, apoptosis, proliferation and hyperplasia) and cellular components (e.g. fibroblasts) were most critical when modeling ER+ breast cancer progression, a concept we have published previously [467]. While previous studies have introduced models that contain components of the *in vivo* mammary microenvironment[75; 98; 367; 379; 469; 481; 482], we are not aware of a breast cancer model that incorporates structure, matrix proteins, and stromal cells into a single system, that is also capable of evaluating molecular, cellular and tissue level estrogenic signaling responses. **Table 3.1** compares characteristics of the MCF7-derived duct model introduced in this paper to organotypic mammary models described in previous publications.

| Publication | Estrogen Responsive | Presence of Hyperplasia | Polarized Epithelial Cells | Stromal Component | Method Used to Acquire Tissue Structure | ECM |
|---|---|---|---|---|---|---|
| **Morgan, 2018** | Yes. | Yes | Yes | Fibroblasts | Seeded into engineered lumen; formed lumen structure | Yes; Collagen |
| **Bischel, 2015** | Not evaluated | Not evaluated | Yes | Fibroblasts | Seeded into engineered lumen; formed lumen structure | Yes; Mixture of collagen and Matrigel |
| **Carter, 2017** | Not evaluated | Yes | Yes | Myoepithelial cells | Self-assembled into bilayered luminal structures | Yes; Collagen or matrigel |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Debnath, 2003** | Not evaluated | Not evaluated | Yes | No | Self-assembled into luminal structures | Yes; Matrigel |
| **Krause, 2010** | Not evaluated | Yes | Polarized in Matrigel or in co-culture. | Fibroblasts | Self-assembled into spheroids or luminal structures. | Yes; Collagen or Matrigel |
| **Marchese, 2012** | Yes | Yes | Yes | No | Self-assembled into luminal structures | Yes; Matrigel |
| **Vantangoli, 2015; Vantangoli, 2017** | Yes | Yes | Hemi-polarized | No | Self-assembled into microtissues that contain a luminal space | No; Agarose scaffold |

**Table 3.1. Comparison of MCF7-derived duct model to previously published organotypic breast models.**

Previous publications have found that MCF7s plated on plastic lack polarity but can become polarized when cultured in a tissue relevant environment[469]. For example, one study found that MCF7s embedded in a collagen matrix were not polarized, however, the introduction of a reconstituted basement membrane (e.g. Matrigel) and stromal cells induced polarity[98]. In our platform, MCF7-derived ducts grown in normal growth media exhibited apical-basal polarity, suggesting that the ductal structure is important for cell polarization, which has been previously reported[379]. The specific mechanisms that dictate how the culture environment induces polarization have been widely discussed, and while some pathways have been identified, these interactions are not completely understood [483; 484].

The ER status of mammary fibroblasts varies between species, as well as within an individual's mammary tissue. For example, in the adult human mammary gland, ERα is expressed in the epithelium but not the stroma[471]. In contrast, ERα is expressed in both the epithelium and stroma of the mouse mammary gland[472]. These species differences become critical when evaluating ER-driven responses, as ERα ligands could target both the stroma and the epithelium in the mouse, in contrast to just the epithelium in the human. HMFs grown in our system did not stain positively for ERα protein and did not show induction of ERα-driven genes after E2 treatment, suggesting that the fibroblasts used in our system do not express ERα. The HMFs also did not show induction of ERβ-driven genes after E2 treatment, which was evaluated because a portion of fibroblasts in the human breast expresses ERβ[485].

Overall, these results suggest that the differences in estrogenic response observed in co-culture were not due to E2 stimulating ER in the HMFs.

Previous studies using conventional 2D platforms have found that stromal cells such as cancer-associated mammary fibroblasts or bone marrow stromal cells regulate ER protein expression in mammary epithelial cells[122; 126]. In this study, we found that fibroblasts also regulate ER protein in MCF7s grown as ductal structures, as demonstrated by reduced ER protein in the vehicle-treated co-cultures compared to the vehicle-treated monocultures. As ER protein is downregulated upon ER activation[479], we suspect that the increased ER transactivation observed in the vehicle-treated co-cultures was responsible for the reduction in ER protein. Surprisingly, ER protein was similar in both the estrogen-treated monoculture and co-cultures, which is notable because ER transactivation was significantly higher in the estrogen-treated co-cultures compared to the estrogen-treated monocultures. This suggests that ER signaling dynamics are distinctly different in MCF7s cultured with fibroblasts, compared to MCF7s cultured alone. These differences could become critical when evaluating breast cancer therapeutics and risk factors because drug resistance/sensitivity could change in the presence of stromal cells.

Our finding that MCF7 cell density was increased in the presence of fibroblasts is consistent with previous findings[122], and led us to hypothesize that fibroblasts were influencing proliferation and/or apoptosis. Upon characterization of the cell proliferation marker Ki67, we found the percentage of Ki67+ cells in the MCF7-derived ducts (~2-4% Ki67+ cells) was comparable to the percentage of Ki67+ cells in normal human breast tissue (~3% Ki67+ cells)[486]. These percentages are dramatically reduced compared to cells cultured in 2D, where one group found that 90% of MCF7s plated on plastic are Ki67+[487]. Similar to previous reports[488], E2 increased MCF7 proliferation, as indicated by a 2-3 fold increase in Ki67 expression. Despite finding an increased cell density in our co-culture platform, we did not observe a

difference in Ki67+ expression in our monoculture vs. co-culture, leading us to suspect that the differences in cell density were independent of proliferation.

Previous publications have found that some ER+ breast cancer cells rely on estrogen for survival, and undergo apoptosis when grown in estrogen-starved conditions[480; 489]. Therefore, it was not surprising that a portion of the MCF7s treated with a vehicle control, and consequently estrogen-starved, were dead or undergoing apoptosis. Also in agreement with previous publications[184], E2 treatment improved cell viability and reduced apoptosis in our monoculture platform. When fibroblasts were included in the matrix, there were low levels of apoptosis and high cell viability even in the absence of estrogen. We did not observe significant E2-induced differences in cytotoxicity or apoptosis in co-culture; however, we suspect that E2 had no effect on cell viability in the co-cultures because the paracrine signals from the fibroblasts increased viability to such high levels (approaching 100%) that viability could not be further increased by estrogen treatment. This suspicion is supported by the concomitant low levels of apoptosis (~3% cells) in both the vehicle and estrogen-treated co-cultures. Future studies are needed to determine if the estrogen- and paracrine-induced reduction of apoptosis occur through shared or distinct mechanisms, however, the increased ER activity in conjunction with the reduced apoptosis in our co-cultures suggests an ER-dependent mechanism. Upon examination of MCF7s that detached from the lumen due to cell death, we found that significantly less cells left the lumen in the E2-treated co-cultures compared to the E2-treated monocultures. We suspect that this reduction in cell loss, along with the maintenance of E2-induced proliferation, could explain the increased cell density observed in the co-cultures.

We characterized ductal hyperplasia, which refers to the abnormal growth of epithelial cells into the center of a breast duct, because ductal hyperplasia is commonly used in a clinical setting as it is a defining feature of premalignant breast lesions[490]. 2D *in vitro* models are unable to evaluate hyperplasia due to the absence of tissue structure. In the monoculture platform, hyperplasia was observed after 10

days of E2 exposure, which agrees with other *in vitro* organotypic studies that reported ductal hyperplasia in the presence of E2[75; 469]. When stromal cells were incorporated into the matrix, E2-induced hyperplasia was detected sooner and was more prominent than in our monoculture model, likely due to reduced apoptosis and consequently the increased cell density observed in the co-culture platform.

Our finding that HMFs modulate both ER protein and apoptosis in MCF7 cells may shed light into the perplexing role of ER-regulated apoptosis. Treatment with anti-ER therapies typically induces apoptosis in ER+ breast tumors, and exposure to ER agonists reduces apoptosis. However, breast tumors can become resistant to anti-ER therapies, and in these tissues, exposure to ER agonists can increase apoptosis[491; 492]. It is not clear what causes a tumor to respond differently to ER ligands, however, some have speculated that the interactions between the tumor cell and the surrounding stroma play a role[493; 494]. Therefore, future studies that evaluate how primary fibroblasts or other mammary fibroblast cell lines affect ER-driven responses in the MCF7-derived duct system may provide insight into the role of the stroma in mediating therapy resistance and its relation to ER-regulated apoptosis. Of course, these future studies must consider the shortcomings associated with the MCF7-derived duct model. For example, the model is less throughput than traditional *in vitro* models, due to the incorporation of the biomimetic ductal structure, matrix proteins and additional cell types. Additionally, the overall complexity of the model introduces additional variables that must be considered when investigating biological or chemical mechanisms.

In summary, MCF7s cultured in a mammary duct structure exhibited apical-basal polarity, cellular proliferation levels similar to the ducts of a normal breast *in vivo*, and growth into the center of the lumen resembling atypical ductal hyperplasia. We confirmed that the MCF7-derived duct model responds to estrogens similarly to what has been previously shown both *in vitro* and *in vivo*, where E2 exposure induced ductal hyperplasia, increased proliferation, decreased apoptosis, increased ER

transactivation and reduced ER protein. Incorporation of fibroblasts into the surrounding matrix sped the onset and increased the severity of estradiol-induced hyperplasia. We found the increased hyperplasia was due to MCF7s exhibiting lower levels of apoptosis coupled with similar levels of estradiol-induced proliferation in co-culture, which led to an increased cell density. Furthermore, the reduction in apoptosis may have occurred through an ER-dependent mechanism, as we observed a reduction in ER protein and an increase in ER transactivation in the co-culture model. Overall, this study introduces a new platform that can be used to evaluate the role of the mammary microenvironment in ER+ breast cancer progression, and provides insight into the importance of fibroblasts when evaluating environmental chemicals or candidate therapeutics.

### 3.5 Acknowledgements

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## Chapter 4. Mammary adipose stromal cells derived from obese women reduce sensitivity to the aromatase inhibitor anastrazole in an organotypic breast model

Aromatase inhibitors are the preferred treatment for certain women with estrogen receptor (ER) positive breast cancer, but evidence suggests that obese women experience aromatase inhibitor resistance at higher rates. To compare how stromal cells derived from lean and obese women influence response to the aromatase inhibitor, anastrazole, we incorporated patient-derived stroma in a previously characterized MCF7-derived *in vitro* duct model. Co-culture with adipose stromal cells enabled the metabolism of testosterone to 17β-estradiol, which induced estrogen response element activity, epithelial proliferation, and hyperplasia in MCF7 cells. Testosterone-induced effects were inhibited by the ER antagonist tamoxifen and aromatase inhibitor anastrazole and were increased by the aromatase inducer dexamethasone. Primary mammary adipose stromal cells derived from obese women displayed increased aromatase mRNA compared to lean controls. MCF7-derived ducts co-cultured with obese stromal cells exhibited higher maximal aromatization-induced ER transactivation and reduced anastrazole sensitivity, a difference not seen in two-dimensional co-culture. Finally, tamoxifen was more effective than anastrazole at reducing aromatization-induced ER transactivation and proliferation. These findings suggest that patient-specific responses to hormone therapies can be modeled and studied organotypically *in vitro* and add to evidence advocating obesity as a parameter to consider when identifying treatments for ER+ breast cancer patients.

This chapter has been adapted from the manuscript that is under revision in *FASEB*: "Mammary adipose stromal cells derived from obese women reduce sensitivity to the aromatase inhibitor anastrazole in an organotypic breast model." The manuscript was authored by Molly M. Morgana, Lisa M. Arendt, Elaine T. Alarid, David J. Beebe, Brian P. Johnson.

## 4.1 Introduction

Approximately two thirds of all breast cancer cases are estrogen receptor alpha (ER) positive. ER is thought to regulate the progression of ER+ breast cancer by controlling the growth and death of breast cancer cells through estrogen-regulated signaling [467]. In postmenopausal women, treatment of ER+ breast cancer typically involves directly targeting ER-mediated signaling with ER antagonists such as tamoxifen, or indirectly limiting local estrogen by suppressing the conversion of testosterone to 17β-estradiol with the use of aromatase inhibitors such as anastrazole [439]. While both tamoxifen and aromatase inhibitors are effective treatments, clinical studies indicate that aromatase inhibitors are more effective at reducing recurrence and mortality rates than tamoxifen [150; 151; 158; 238; 239].

Unfortunately, the patient response rates using aromatase inhibitors range from 20-50%, and understanding the underlying causes of treatment resistance is a persistent challenge [495]. In particular, obesity is a risk factor for aromatase inhibitor resistance. Clinical trials have found that in comparison to lean patients, obese breast cancer patients treated with aromatase inhibitors are at higher risk of recurrence and/or are less responsive to treatment [247; 248; 249]. There is some evidence that obese postmenopausal women may benefit from other therapies such as tamoxifen [247; 496], although these findings are controversial and warrant further investigation [497; 498]. Understanding the mechanisms that predispose obese individuals to aromatase inhibitor resistance may increase our ability to predict which patients are poor candidates for aromatase inhibitors, as well as pinpoint alternative therapeutic strategies.

A major challenge with studying aromatase inhibitor resistance is a lack of suitable model systems. Studies examining aromatase inhibitor resistance in obese versus lean individuals typically rely on mouse models where deciphering mechanisms can be challenging due to difficulties with pinpointing specific cell:cell or chemical:cell interactions [159; 499]. Additionally, extrapolating data between species is particularly difficult due to the differences between the physiology of the mouse and human mammary

gland. For instance, estrogen receptor expression differs in the mammary gland of the mouse and human; ER is expressed in the stroma of the mouse mammary gland, but not in the stroma of the human mammary gland [500]. Unlike humans, mice lack the promoters that regulate aromatase expression in peripheral tissues, such as the breast [14; 15]. While using an *in vitro* model mitigates these issues, traditional *in vitro* breast cancer cell models neglect to include stromal cells. Stromal cells are essential for studying aromatase inhibitor resistance because breast stromal cells are primarily responsible for producing aromatase [148; 149]. Researchers have started to incorporate mammary stroma into *in vitro* breast cancer platforms, which have revealed striking differences in how obese and lean stromal cells influence the behavior of breast cancer cells [250], and have confirmed that mammary stromal cells can induce ER-driven responses by metabolizing testosterone to estrogen [501]. However, these studies cultured cells in platforms that did not include an extracellular matrix or tissue geometry, both of which have been shown to be important to recapitulating *in vivo* responses [502]. These studies also did not compare resistance to aromatase inhibitors in obese and lean patients, and consequently, the increased risk of aromatase inhibitor resistance in obese individuals remain largely understudied. Altogether, an *in vitro* breast model that incorporates aromatase signaling would be useful for deciphering mechanisms of aromatase inhibitor resistance.

To this end, we utilized a previously characterized organotypic mammary duct model to investigate how the mammary stromal cells of lean and obese women differentially influence response to the aromatase inhibitor, anastrazole, *in vitro*. We chose to use the organotypic model as it enables the study of stromal:epithelial interactions in a more physiologically relevant environment than a traditional 2D model [116]. Importantly, we found that the organotypic co-culture system was able to segregate differences in anastrazole sensitivity between lean and obese patients while a two-dimensional co-culture model could not. Our results suggest that 1) the mammary stroma regulates resistance to aromatase inhibitors, 2) patient-specific responses to anastrazole can be modeled and

studied *in vitro,* and 3) that obesity may be a useful parameter to consider when choosing hormone therapies for breast cancer patients.

## 4.2 Materials and Methods

**4.2.1 Primary Human Tissue Isolation.** All breast tissue procurement for these experiments was obtained in compliance with the laws and institutional guidelines, as approved by the Institutional Review Board (IRB) committee from the University of Wisconsin-Madison. Disease-free, de-identified breast tissues were obtained from female patients undergoing elective reduction mammoplasty with informed consent through the Translational Science BioCore BioBank at the Carbone Cancer Center at the University of Wisconsin-Madison. This research study was approved by IRB as Not Human Subject Research with a limited patient data set including patient age, date of surgery, and body mass index (BMI). All mammary stromal cells used in the manuscript were isolated from the stromal vascular fraction of breast tissue of premenopausal patients undergoing reduction mammoplasty, as described previously [503]. Briefly, after collection breast tissue was incubated for eight hours with 1.5 mg/mL collagenase I (Sigma Aldrich, St. Louis, MO, USA) diluted in DMEM:F12 media (Gibco, Gaithersburg, MD, USA) supplemented with 5% calf serum (Gibco). After digestion, the tissue was incubated for ten minutes at room temperature then the lipid-rich portion was discarded. The stromal fraction was incubated with red blood cell lysis buffer (ACK Lysing Buffer; Lonza, Basel, Switzerland) then plated in DMEM supplemented with 10% fetal bovine serum (Gibco) and 1% antibiotic/anti-mycotic solution. For experiments that evaluated the effect of cell density on anastrazole resistance, we used mammary stromal cells derived from obese patients that were at a late passage ($p > 7$). Unless otherwise indicated, when comparing lean and obese patients, we used mammary stromal cells at an early passage ($p \leq 3$). **Table 4.1** lists the BMI and age of the 12 patients (6 lean and 6 obese) used in the obese vs. lean comparison studies. All patients in the study who reported their ethnicity were non-Hispanic white; one patient did not include race.

| Variables | All (n = 10) | Lean (n = 5) | Obese (n = 5) | P value |
|---|---|---|---|---|
| Age (median, range) | 33.5 (20 to 52) | 33 (20 to 46) | 35 (26 to 52) | 0.24 |
| BMI (median, range) | 27.5 (23 to 36) | 24 (23 to 25) | 34 (30 to 36) | 0.00001 |

**Table 4.1. Patient Demographics.** Table 1 lists the age and BMI for each of the 12 patients (6 lean, 6 obese) evaluated for aromatase resistance. All patients who reported ethnicity were non-Hispanic white; one patient did not disclose ethnicity.

**4.2.2 Cell culture.** Adipose derived mesenchymal stem cells (AdMSCs) were purchased from ATCC. The MCF7 cells were previously transfected with an estrogen response element (ERE)-luciferase reporter to detect ER activation using luminescent activity [394]. All cell types were maintained in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin (PS; Thermo Fisher, Waltham, MA, USA). 48 hours before experiment seeding, culture flasks were washed with PBS then replenished with estrogen-free media, which contains phenol-red free DMEM (Thermo Fisher) with 10% charcoal-stripped FBS, 2 mM glutamine, and 1% PS. All cultures were maintained in an incubator at 37°C and 5% $CO_2$.

**4.2.3 Generation of MCF7-derived ducts.** MCF7-derived ducts were generated as described previously [116]. Briefly, the two-layered microfluidic devices were constructed from polydimethylsiloxane (PDMS) using the Sylgard 184 Silicone Elastomer Kit (Dow Corning, Auburn, MI, USA) and standard photolithography techniques. After PDMS devices were treated with 2% poly(ethyleneimine) (Sigma Aldrich) and 0.4% glutaraldehyde (Sigma Aldrich) for 10 minutes and 30 minutes, respectively, devices were washed three times with water. Each device was loaded with 6.5 µl of a 4.5 mg/ml neutralized rat-tail collagen I solution (Corning, Corning, New York, USA) that contained media or stromal cells in media. 4,000 AdMSCs per device were used for all experiments validating metabolism of testosterone to 17β-estradiol, except for experiments evaluating aromatase induction via dexamethasone; dexamethasone experiments used 2,000 AdMSCs per device. Experiments that evaluated different AdMSC densities used approximately 2,000, 8,000, and 16,000 AdMSCs per device for the low, medium, and high concentrations, respectively. Experiments comparing lean to obese cultures used approximately 4,000

stromal cells per device. After loading, collagen was polymerized at room temperature for ten minutes, and then transferred to the incubator for one hour. Afterward, the sacrificial rod was removed and the resulting luminal structure was filled with 1.5 µl of MCF7s at 50,000/µl. Cultures were flipped every 20 minutes for one hour then excess cells were aspirated from the large port and media was replenished through the small port. An evenly seeded confluent lumen contains approximately 4,000 MCF7 cells. All organotypic cultures were seeded in estrogen-free media supplemented with serum free fibroblast growth supplement (ScienCell, Carlsbad, CA, USA). Experiments were dosed with 17β-estradiol (E2), testosterone (T), anastrazole, dexamethasone (DEX), 5α-dihydrotestosterone (DHT), or 4-hydroxytamoxifen (OHT). All chemicals were dissolved in ethanol and were bought from Sigma Aldrich, except for DHT which was bought from Cerilliant (Round Rock, Texas, USA). Drugs were dosed in organotypic culture media containing 2 mM of VivoGlo luciferin (Promega, Madison, WI, USA), which cleaves luciferase to produce a luminescent signal linear to ER transactivation. Cultures were exposed to test chemicals the day after seeding and dosing media was replenished daily.

**4.2.4 2D co-culture.** We used a co-culture approach similar to a previous study [156]. Mammary stromal cells were seeded in a 384 well plate at 2,500 cells/well and allowed to adhere for 3 hours. 2,500 MCF7s were then seeded on top of the stromal cells. Similar to the organotypic system, we used a 1:1 ratio of adipose stromal cell:MCF7 cell and dosed the cultures the day after seeding. Additionally, the same types of media were used for seeding and dosing as in the organotypic system.

**4.2.5 ER transactivation assay.** The MCF7s used in the model were previously transfected with a reporter placing a luciferase gene downstream of the ERE located in the vitellogenin gene; therefore, the luciferase produced by the cells is linear to ER transactivation [394]. Luminescence produced in organotypic cultures was measured with a Biorad imager, while luminescence in well plates was measured by a Pherastar plate reader.

**4.2.6 RNA isolation and RT-qPCR**. To lyse cells grown on 96 well plates, media was removed from each well and replaced with the lysis binding buffer of the Dynabead mRNA Direct Purification Kit (Thermo Fisher). To lyse cells from organotypic cultures, the top layer of the PDMS device was removed and the collagen/cell mixture was transferred to a tube containing lysis buffer. Collagen was then pulverized with a 23-gauge needle (Fisher Scientific, Waltham, USA, MA). RNA was isolated from the lysates using the Dynabeads mRNA Purification Kit according to the manufacturer's protocol, and reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher). PCR was conducted using the Light Cycler 480 Probes Master Mix and Taqman primers to TFF1 (Hs00907239_m1), PGR (Hs01556702_m1) or CYP19A1 (Hs00903411_m1) and normalized to HPRT (Hs02800695_m1) and RPLP0 (Hs99999902_m1). We used HPRT and RPLP0 as housekeeping genes because they have been used previously as housekeeping genes for breast cancer studies [504; 505] and are a lowly (HPRT) and highly (RPLP0) expressed housekeeping gene. Relative gene expression was determined using the ΔΔCT method.

**4.2.7 Evaluation of cell density.** Cell density was quantified as described previously [116]. Cells were fixed with 4% paraformaldehyde (Alfa Aesar, Tewksbury, MA, USA) for 15 minutes then washed twice with PBS [116]. To examine cell density, cells were stained with Hoescht (Thermo Fisher) and Texas red phalloidin (Thermo Fisher) to visualize nuclei and F-actin, respectively. The semi-automated Image J based program, JEXperiment was used for image quantification [506], where regions of interests of equal size were drawn over the ducts of each image, and a rolling ball background subtraction was used. The number of nuclei was then identified.

**4.2.8 Cross sectioning.** After 5 days in culture, lumens were fixed and stained for nuclei and F-Actin, and then embedded in agarose and cross-sectioned with a Compresstome (Precisionary Instruments, Greenville, North Carolina, USA) as described previously [116]. To quantify ductal thickness, the number of nuclei was counted at six evenly spaced points along each duct.

**4.2.9 Statistics.** Excel and GraphPad Prism were used to conduct statistical tests. Nonlinear regression was used to calculate dose response curves and EC50s/IC50s, which is the concentration that induces, or represses, a response by 50%. Specifically, Prism models log(agonist) vs. response (three parameters) and log(inhibitor) vs. response (three parameters) were used. Error bars in all graphs represent standard error and significance is defined as $p < 0.05$. A student's t test was used to evaluate significance in experiments comparing two conditions. Experiments evaluating more than two conditions used a one-way ANOVA to determine significance. A two-way ANOVA followed by a multiple comparisons test was used when evaluating the effect of adipose stromal cells on responses to testosterone.

## 4.3 Results

### 4.3.1 Testosterone induces ER-driven responses when AdMSCs are in the matrix surrounding MCF7-derived ducts

In the mammary gland, adipose stromal cells-- a mixture of adipose stem cells, fibroblasts, and other stromal cell types [191]-- produce the enzyme aromatase which metabolizes testosterone (T) and androstenedione to 17β-estradiol (E2) and estrone (E1), respectively [148; 149]. To mimic the conversion of androgens to estrogens that occurs in breast tissue *in vivo*, we incorporated a commercially available source of primary human adipose stromal cells, AdMSCs, into the collagen matrix that surrounds a ductal structure lined with MCF7 breast cancer cells.

To validate the conversion of T to E2, MCF7-derived ducts cultured alone or with AdMSCs in the matrix were evaluated for ER transactivation after a 48-hour exposure to five concentrations of T; we chose to evaluate T rather than androstenedione because a previous *in vitro* study demonstrated that breast adipose fibroblasts could metabolize T to E2 which induced ER-driven responses in MCF7 cells [156]. A dose-dependent relationship was observed between T exposure and ER transactivation in the co-culture, but not in the monoculture **(Fig. 4.1A)**.

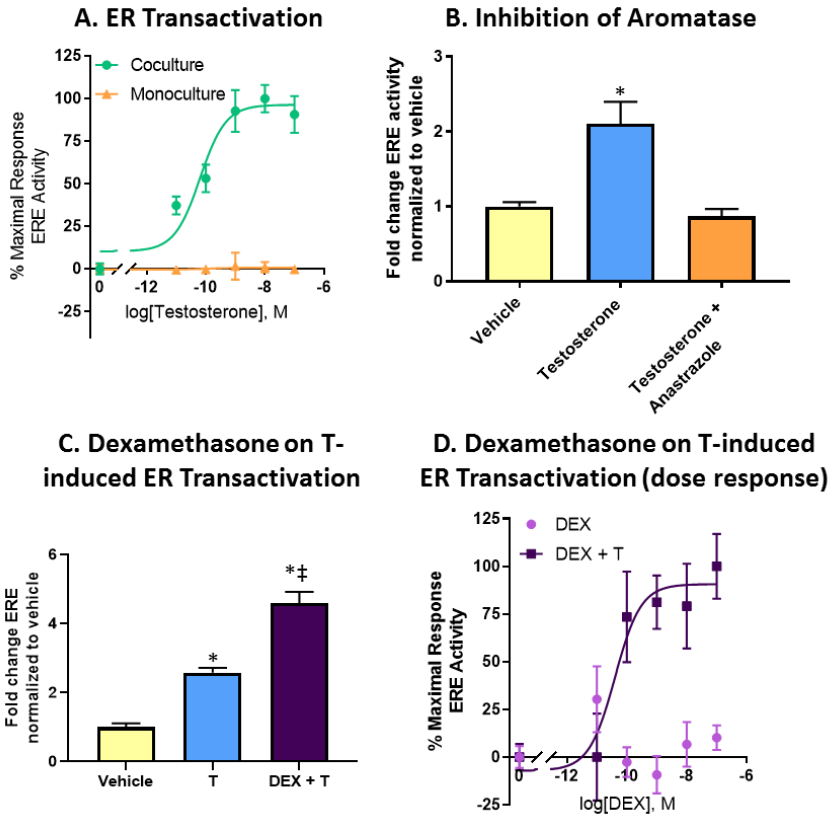


**Fig. 4.1. Testosterone induces ER transactivation in MCF7-derived ducts co-cultured with AdMSCs. (A)** ER transactivation was evaluated in MCF7-derived ducts grown alone or with AdMSCs after a 48-hour exposure to five doses of testosterone. **(B)** MCF7-derived ducts co-cultured with AdMSCs were exposed to a vehicle control, testosterone (100 nM), or testosterone and anastrazole (1 µM) for 24 hours then evaluated for ER transactivation. **(C)** MCF7-derived ducts co-cultured with AdMSCs were exposed to a vehicle control, testosterone (T), or T in combination with dexamethasone (DEX) then evaluated for ER transactivation. **(D)** MCF7-derived ducts co-cultured with AdMSCs were exposed to five doses of DEX, alone or in combination with T then evaluated for ER transactivation. * = vs. vehicle ($p < 0.05$) and ‡ = vs. testosterone ($p < 0.05$).

To confirm that the T-induced ER transactivation was due to estrogen production via aromatase, we evaluated how ER transactivation was affected by testosterone when co-treated with an aromatase inhibitor or inducer. First, we exposed MCF7-derived ducts co-cultured with AdMSCs to a vehicle control, 100 nM T, or 100 nM T and 1 µM of the aromatase inhibitor anastrazole. As expected, while T increased ER-transactivation two-fold, co-treatment with anastrazole inhibited this effect **(Fig. 4.1B)**. Next, MCF7-derived ducts co-cultured with AdMSCs were exposed to a vehicle control, 100 nM T, or 100 nM T and 1 µM of the aromatase inducer dexamethasone. ER transactivation was significantly higher when the cultures were exposed to dexamethasone and testosterone (4.6-fold relative to vehicle),

compared to when exposed to testosterone alone (2.5-fold relative to vehicle) **(Fig. 4.1C)**. Next, MCF7-derived ducts co-cultured with AdMSCs were exposed to 5 doses (0.1 – 1000 nM) of dexamethasone, alone or in combination with testosterone. While dexamethasone had no effect on ER transactivation in the absence of testosterone, when exposed in the presence of testosterone dexamethasone increased ER transactivation in a dose-dependent manner **(Fig. 4.1D)**.

To evaluate if testosterone induces proliferation, cell number was evaluated after MCF7-derived ducts cultured alone or with AdMSCs were exposed to a vehicle control, 100 nM of 17β-estradiol (E2), or 100 nM of T for 5 days. While E2 significantly increased cell number in both culture conditions, T treatment showed no effect in the monoculture. In contrast, exposure to T led to a significant increase in cell number in the co-culture **(Fig. 4.2A and B)**, which we suspect was due to the conversion of testosterone to estrogen. The vehicle and T-treated co-cultures were cross-sectioned to evaluate the presence of hyperplasia, a defining feature of preinvasive breast lesions **(Fig. 4.2C)**. Quantification revealed a single cell layer in the vehicle-treated co-culture, while the testosterone-treated co-culture had approximately 2.5 cell layers per lumen **(Fig. 4.2D)**.

Previous studies have reported that androgens can influence the growth of breast cancer cells by acting on the androgen receptor (AR) [501; 507]. To examine if some of the effects of testosterone are mediated through AR, we exposed MCF7-derived ducts co-cultured with AdMSCs to a vehicle control, testosterone, or DHT. DHT is a potent androgen but is not a substrate for aromatase. ER transactivation was evaluated after 48 hours **(Fig. 4.3A)** and lumen confluency after 5 days **(Fig. 4.3B)**. Consistent with our previous findings, testosterone induced ER transactivation and increased lumen confluency. However, no detectable response was observed after DHT treatment.

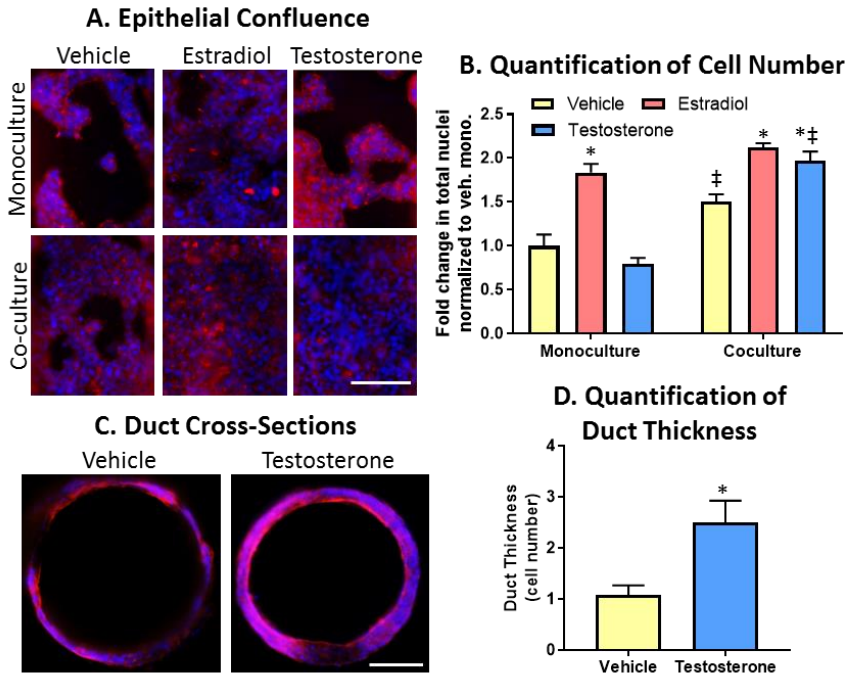


**Fig. 4.2. Testosterone induces proliferation and hyperplasia in MCF7-derived ducts co-cultured with AdMSCs.** **(A)** After a 5-day exposure to a vehicle control,17β-estradiol (100 nM), or testosterone, MCF7-derived ducts cultured alone or in co-culture with AdMSCs were fixed then stained for nuclei (blue) and F-Actin (red). **(B)** Cell number was quantified by counting the number of nuclei within each duct. * = vs. vehicle ($p < 0.05$) and ‡ = vs. respective monoculture ($p < 0.05$). **(C)** After a 5-day exposure to a vehicle control or testosterone, MCF7-derived ducts co-cultured with AdMSCs were fixed, stained for nuclei and F-actin and cross-sectioned to evaluate hyperplasia. **(D)** Hyperplasia was quantified by counting the number of cells lining the duct at six evenly spaced points along each duct.

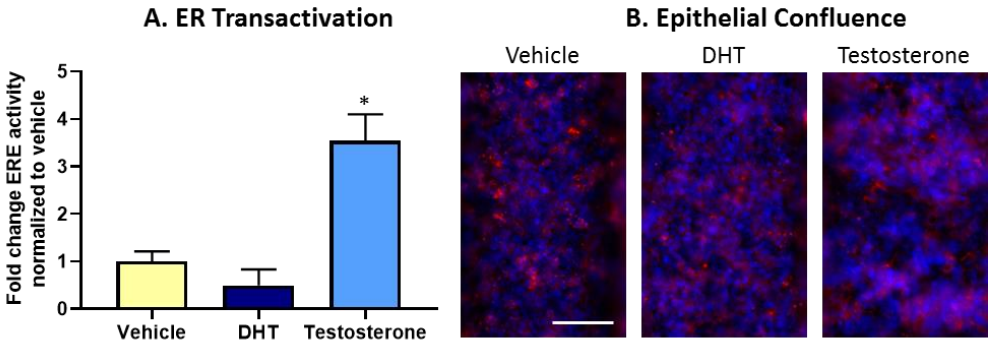


**Fig. 4.3. DHT does not affect ER transactivation or epithelial confluence.** MCF7-derived ducts co-cultured with AdMSCs were exposed to a vehicle control, 5α-dihydrotestosterone (DHT), or T then evaluated for ER transactivation after 48 hours **(A)** or cell confluency after 5 days **(B)**. Cell confluency was assessed by staining for nuclei (blue) and filamentous actin (red). The scale bar represents 100 microns.

### 4.3.2 Breast cancer cells cultured with a higher concentration of AdMSCs exhibit decreased sensitivity to anastrazole

We hypothesized that increased adipose volume, and consequently increased number of adipose stromal cells, would reduce anastrazole sensitivity in breast cancer cells due to enhanced levels of aromatase. We measured ER transactivation when MCF7-derived ducts were co-cultured with a medium (2:1 AdMSC:MCF7) or high concentration (4:1 AdMSC:MCF7) of AdMSCs and exposed to 10 nM T, in the presence of five concentrations of anastrazole. MCF7-derived ducts co-cultured with a high concentration of AdMSCs exhibited an IC50 of 1.4E-8 M compared to an IC50 2.5e-10 M for the medium concentration co-culture **(Fig. 4.4A)**. The IC50 is a measure of drug potency, as it describes the concentration needed to inhibit a response by 50%. The dose response experiment was repeated using human adipose stromal cells derived from the stromal vascular fraction of reduction mammoplasty breast tissues. Similar to our findings with the AdMSCs, an increased concentration of human mammary stromal cells was associated with a decreased sensitivity to anastrazole, as indicated by a significantly increased IC50 (IC50 of high concentration cultures was 1.9e-8 M compared to 4e-11 M of medium concentration cultures) **(Fig. 4.4B)**.

To confirm that testosterone to estrogen metabolism is dependent on the number of adipose stromal cells, ER transactivation was evaluated for MCF7-derived ducts co-cultured with a low (0.5:1 AdMSC:MCF7), medium (2:1 AdMSC:MCF7), or high concentration of AdMSCs (4:1 AdMSC:MCF7) **(Fig. 4.4C**), when exposed to a vehicle control, 100 nM of T, or 100 nM of E2 **(Fig. 4.4D)**. T-induced ER transactivation was significantly lower when MCF7s were co-cultured with a low concentration of AdMSCs, compared to when co-cultured with a medium or high concentration of AdMSCs. There was no difference between the T-induced ER transactivation in the medium and high concentration of AdMSCs, and E2-induced ER transactivation was the same across the three culture conditions.

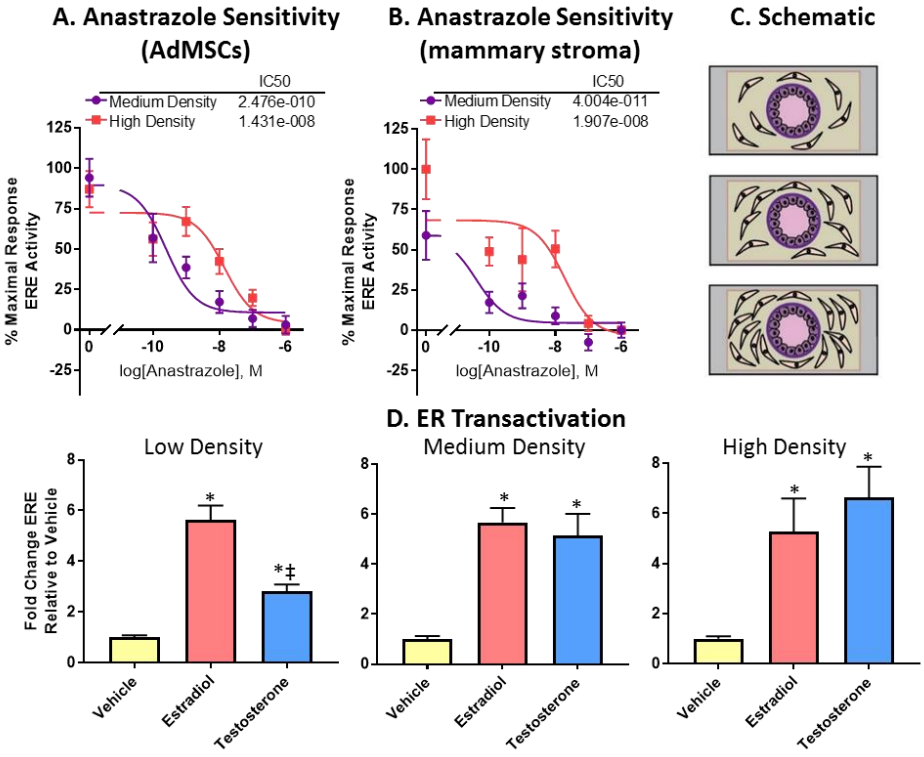


**Fig. 4.4. Anastrazole sensitivity and testosterone metabolism are dependent on the concentration of AdMSCs. (A)** MCF7-derived ducts cultured with a medium and high concentration of AdMSCs in the presence of 10 nM testosterone were exposed to five concentrations of anastrazole for 48 hours then evaluated for ER transactivation. **(B)** MCF7-derived ducts cultured with a medium and high concentration of late passage primary human mammary adipose stromal cells derived from obese women in the presence of 10 nM testosterone were exposed to five concentrations of anastrazole for 48 hours then evaluated for ER transactivation. **(C)** Schematic showing MCF7-derived ducts cultured with a low, medium, or high concentration of AdMSCs; the ratio of cancer cells to AdMSCs for the low, medium and high concentration conditions are 1:2, 2:1, and 4:1, respectively. **(D)** MCF7-derived ducts cultured with different concentrations of AdMSCs were exposed to a vehicle control,17β-estradiol, or testosterone for 48 hours then evaluated for ER transactivation. * = vs. vehicle ($p < 0.05$) and ‡ = vs.17β-estradiol ($p < 0.05$).

### 4.3.3 Breast cancer cells cultured with obese-derived stromal cells exhibit decreased sensitivity to anastrazole compared to when cultured with lean-derived stromal cells

Accumulating evidence suggests that obese women are less sensitive to anastrazole than lean women [247; 248; 249]. To test this *in vitro*, we acquired mammary adipose stromal cells derived from the reduction mammoplasties of lean (BMI ≤ 25) and obese (BMI ≥ 30) women. Obese stromal cells and lean stromal cells were cultured in a 2D monoculture for 48 hours then evaluated for CYP19A1, the gene

encoding aromatase. RT-qPCR revealed that obese stromal cells had 1.5-fold higher levels of CYP19A1 mRNA than lean stromal cells **(Fig. 4.5A)**. To compare the conversion of testosterone to E2, MCF7-derived ducts co-cultured with lean or obese stromal cells at a low passage number ($p \leq 3$) were exposed to a vehicle control or testosterone for 48 hours. Evaluation of ER transactivation revealed that obese co-cultures converted more testosterone to estrogen, as T-induced ER transactivation was 3-fold higher than the vehicle in obese co-cultures, compared to 2-fold in the lean co-cultures **(Fig 4.5B)**. As studies have found that the molecular profiles of primary cells can change over time in culture [68; 69], aromatization-induced ER transactivation was compared again when the lean and obese stromal cells were at a late passage number ($p > 7$). While obese co-cultures exhibited slightly increased ER transactivation compared to lean co-cultures, the difference was not significant **(Fig. 4.5C)**.

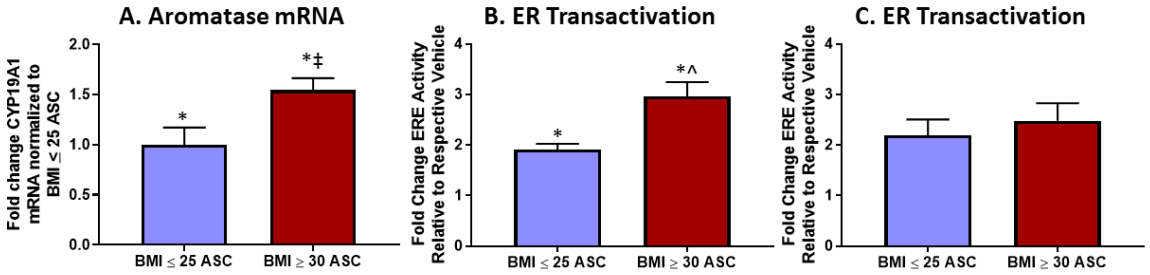


**Fig. 4.5. Mammary stromal cells derived from obese women exhibit increased aromatase expression and aromatization-induced ER transactivation. (A)** The expression of CYP19A1 mRNA was evaluated in the mammary stroma of lean female donors and the mammary stroma of obese female donors. MCF7-derived ducts were exposed to a vehicle control or testosterone when co-cultured with mammary stromal cells of lean and obese patients at an early passage **(B)** ($p \leq 3$) and a late passage **(C)** ($p > 7$) then evaluated for ER transactivation. Testosterone treated cultures were normalized to the vehicle treated cultures of each respective patient. * = vs. respective vehicle ($p < 0.05$) and ^ = vs. lean cultures ($p < 0.05$).

To assess response to anastrazole, we evaluated ER transactivation after MCF7-derived ducts cultured with mammary stromal cells derived from either lean or obese women were exposed to five concentrations of anastrazole. All cultures were seeded with the same concentration of stromal cells (1:1 adipose stromal cell:MCF7; passage $\leq 3$) and supplemented with 10 nM of T. ER transactivation was compared for six obese patients and six lean patients **(Fig. 4.6A).** Evaluation of dose response curves

revealed a dramatically increased IC50 ($p < 0.01$) and increased maximal ER transactivation ($p < 0.0001$) in the obese cultures compared to the lean cultures. The vehicle-treated organotypic cultures (e.g. cultures treated with 10 nM T but no anastrazole) were evaluated at day 4 for ER-driven genes TFF1 and PGR, and there was an approximately 2.9-fold and 2.5-fold higher expression of each gene, respectively, in the obese cultures **(Fig. 4.6B)**. Anastrazole IC50 was graphed against BMI, which revealed a weak positive trend ($r^2 = 0.34$; $p < 0.05$) **(Fig. 4.6C)**. For 8 of the 12 patient samples (4 lean and 4 obese), sensitivity to anastrazole was also compared when MCF7 cells were co-cultured with mammary stromal cells on a 2D well plate. The 2D co-culture experiment used the same ratio of stromal cells to MCF7 cells (1:1) and was seeded, dosed, and evaluated at the same time points as the organotypic cultures. In contrast to the organotypic platform, the obese and lean dose response curves did not segregate or differ in IC50 **(Fig. 4.6D)**.

### 4.3.4 Tamoxifen is more effective than anastrazole at reducing ER transactivation and proliferation

While anastrazole has been found to be as effective or more effective than tamoxifen, there is evidence that tamoxifen may be more effective in obese women [247; 496]. To examine this hypothesis *in vitro*, MCF7-derived ducts co-cultured with stromal cells derived from obese women were exposed to a vehicle control, 10 nM T, 10 nM T in combination with 1 nM anastrazole, or 10 nM T in combination with 1 nM of 4-hydroxytamoxifen (OHT—the active metabolite of tamoxifen). Evaluation of ER transactivation after 48 hours revealed that tamoxifen prevented T-induced ER transactivation, while anastrazole did not **(Fig. 4.7A).** Similarly, quantification of cell number after a 5-day exposure showed a similar number of cells in the vehicle and tamoxifen treated cultures, while the testosterone and anastrazole treated cultures were significantly increased relative to the vehicle (**Fig. 4.7B).**

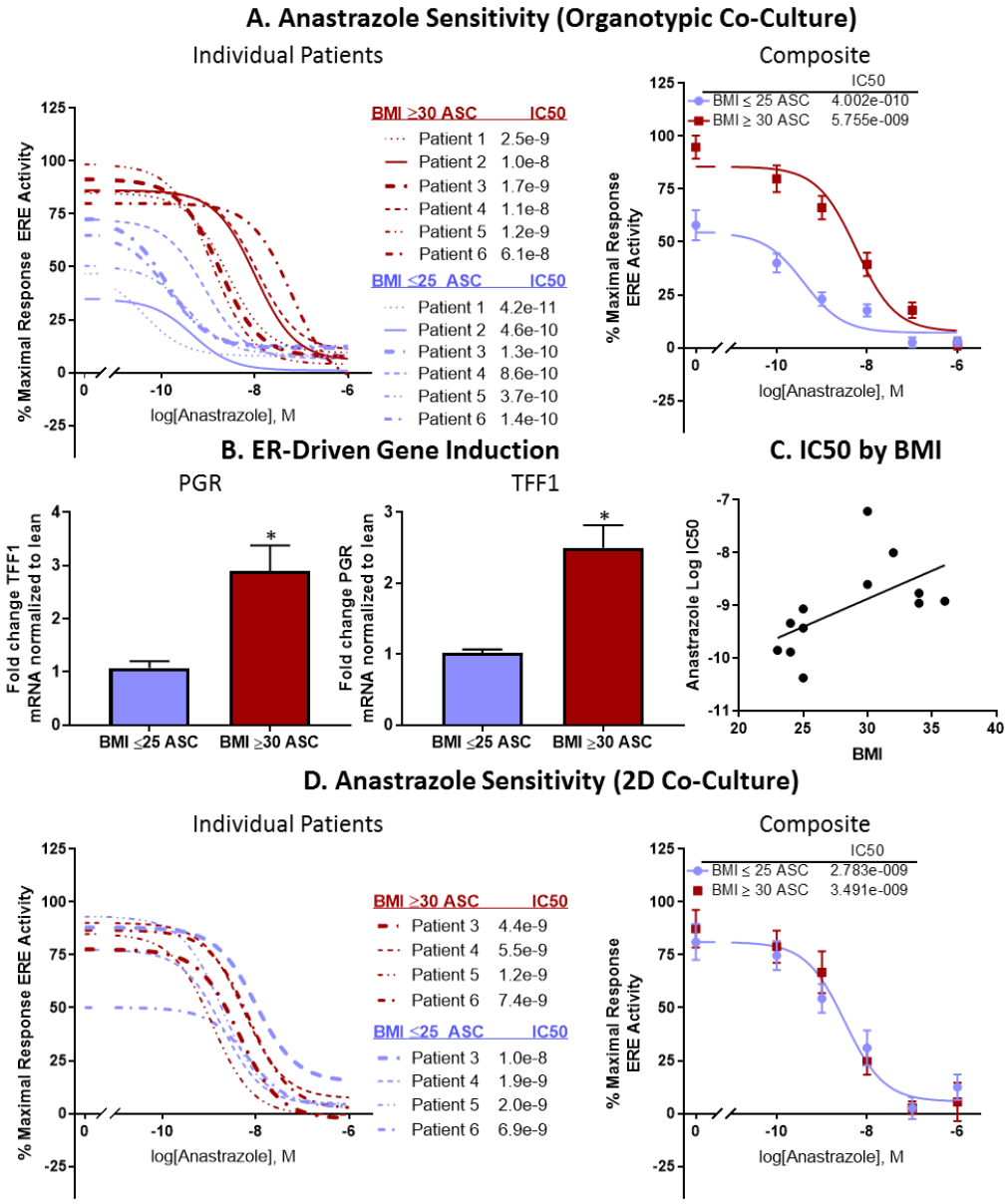


**Fig. 4.6. MCF7-derived ducts co-cultured with stromal cells derived from obese individuals are more resistant to anastrazole treatment. (A)** MCF7-derived ducts co-cultured with lean or obese mammary stromal cells in the presence of 10 nM T were exposed to five concentrations of anastrazole for 48 hours then evaluated for ER transactivation. Right panel shows individual patients and left panel shows patients merged into a single curve. **(B)** The vehicle treated (e.g. no anastrazole but exposed to 10 nM of testosterone) cultures of panel D were lysed and evaluated for ER-driven genes TFF1 and PGR. **(C)** Patient BMI was graphed against their correlating anastrazole IC50. **(D)** MCF7 cells co-cultured with lean or obese mammary stromal cells in a 2D well plate in the presence of 10 nM T were exposed to five concentrations of anastrazole for 48 hours then evaluated for ER transactivation. Right panel shows individual patients and left panel shows patients merged into a single curve.

## 4.4 Discussion

The effectiveness of aromatase inhibitors may vary depending on adiposity, although the mechanisms are not fully understood. Using an organotypic mammary model, we found that adipose stromal cells converted testosterone to estrogen via aromatase and induced ER-driven responses proliferation and hyperplasia in breast cancer cells, and that testosterone metabolism and anastrozole resistance were dependent on the concentration of adipose stromal cells. We also examined how sensitivity to the aromatase inhibitor anastrazole differs in lean and obese women. We provided *in vitro* evidence that anastrazole is less effective in obese individuals compared to lean individuals. Importantly, when MCF7 cells were cultured with adipose stromal cells in a conventional 2D co-culture system, we did not detect differences in anastrazole sensitivity of obese and lean patients. This data supports the use of organotypic models for future *in vitro* breast cancer studies and introduces an *in vitro* system that can be used to study the mechanisms of aromatase inhibitor resistance. Together, this data suggests that patient-specific responses to hormone therapies can be modeled *in vitro*, and that tamoxifen may be a more effective treatment for obese women.

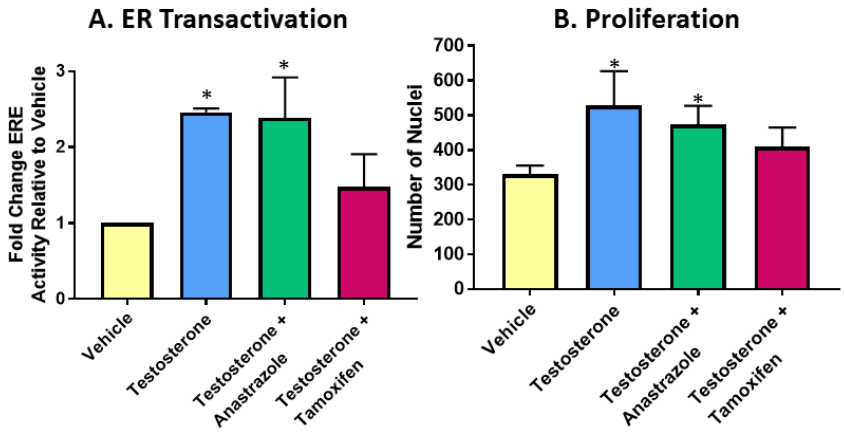


**Fig. 4.7. Tamoxifen is more effective than anastrazole at reducing T-induced ER transactivation and proliferation.** MCF7-derived ducts cultured with obese stromal cells were exposed to a vehicle control, 10 nM testosterone, 10 nM testosterone with 1 nM anastrazole, or 10 nM testosterone with 1 nM of the active metabolite of tamoxifen, 4-hydroxytamoxifen. **(A)** After 48 hours, ER transactivation was evaluated. **(B)** After 5 days, cultures were fixed and evaluated for nuclei.

While testosterone is an AR ligand and AR has been shown previously to influence the growth of breast cancer cells [501; 507], our data suggests that the effects induced by testosterone were not induced by AR, and instead occurred through ER. If some of the effects of testosterone were mediated through AR, we would expect that testosterone should have induced responses in the MCF7 monocultures; however, we did not observe significant responses. Breast epithelial cells produce little aromatase except for in very advanced breast cancers [195; 508], suggesting that testosterone was not metabolized to estrogen when MCF7s are grown alone. When co-cultured with AdMSCs, testosterone exposure induced ER transactivation and increased proliferation in MCF7 cells. This is in agreement with a previous study that co-cultured adipose stromal cells with ER+ breast cancer cells in 2D, which revealed that adipose stromal cells metabolize testosterone to estrogen and induce proliferation in breast cancer cells [501]. We also found that induction of aromatase via dexamethasone increased testosterone-induced ER transactivation, and inhibition of aromatase via anastrazole prevented testosterone-induced ER transactivation, suggesting that the effects observed were related to the metabolism of testosterone to17β-estradiol via aromatase. When estrogen signaling was antagonized via tamoxifen, testosterone had no effect on ER transactivation or proliferation. In addition, exposure to the non-aromatizable androgen DHT had no effect on ER transactivation or proliferation. Together, these results suggest that testosterone was metabolized to estrogen via aromatase and exerted effects through ER. Our data also validates that AdMSCs can metabolize testosterone to 17β-estradiol and are thus a commercially available cell line that can be used for aromatase studies.

Previous studies have speculated that increased adipose volume, and consequently, increased number of adipose stromal cells, in the breast tissue of obese individuals contributes to aromatase inhibitor resistance [158]. Our finding that the conversion of testosterone to 17β-estradiol and anastrazole sensitivity are dependent on the number of AdMSCs supports the idea that the number of adipose stromal cells can influence aromatase concentration. These findings also suggest that the ratio of

adipose stromal cells to cancer cells should be optimized when conducting aromatase experiments, as too few or too many adipose stromal cells may complicate detection of aromatase inhibitors or inducers, respectively. However, clinical studies have found that aromatase expression and activity increases with BMI [162; 251], and *in vitro* and *in vivo* studies have reported increased CYP19A1 mRNA and activity in obese stromal cells compared to lean stromal cells [159; 250]. In concordance with these studies, we found that stromal cells derived from obese individuals exhibited increased aromatase mRNA compared to stromal cells derived from lean individuals. We have expanded upon these studies to demonstrate that the mammary stromal cells of obese and lean women differently affect aromatase-driven responses in breast cancer cells *in vitro*. Specifically, we showed that obese stromal cells conferred higher rates of aromatization-induced ER transactivation and decreased sensitivity to the aromatase inhibitor anastrazole, as indicated by an increased IC50. We suspect that the increased aromatase expression is likely responsible for the increased testosterone-induced maximal ER transactivation in the obese co-cultures, as well as the decreased sensitivity to anastrazole; we hypothesize that the heightened aromatase expression in obese cultures increased the conversion of testosterone to 17β-estradiol. This hypothesis is supported by clinical data that found that aromatase inhibitors are less effective at reducing serum estrogen levels in obese women than in lean women [253; 509]. Together, these findings support the hypothesis that aromatase inhibitor resistance is mediated by changes in the mammary stroma and introduce an *in vitro* method that can be used to study the mechanisms responsible for aromatase inhibitor resistance.

Our finding that the increased testosterone-induced ER transactivation observed in the obese compared with lean cultures is diminished at later passages is consistent with studies that have reported changes in the molecular and functional profiles of primary cells that are cultured for several passages [68; 69; 70]. This data underscores the challenges with using primary cells and highlights the importance of using freshly isolated cells.

A major obstacle to studying the mechanisms of drug resistance is that traditional *in vitro* models poorly recapitulate *in vivo* biology. Several authors have argued that increasing the physiological relevance of *in vitro* platforms may improve the ability to predict drug responses [76; 467; 510]. In support of this, we found that a 2D co-culture system did not segregate the anastrazole responses of lean and obese patients as did our organotypic culture platform. These findings are important as they suggest that some aspect of the organotypic culture is needed to recapitulate *in vivo* responses to aromatase inhibitors. Several variables that differ between the platforms, including material (PDMS vs. polystyrene), culture volume (5 uL vs 40 uL), matrix proteins (collagen vs. plastic), confluency (confluent vs. not confluent) and structure (lumen vs. flat surface), have been previously shown to influence the behavior of stromal cells and/or breast cancer cells, paracrine signaling and/or drug sensitivity. For instance, microfluidic systems are thought to be more sensitive at detecting stromal:epithelial interactions because the higher surface area to volume ratio inherent in microfluidics increases the concentration of secreted factors [119]. Previous studies from our lab and others have reported striking differences in cell phenotype and behavior when cells are cultured in conventional 2D platforms, compared to when cultured in 3D matrices and/or organotypic models [92; 482; 511; 512]. Therefore, we suspect that the organotypic co-culture platform modulated the function of the stromal and/or epithelial cells, which enabled the model to recapitulate the differences in anastrazole resistance in obese and lean patients. Additional studies are needed to clarify the mechanism(s) responsible for the different anastrazole responses observed in the 2D and organotypic system.

One limitation of the study was that due to sample availability, these experiments were conducted using normal cells from premenopausal women. To better understand the interactions between obesity and aromatase inhibitor resistance in breast cancer, future studies could integrate the stroma of postmenopausal breast cancer patients. The proposed study should also include individuals of varying ethnicities, as in this study all patients who reported their ethnicity were non-Hispanic white.

We suspect that a similar trend will be observed, as a previous study that included women of various ethnicities found that BMI correlated with increased aromatase expression in both pre- and post-menopausal women [251].

Multiple clinical trials have reported a reduced efficacy of anastrazole in obese breast cancer patients compared to lean breast cancer patients, suggesting that higher doses or alternative therapies may be beneficial for overweight women [248]. However, body weight is not considered when choosing therapies for ER+ breast cancer patients [496]. In contrast to aromatase inhibitors, clinical evidence suggests that other breast cancer therapies such as tamoxifen [513] are not influenced by BMI. Our data supports this hypothesis, as we found that tamoxifen was more effective than anastrazole at reducing aromatization-induced ER transactivation and proliferation. While our preliminary results are promising, a prospective clinical study is needed to verify if the MCF7-derived co-culture model predicts responses to hormone therapies.

Altogether, this study adds to evidence that suggests obese individuals may be less responsive to anastrazole compared to lean patients. These findings support the notion that body weight may be a useful parameter when choosing therapies for ER+ breast cancer patients [247; 249], and suggest that patient-specific responses to hormone therapies can be modeled and studied *in vitro*.

## 4.5 Acknowledgments

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equity in Onexio Biosystems L.L.C. MM is an employee at Salus Discovery where she has received compensation.

## Chapter 5: The effect of the culture environment on the toxicity of tributyltin chloride

Exposure to environmental chemicals is a major preventable factor that contributes to breast cancer risk. However, standard methods to test the effect of chemicals on breast cancer risk *in vitro* focus solely on the breast cancer cell, and neglect to consider other components of the mammary microenvironment. We hypothesized that evaluating chemicals in a traditional *in vitro* platform would lead to an oversimplification of chemical toxicity, as chemicals could target stromal cells, disrupt stromal:epithelial interactions, or affect hormone metabolism. To this end, we compared how the organotin compound tributyltin chloride (TBT) influences ER transactivation in MCF7 breast cancer cells grown in a 2D monoculture, in co-culture with human adipose stromal cells in 2D, or in co-culture with human adipose stromal cells in an organotypic platform. TBT was dosed in a hormone-deprived environment, and in the presence of testosterone. Short term exposure to TBT induced mRNA aromatase expression in adipose stromal cells, which is the enzyme that metabolizes testosterone to estradiol. However, TBT also inhibited the induction of ER-driven genes. Consequently, depending on the culture condition, TBT had no effect, reduced, or inhibited ER transactivation. As previous studies showed that TBT increases the differentiation of murine pre-adipocytes into adipocytes, we hypothesized that chronic exposure to TBT would indirectly reduce aromatase mRNA expression in human adipose stromal cells because mature adipocytes do not express aromatase. Adipose stromal cells exposed to TBT for 12 days exhibited increased lipogenesis and reduced aromatase expression. Altogether, this study demonstrates the challenges associated with identifying what model systems to use when screening chemicals, as the complex actions of chemicals can be overlooked when evaluated in simple *in vitro* platforms.

## 5.1 Introduction

While inherited genetic factors are estimated to account for 5-25% of breast cancers, the remainder of cases are thought to have a multifactorial pathogenesis involving genetics, lifestyle choices, and environmental factors[514; 515]. *In vivo* and *in vitro* studies have found that some chemicals increase the proliferation of breast cancer cells[5], and epidemiological data has correlated serum levels of multiple chemicals to an increased breast cancer risk[4; 5]. However, most common environmental chemicals and consumer goods have not been evaluated for effects on breast cancer progression.

In recent years, both the scientific community and government agencies have called for the evaluation of high-risk environmental chemicals, with initial screens focusing on evaluating chemical effects *in vitro*[221; 516]. Typically, assays that evaluate breast cancer risk *in vitro* monitor chemical effects on breast cancer cells cultured in isolation on a 2D plastic surface, and do not include aspects of the mammary microenvironment. Though valuable, these platforms are inherently unable to assess how breast cancer progression is affected by chemicals that target stromal cells or disrupt stromal epithelial interactions[517]. Likewise, cell signaling differs when cells are cultured on plastic as compared to when cells are cultured in platforms that incorporate biologically relevant geometries and/or matrices[502; 518; 519]. To address these shortcomings, researchers have developed *in vitro* platforms that incorporate stromal cells, biologically relevant geometries, and/or matrix proteins[116; 469; 501]. As expected, when chemical hits are evaluated in more complex *in vitro* platforms, their effects can vary dramatically compared to when evaluated in a traditional *in vitro* model, leading to conflicting reports on how chemicals affect disease progression. The associated challenge is that we lack strategies to pinpoint which chemical hits are predictive of *in vivo* responses, and consequently are unaware of which *in vitro* models should be used to test chemicals.

To illustrate the lack of consistency in findings across platforms and across the literature, we focus here on tributyltin chloride (TBT). TBT is an organotin compound that was once widely used in

anti-fouling paints as it is an effective biocide that prevents the growth of marine organisms on ships[520]. However, TBT was eventually banned in most countries due to its endocrine disrupting effects in several marine species. TBT is an ideal case study because it has been found to modulate multiple signaling pathways important to mammary gland physiology, however its exact effects are controversial. For example, previous studies have found that TBT increases[521; 522] or decreases[523] the estrogen receptor (ER) signaling pathway; ER signaling controls the proliferation of ER+ breast cancer cells. There are also conflicting reports on how TBT influences aromatase, which is an enzyme produced by adipose stromal cells that metabolizes testosterone to estradiol. Some studies report that TBT is an aromatase inducer[524; 525] while others report that TBT is an aromatase repressor[521; 526; 527]. TBT has also been found to increase the differentiation of murine pre-adipocytes into adipocytes as it is a potent activator of the PPARy signaling pathway[528]; interestingly, long term activation of the PPARy signaling pathway reduces aromatase activity in adipose stromal cells as mature adipocytes do not express aromatase[529; 530]. To summarize, TBT has been shown to target multiple cell types that are present in the breast cancer microenvironment and consequently, likely has conflicting effects depending on what cell types are included in a platform.

We hypothesized that TBT would differentially affect pathways relevant to breast cancer progression depending on the culture platform. We evaluated the effect of TBT on the ER+ breast cancer cell line MCF7 grown either in a 2D monoculture, a 2D co-culture with adipose stromal cells, or in an organotypic co-culture with adipose stromal cells. We also evaluated how the length of TBT exposure affected aromatase activity in adipose stromal cells. Altogether, this chapter demonstrates that chemicals can exert diverse effects on the same pathway depending on the culture platform. Further, it provides insight into the value of including the breast cancer microenvironment in chemical screening platforms and highlights the need for strategies to validate if chemical hits are predictive of *in vivo* responses.

## 5.2 Materials and Methods

**5.2.1 Chemicals.** 17β-estradiol, testosterone, rosiglitazone, fenarimol, bisphenol-A, fulvestrant, genistein, diethylstilbestrol, estrone and tributyltin chloride were bought from Sigma Aldrich (St. Louis, MO, USA) and dissolved in ethanol.

**5.2.2 Cell Culture.** Adipose derived mesenchymal stem cells, referred to as adipose stromal cells, were purchased from ATCC (Manassas, VA, USA). The MCF7s used in the paper were previously stably transfected with a construct to produce a luminescent signal when ER is activated[394]. All cell lines were maintained in high glucose DMEM (Thermo Fisher; Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; VWR, Radnor, PA) and 1% penicillin-streptomycin (Thermo Fisher, Waltham, MA, USA). For experiments evaluating ER-driven responses, 48 hours prior to experiments, cell culture flasks were washed with PBS then cells were grown in estrogen-free media, which contains phenol-red free DMEM (Thermo Fisher) with 10% charcoal-stripped FBS, 2 mM glutamine, and 1% penicillin-streptomycin. All cultures were maintained at 37C in a 5% CO2 incubator. When seeding/feeding/dosing estrogen-free experiments, estrogen-free media supplemented with fibroblast growth supplement (ScienCell #2352) was used.

**5.2.3 Generation of MCF7-derived ducts.** MCF7-derived ducts were generated as described previously[116]. Briefly, microfluidic devices were constructed from polydimethylsiloxane (PDMS; Dow Corning, Auburn, MI, USA), bonded to a glass slide, then UV sterilized. Devices were treated with 2% poly(ethyleneimine) in water (Sigma Aldrich) for 10 minutes, followed by 0.4% glutaraldehyde (Sigma Aldrich) in water for 30 minutes, and then washed three times with water. The devices were loaded with a 4.5 mg/ml neutralized collagen solution (Corning; Corning, New York, USA) that was diluted with media or stromal cells. Devices were incubated at room temperature for 10 minutes to facilitate collagen polymerization, then incubated at 37C for 30 minutes. Afterwards, a small drop of media was placed into the small port of the device and the PDMS rod in the chamber of the device was removed,

leaving a luminal structure. The input port was loaded with 1.5 ul of a cell solution containing 50,000 MCF7s/ul. Devices were flipped every 20 minutes over a 100-minute period, then media in the devices was refreshed. Experiments were dosed the morning after seeding, and cells were fed daily.

**5.2.4 Seeding of 2D ER Transactivation experiments.** 2D experiments were performed in 96 well plates. For 2D monoculture experiments, MCF7 cells were seeded at 10,000 cells/well. For 2D co-culture experiments, adipose stromal cells were seeded at 7,500 cells/well then allowed to adhere for 2 hours. Then, MCF7 cells were seeded on top of the adipose stromal cells at 7,500 cells/well. Experiments were dosed the morning following seeding and replenished daily.

**5.2.5 ER Transactivation Assay.** The MCF7s used in the model were previously transfected with a reporter placing a luciferase gene downstream of estrogen response element in the vitellogenin gene; therefore, the luciferase produced by the cells is linear to ER transactivation[394]. Drugs were dosed in media containing 2 mM of the endotoxin-free luciferin VivoGlo (Promega, Madison, WI, USA), which cleaves luciferase producing a luminescent signal. A Biorad imager was used to image luminescence produced in the organotypic cultures, and a Pherastar plate reader was used to record luminescence produced in well plates.

**5.2.6 RT-qPCR.** For RT-qPCR experiments, MCF7 cells and adipose stromal cells were each seeded at 10,000 cells per well of a 96 well plate. RNA was isolated from cells grown on 96 well plate using the Dynabeads mRNA Direct Purification Kit (Thermo Fisher). Reverse transcription and PCR were performed using Taqman Fast Virus 1-Step Master Mix (Thermo Fisher) along with the following Taqman primers: TFF1 (Hs00907239_m1), PGR (Hs01556702_m1), CYP19A1 (Hs00903411_m1), PPARγ (Hs01115513_m1), HPRT (Hs02800695_m1) and PO (Hs99999902_m1). Expression of experimental genes was calculated using ΔΔCT method and was normalized to housekeeping genes HPRT and PO.

**5.2.7 Adipocyte Differentiation.** Cells were seeded in 96 well plates at confluency (15,000 cells/well) in DMEM with 10% FBS and 1% penicillin-streptomycin then cultured for an additional 48 hours to

induce senescence. Media was then replaced with Preadipocyte Differentiation Medium (ScienCell, Carlsbad, CA, USA) along with the test chemical—vehicle (0.1% ethanol), TBT, or rosiglitazone at the indicated concentrations—for 48 hours. Then media was replaced with Adipocyte Medium (ScienCell) containing each test compound and incubated for 10 days. Media was refreshed every 2-3 days and cultures were differentiated for 12 days.

**5.2.8 Oil Red O Staining.** Oil Red O (Sigma Aldrich) was dissolved in 2-proponol to a concentration of 0.5% weight by volume. To create a working solution, the stock solution was diluted 2:3 in deioinized water then filtered with a 0.2-micron filter. Cells were fixed with 4% paraformaldehyde (Alfa Aesar, Tewksbury, MA, USA), washed with PBS twice, then incubated with the working solution of Oil Red O at room temperature for 30 minutes. The wells were then washed with deionized water three times. A Pherastar plate reader was used to quantify absorbance at 510 nm.

**5.2.9 Statistics.** Error bars in all graphs represent standard error and significance is defined as $p < 0.05$. A one-way ANOVA was used to determine significance in experiments that compared more than two conditions.

## 5.3 Results

### 5.3.1 Organotypic model can detect lowly estrogenic compounds

To study the effect of TBT in a platform that more closely recapitulates the physiology of the mammary gland, we used an organotypic model consisting of a ductal structure lined with ER+ MCF7 breast cancer cells surrounded by a collagen matrix containing primary adipose stromal cells. Adipose stromal cells were included as we showed in Chapter 4 that they produce aromatase, the enzyme that metabolizes testosterone to estradiol.

We have used the organotypic model previously to study potent ER agonists[116] and aromatase modulators (Chapter 4), but have not used the system to evaluate weak ER agonists. To confirm that weakly estrogenic compounds can induce ER-driven responses in the MCF7-derived duct model, we

exposed the model to 1000 nM of chemicals with varying levels of estrogenic activity (listed from least to greatest): fenarimol, bisphenol-A, genistein, estrone (E1), and 17β-estradiol (E2), and evaluated ER transactivation after 48 hours. Relative to vehicle, fenarimol, bisphenol-A, and genistein increased ER transactivation approximately 2.5-fold, while E1 and E2 increased ER transactivation 3.8-fold and 4.5-fold, respectively **(Fig. 5.1A)**. Next, proliferation was evaluated by staining for the proliferation marker, Ki67. While the percentage of Ki67 positive cells was increased by all chemicals, it was only significant for E1 and E2 **(Fig. 5.1B)**.

### 5.3.2 Organotypic model is more sensitive to ER ligands than 2D monoculture

To investigate the influence of the culture environment on the response to ER ligands, we characterized MCF7 response to a strong ER agonist, diethylstilbestrol (DES), as well as to a weak ER agonist, fenarimol, in a 2D monoculture and in the organotypic co-culture model. Researchers typically evaluate ER ligands in an *in vitro* environment devoid of hormones. While this approach increases sensitivity to estrogens, it is not physiologically relevant. Levels of testosterone in post-menopausal women range from 0.6-2.5 nM[531], therefore we performed experiments in both hormone-deprived conditions as well as in the presence of 1 nM testosterone.

DES acted as an ER agonist when cells were grown in a hormone-deprived environment in both culture platforms, although the relative fold change was higher in the organotypic platform. When co-exposed with testosterone, DES exhibited a reduced potency in the organotypic platform while the potency was unchanged in the 2D platform **(Fig. 5.1C)**. Fenarimol also acted as an ER agonist in both culture platforms, but its potency was reduced in the presence of testosterone in the organotypic model **(Fig. 5.1D)**.

### 5.3.3 Exposure to tributyltin chloride antagonizes ER in breast cancer cells and induces aromatase expression in adipose stromal cells

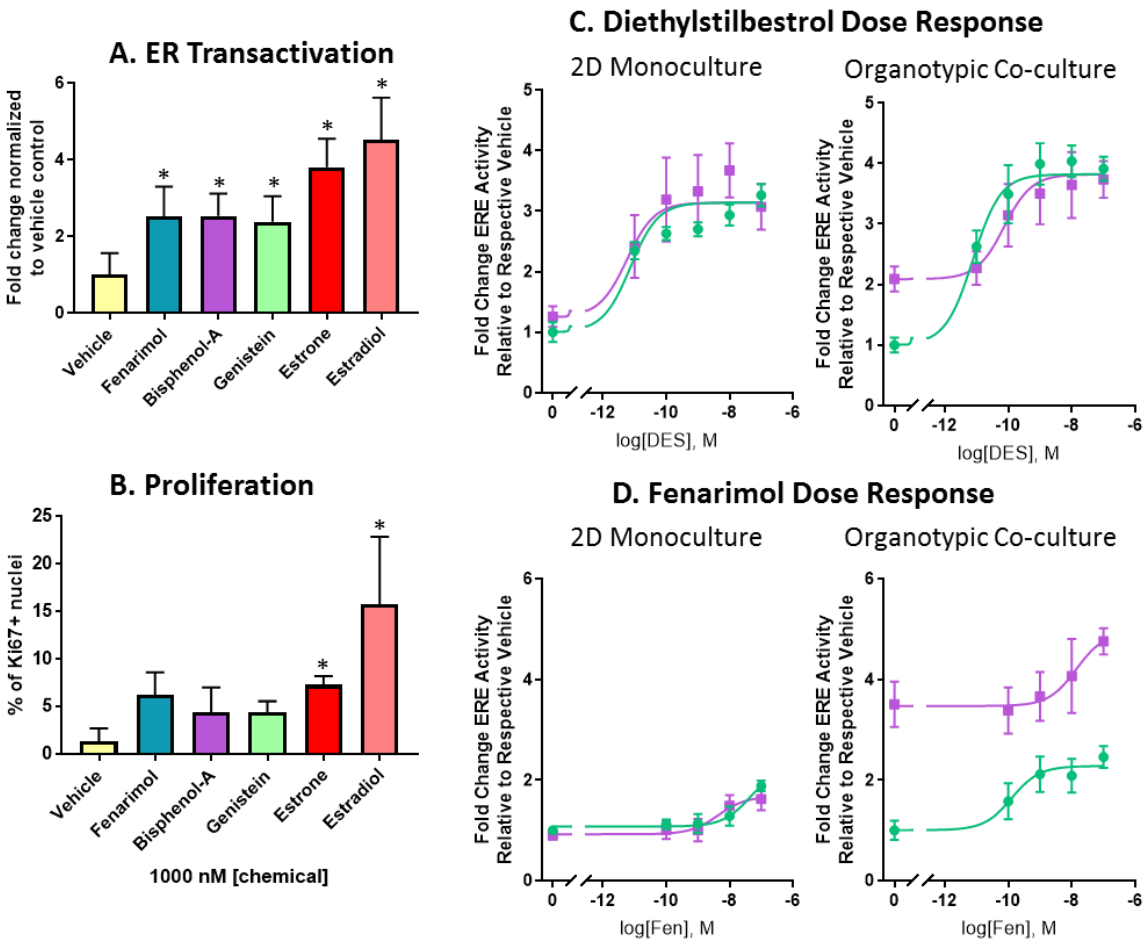


**Figure 5.1. MCF7 cells are more sensitive to ER ligands inducing ER transactivation when grown in an organotypic co-culture.** MCF7s grown in an organotypic co-culture system were exposed to a vehicle control, fenarimol, bisphenol-A, genistein, estrone, or estradiol for 48 hours, then evaluated for **(A)** ER transactivation and **(B)** proliferation. ER transactivation was measured by quantifying the luminescence produced by the cells, as the MCF7s used were stably transfected with a reporter that produces luminescent signal when EREs are activated. Proliferation was evaluated by measuring the number of nuclei positive for the proliferation marker, Ki67. MCF7s were cultured as a 2D monoculture or in the organotypic co-culture platform in the presence and absence of testosterone then evaluated for ER transactivation after exposed to **(C)** diethylstilbestrol and **(D)** fenarimol for 48 hours. The green curves show the test chemicals exposed in the absence of testosterone, while the purple curves show the test chemicals exposed in the presence of 1 nM of testosterone.

As previous reports have found that chemical effects differ depending on the culture platform, we tested if TBT would differentially affect ER transactivation when MCF7 cells were grown as a 2D monoculture, a 2D co-culture or in an organotypic co-culture. TBT had no effect on ER transactivation when MCF7s were grown as a 2D monoculture, in the presence or absence of testosterone. However,

when grown in both the 2D co-culture and the organotypic co-culture model in the absence of testosterone, TBT acted as an ER antagonist. When exposed in the presence of testosterone, TBT increased ER transactivation in a dose-dependent manner **(Fig. 5.2)**. These results suggest that TBT is both an aromatase inducer and an ER antagonist.

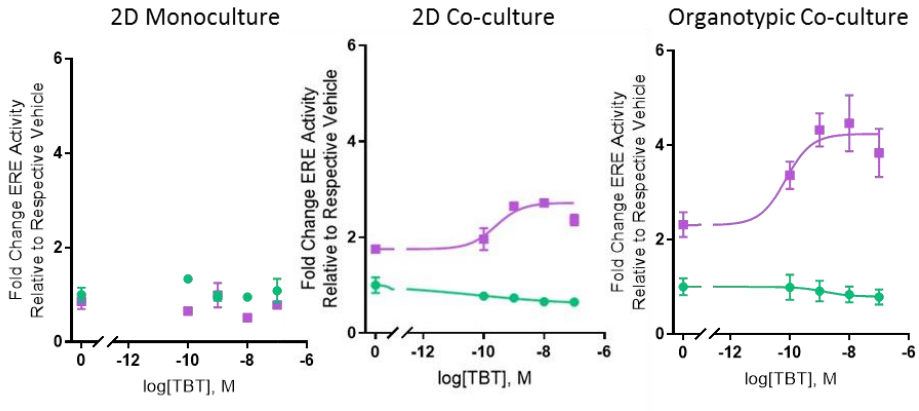


**Figure 5.2. TBT differently affects ER transactivation depending on the culture platform.** MCF7s grown as a 2D monoculture, a 2D co-culture, or an organotypic co-culture platform in the absence and presence of testosterone were exposed to tributyltin chloride for 48 hours, then evaluated for ER transactivation. The green curves show the test chemicals exposed in the absence of testosterone, while the purple curves show the test chemicals exposed in the presence of 1 nM of testosterone.

To confirm that TBT is an ER antagonist, MCF7 cells were grown in 2D and exposed to: a vehicle control, estradiol, estradiol with the potent ER antagonist fulvestrant (ICI), or estradiol with 10, 100, or 1000 nM TBT. After 24 hours, the expression of ER-driven genes TFF1 **(Fig. 5.3A)** and PGR **(Fig. 5.3B)** was assessed. As expected, estradiol induced TFF1 3-fold and PGR 10-fold, and co-treatment with ICI completely inhibited this effect. TBT inhibited the induction of ER-driven genes in a dose-dependent manner.

To evaluate the ability of TBT to induce aromatase expression, adipose stromal cells were grown in 2D then exposed to the potent aromatase inducer dexamethasone (100 nM) or TBT at 10, 100, or 1000 nM for 24 hours then evaluated for aromatase (CYP19A1) mRNA. As expected, dexamethasone significantly induced aromatase expression and TBT had a dose-dependent effect on CYP19A1 mRNA expression **(Fig 5.3C)**.

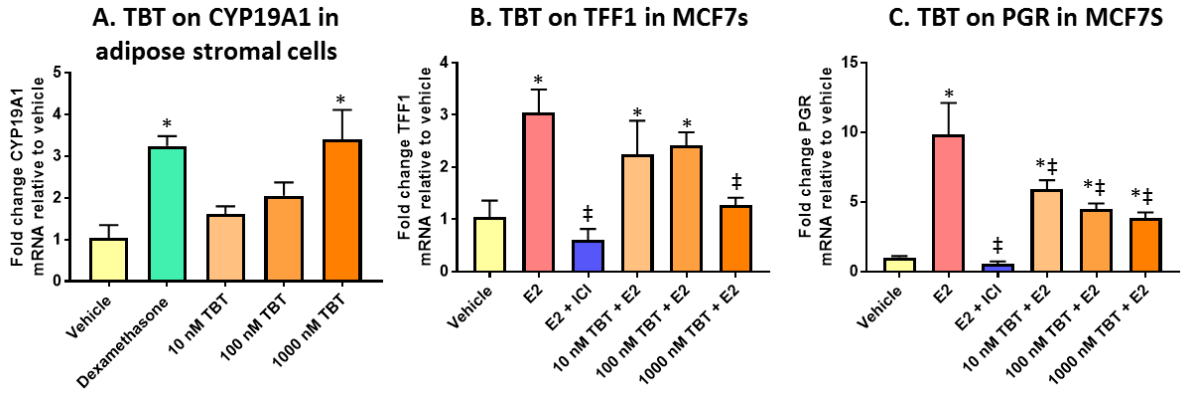


**Figure 5.3. TBT inhibits estradiol-induced gene expression and increases aromatase mRNA expression.** MCF7s were exposed to a vehicle control, E2, E2 and fulvestrant (ICI), or E2 and TBT then evaluated for the mRNA expression of **(A)** TFF1 and **(B)** PGR. **(C)** Adipose stromal cells were exposed to a vehicle control, the known aromatase inducer dexamethasone, or TBT then evaluated for CYP19A1 mRNA. * = vs. vehicle ($p < 0.05$); ‡ = vs. estradiol ($p < 0.05$)

### 5.3.4 Chronic exposure to tributyltin chloride increases adipocyte differentiation, and reduces aromatase expression

As described above, TBT has previously been shown to increase lipogenesis in murine pre-adipocytes by activating PPARγ[528]. Mature adipocytes do not produce aromatase[532], so we hypothesized that chronic exposure to TBT would indirectly reduce aromatase expression through differentiation of pre-adipocytes into adipocytes. As the majority of data that found TBT to be obesogenic was collected from studies that used mouse models or cells from fetal mice[520], we first evaluated if TBT is obesogenic towards adipose stromal cells derived from mature humans. Lipid production was measured after a 12-day exposure to 1 uM of a known obesogenic compound (rosiglitazone), as well as to 10 and 100 nM of TBT. Exposure to rosiglitazone and 100 nM TBT significantly increased lipid production, 2.8-fold and 1.9-

fold, respectively (**Fig. 5.4A**, **Fig. 5.4B**)**.** Next, the mRNA expression of aromatase and PPARy were measured in adipocytes after a 12-day exposure to rosiglitazone or 10 nM and 100 nM TBT. Exposure to TBT and rosiglitazone significantly decreased the expression of CYP19A1 **(Fig. 5.4C)** and increased the expression of PPARy **(Fig. 5.4D)**, which was consistent with our hypothesis that long-term exposure to TBT would induce PPARy, and thereby increase lipogenesis and reduce aromatase.

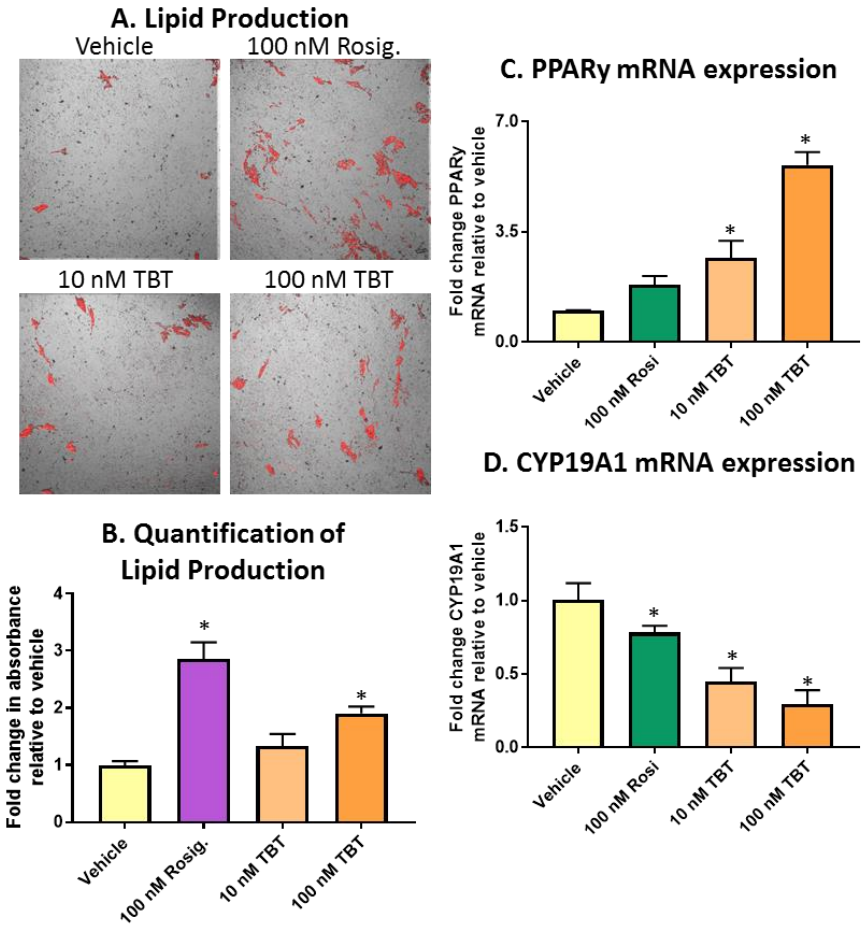


**Figure 5.4**. **TBT increases lipogenesis and reduces aromatase expression in adipocytes.** After adipose stromal cells were differentiated for 12 days in the presence of a vehicle control, rosiglitazone, 10 nM tributyltin chloride, or 100 nM tributyltin chloride, lipid production was evaluated by conducting an oil red o assay. **(A)** shows representative images while **(B)** shows quantification. Adipocytes were lysed and evaluated for **(C)** PPARy and **(D)** CYP19A1 mRNA.

## 5.4 Discussion

In this chapter, we evaluated how the organotin biocide, tributyltin chloride, differentially affects ER transactivation in breast cancer cells, as well as aromatase expression in adipose stromal cells. We found that TBT activated multiple pathways that are relevant to breast cancer progression, all of which can directly or indirectly have effects on the activation of ER.

In the absence of testosterone, TBT had no effect on the 2D monoculture but acted as an ER antagonist in the 2D co-culture and organotypic co-culture platforms. When researchers evaluate the ability of a compound to antagonize ER, cultures are exposed to estradiol or another ER agonist because ER is generally not activated in the absence of a ligand. However, multiple studies have found that fibroblasts can activate ER in the absence of a ligand[116; 126]; therefore, we suspect that the ability of the co-culture platforms to detect antagonistic effects in the absence of an ER ligand was due to the ligand-independent activation of ER. As MCF7s do not express aromatase[501], we were not surprised that TBT also had no effect in the 2D monoculture when exposed in the presence of testosterone. In contrast, when MCF7 cells were exposed to TBT in the presence of testosterone when cultured in the 2D co-culture platform and the organotypic co-culture platform, TBT increased ER transactivation in a dose-dependent manner. This data, as well as qPCR data confirming that TBT increased CYP19A1 mRNA expression in adipose stromal cells, implicate TBT as an aromatase inducer. Finally, our findings suggest that the 2D co-culture platform is as sufficient as the organotypic co-culture platforms for flagging compounds that affect aromatase and estrogen receptor signaling. These findings are important as the 2D co-culture system is more resource efficient than the organotypic co-culture platform and is consequently more amenable to chemical screening. Future studies that validate a broader chemical list will be needed to confirm this hypothesis.

How TBT influences aromatase differs on the species as well as cell type. For example, TBT was initially studied due its catastrophic effects on marine life; TBT induced imposex in gastropods as it

inhibited aromatase and consequently increased testosterone levels[533], and was later shown to have similar effects in zebrafish[534]. While TBT has been shown to consistently decrease aromatase activity in aquatic life, its effects on mammalian species varies. *In vivo* studies have found that TBT represses aromatase, while *in vitro* studies have found that it induces[524; 525] or inhibits aromatase expression[526; 527]. How aromatase differs in *in vitro* studies can be partially explained by the complexity of the aromatase promoter[535]. However, in addition to cell-specific differences in transcriptional regulation, the length of exposure could also explain how TBT affects aromatase induction; in this study, we found that short term exposure to TBT induced aromatase expression in adipose stromal cells, however long-term exposure to TBT decreased aromatase expression. We suspect that the reduction in aromatase expression was due to the obesogenic effect of TBT because mature adipocytes do not express aromatase[532]. In fact, multiple studies have found that aromatase expression in pre-adipocytes decreases as they differentiate into adipocytes[529; 530]. Therefore, we conclude that the discrepancies in how TBT influences aromatase expression can also be explained by the length of exposure.

Altogether, these experiments demonstrate how the effect of a chemical can differ dramatically depending on the culture environment. Specifically, we showed that TBT can have no effect, induce, or inhibit ER activation depending on the culture platform. We also found that short-term exposure to TBT induces aromatase expression, while long term exposure reduces aromatase expression. This data underscores the challenges associated with identifying what platform to use when testing chemicals, as chemicals can have complex effects that can only be uncovered if evaluated in multiple platforms. Moving forward, we must develop strategies to help identify what culture conditions chemicals should be tested in, as well as approaches to translate how contradictive chemical hits relate to *in vivo* biology.

## 5.4 Acknowledgements

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## Chapter 6. Concluding Remarks and Future Directions

This document focuses on investigating the impact of the mammary microenvironment on how breast cancer cells respond to chemicals related to the estrogen receptor (ER) signaling pathway. In Chapter 1, we overviewed the need for more complex *in vitro* models for chemical testing, described how the microenvironment influences the behavior and chemical responses of breast cancer cells, and introduced estrogen signaling and the chemicals that target ER. In Chapter 2, we discussed the technical evolution of *in vitro* models that are used to predict how patients respond to cancer therapies and described how the adverse outcome pathway (AOP) framework can be used to help build predictive *in vitro* assays. There, we developed the estrogen receptor (ER) positive breast cancer AOP and identified culture conditions and readouts that are important when testing the effect of chemicals on ER+ breast cancer. In Chapter 3, we utilized the suggestions outlined by the AOP to develop a hormonally responsive organotypic breast model that consists of a ductal structure lined with ER+ MCF7 cells. We found that inclusion of immortalized mammary fibroblasts reduced ER protein and increased ER transactivation in breast cancer cells, and increased estrogen-induced hyperplasia by reducing apoptosis. In Chapter 4, we showed that integration of primary adipose stromal cells into the organotypic platform enabled the study of hormone metabolism, as adipose stromal cells metabolize testosterone to estrogen through their production of aromatase. Using this system, we demonstrated that resistance to the breast cancer therapy, anastrazole, differs when cancer cells are grown in the presence of stromal cells derived from lean or obese women. Importantly, we observed no difference in anastrazole resistance in obese and lean women when breast cancer cells were co-cultured with adipose stromal cells in a conventional 2D platform. Finally, Chapter 5 showed that the organotin compound, tributyltin chloride, differently affected ER transactivation when exposed in a monoculture platform, compared to a 2D or organotypic co-culture platform. Altogether, this dissertation demonstrates that the presence of the microenvironment *in vitro* leads to a more complete picture of chemical effects,

suggesting that stromal cells should be included in platforms when evaluating breast cancer cell responses to chemicals. This work has also showed that the ideal model is context dependent; while in some scenarios a simple, 2D *in vitro* model is sufficient for predicting chemical effects, there are other cases where a co-culture model or an organotypic co-culture model is needed. Future studies are needed to better define the scenarios where more complex *in vitro* model systems are needed to recapitulate *in vivo* responses.

### 6.1 Evaluation of additional breast cancer therapies in an expanded patient cohort

In Chapter 5 we showed that breast cancer cells co-cultured with stromal cells derived from obese women were less sensitive to the aromatase inhibitor anastrazole, compared to when co-cultured with stromal cells derived from lean women. While these findings are promising, future studies are needed to confirm the predictive value of the assay. For example, due to patient availability, all patient samples were derived from pre-menopausal women. Currently, anastrozole is only used to treat post-menopausal women. In addition, the study assessed only 6 lean women and 6 obese women, who were all non-Hispanic. Therefore, future studies should use an increased sample size consisting of post-menopausal women of varying ethnicities. The samples were also collected from women who did not have breast cancer. While future studies should certainly use stromal cells derived from breast cancer patients, we suspect that when we use stromal cells derived from breast cancer patients, we will see greater heterogeneity in therapeutic response.

Other breast cancer therapies should also be assessed. While we did compare the effectiveness of tamoxifen and anastazole, we evaluated only one dose and did not compare sensitivity to tamoxifen between obese and lean patients. Letrozole is a more potent aromatase inhibitor than anastrazole and has been shown to be more effective at suppressing estrogen levels than anastrazole [253]. Therefore, the effectiveness of letrozole could also be assessed.

### 6.2 Evaluation of mechanism(s) responsible for increased predictability of organotypic system

As described in Chapter 1, there is a delicate balance between the complexity and simplicity of a model system. When models are simple, chemical mechanisms are straightforward to decipher and the costs and labor requirements are low. However, simple models often fail to predict *in vivo* responses. Ideally, researchers would test chemicals in models that maintain a level of simplicity but are capable of recapitulating *in vivo* responses; unfortunately, the factors that make models predictive are usually unknown. For example, in Chapter 4 we found that the organotypic co-culture model was able to model anastrazole sensitivity in obese and lean women, but the 2D co-culture could not. There were many differences in the 2D co-culture model and organotypic co-culture model that may be responsible, such as cell confluency, culture volume, platform material, presence of a matrix, and presence of a ductal structure. Therefore, future studies could compare anastrazole sensitivity in MCF7 cells cultured with stromal cells derived from lean or obese patients, in various culture conditions. These studies would help pinpoint which factor(s) are responsible for the increased predictability of the organotypic system, and consequently help design a predictive *in vitro* system that is simpler than the organotypic co-culture system but still predictive of *in vivo* responses.

### 6.3 Comprehensive assessment of how chemical hits differ in various culture environments

In Chapter 5, we showed that a single chemical can target pathways present in multiple cell types of the mammary gland and can consequently induce different responses if exposed to cells grown in monoculture, compared to in co-culture with other cell types. Specifically, we demonstrated that the organotin compound tributyltin chloride (TBT) targets the estrogen, aromatase, and lipogenesis pathways, which are active in breast cancer cells, adipose stromal cells, and adipocytes, respectively. This data suggests that conventional screening platforms may over- or understate chemical hits, as environmental chemicals are generally screened using simple *in silico* or *in vitro* models that generally consider one pathway at a time. We argue that co-culture models are especially valuable for understanding the effect of chemicals that hit multiple pathways.

Using publicly available databases such as Toxcast or DSSTox, future studies could identify chemicals that have been shown to interact with the aromatase, lipogenesis, and or/estrogen signaling pathways. The chemicals could be screened in different culture conditions: for example, in monoculture, or in co-culture with immature or mature adipocytes. The results from this study could further bolster the claim that more complex *in vitro* models are needed to validate hits of chemicals that interact with multiple pathways.

While we have focused here on evaluating how the microenvironment affects chemical responses in ER+ breast cancer cells, future studies could also consider other breast cancer subtypes and/or different biological pathways and expand the kinds of readouts. We expect that the importance of each component of the microenvironment will differ depending on the breast cancer subtype and pathway being targeted.

## Appendix A: Integration of primary mammary epithelial cells into organotypic mammary duct model

The majority of work described in this dissertation was done using MCF7 cells, which is an immortalized cell line. While immortalized cell lines are convenient as they can replicate indefinitely and are easy to culture, accumulating evidence suggests that immortalized cell lines poorly recapitulate the tumor of origin[286; 287]. Towards increasing the physiological relevance of our organotypic system, we enriched for primary mammary epithelial cells derived from women undergoing normal reduction mammoplasties (also referred to as HMECs) and evaluated their behavior and estrogen responsiveness when grown in the organotypic mammary duct model.

A major hindrance to using primary mammary epithelial cells is they rapidly lose estrogen receptor (ER) expression when plated *in vitro*. However, a recent paper found that inclusion of TGFB inhibitors in culture medium is sufficient to maintain ER in primary mammary epithelial cells[187]. To evaluate if we could maintain ER in HMECs using the same method, ESR1 mRNA expression was evaluated in HMECs grown in 2D after TGFβ inhibitors SB431542 and RepSox was maintained or excluded from culture media for 24 hours. In agreement with previous findings, HMECs grown in the presence of TGFB inhibitors exhibited a 2-fold higher expression of ESR1 mRNA [data not shown]. Therefore, all studies that evaluated HMECs included TGFB inhibitors in the culture media.

As previous studies found that the culture environment regulates the gene profiles of cells, we hypothesized that HMECs would differ in breast cancer-related genes when grown in 2D, compared to when grown as biomimetic ducts. HMECs were grown in 2D or in ductal structures for 48 hours then evaluated for the expression of ESR1, which encodes ER protein, as well as SPCA1 and SPCA2, which are two calcium pump genes associated with increased calcification found in DCIS[536]. In both patients evaluated, expression of ESR1, SPCA1, and SPCA2 were increased when HMECs were grown as luminal

structures **(Fig. A1)**. These findings add to accumulating evidence that implicates tissue structure as a regulator of cell behavior.

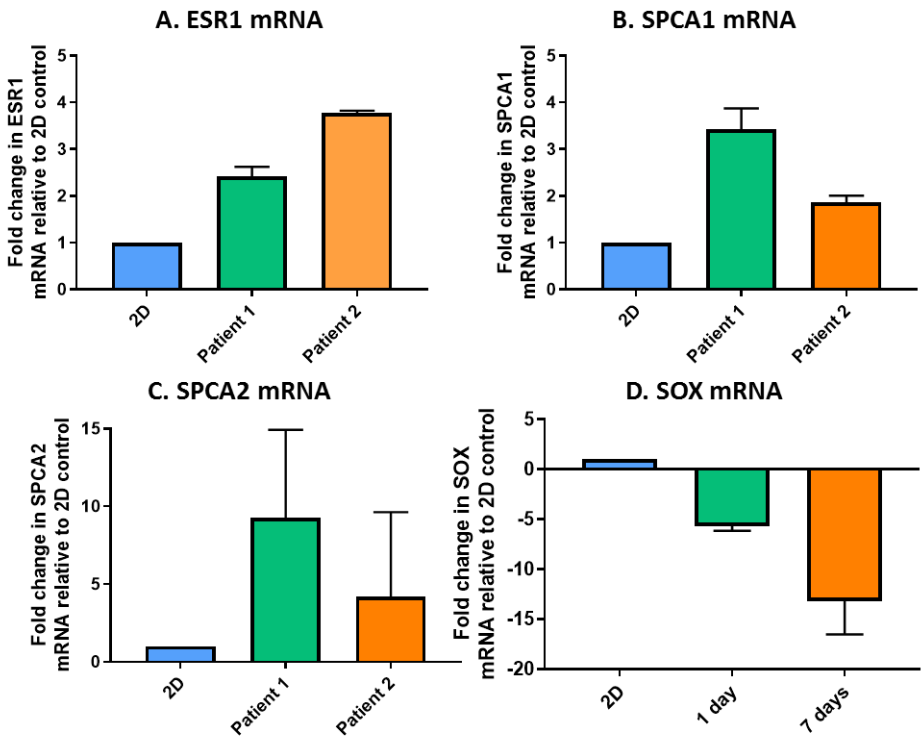



**Figure A.1. The expression of breast cancer-related genes differs in HMECs grown in 2D vs. as biomimetic ducts.** The expression of breast cancer related genes **(A)** ESR1 **(B)** SPCA1 **(C)** SPCA2 were evaluated after HMECs were cultured in 2D or in biomimetic ducts for 48 hours. The data shows two different patients, where each patient was normalized to their own 2D control. **(D)** The mRNA expression of SOX was evaluated after HMECs were cultured in 2D or biomimetic ducts for 24 hours or 7 days. Data is normalized to the 24-hour time 2D timepoint.

Next, we hypothesized that the organotypic culture may select for some types of cells. The expression of the stem-related gene, SOX, was compared when HMECs were grown in 2D for 2 days, in lumens for 2 days, or in lumens for 7 days. HMECs grown in ductal structures expressed 6 log2-fold less SOX mRNA compared to HMECs grown in 2D. After 7 days, the expression of SOX was 13 log2-fold less compared to the mRNA of HMECs grown in 2D for 2 days **(Fig. A2)**. One explanation for the decrease in *SOX* is that the organotypic cultures induce stem-like epithelial cells to differentiate into other cell types, such as basal or luminal epithelial cells. Alternatively, the organotypic cultures may induce the proliferation of SOX- cells or inhibit the growth or SOX+ cells. While SOX expression was even more

decreased at the later time point, we would need to conduct further experiments to determine if this was due to the organotypic culture, or to the length of time the cells were grown; we only had the 2 day time point to compare to, which is not ideal because the gene expression profiles of primary cells might have changed due to time in culture. A more informative comparison would be to compare HMECs grown in organotypic cultures for 7 days compared to HMECs grown in 2D for 7 days.

To evaluate estrogen responsiveness, HMEC-derived ducts were exposed to a vehicle control or the potent estrogen, 17B-estradiol then evaluated for the expression of ER-driven genes *ESR1* and *PGR*. Estradiol decreased *ESR1* mRNA **(Fig. A.2A)** and increased *PGR* mRNA **(Fig. A.2B)**. To further validate that estradiol was including effects through ER, HMEC-derived ducts were exposed to a vehicle control, estradiol, or estradiol and 4-hydroxytamoxifen, which is the active form of the ER antagonist tamoxifen. After 48-hour exposure, cultures were fixed then stained for ER protein and the proliferation marker, Ki67. Similar to normal human breast tissue *(Clarke, 1997; Shoker, 1999a)*, cells rarely co-expressed Ki67 and ER (representative image shown in **Fig. A.2C**), and approximately 10-20% of epithelial cells expressed ER. While estrogen decreased ER protein in HMEC-derived ducts, co-treatment with tamoxifen and estrogen increased ER protein **(Fig. A.2D)**. The percentage of Ki67+ cells decreased with estrogen treatment, while co-treatment with tamoxifen had no effect **(Fig. A.2E)**. This was surprising, as estrogen typically increases the proliferation of ER+ breast cells. We hypothesize that the inclusion of the TGFB inhibitor could have changed the dynamics of estrogen signaling, or that the difference could be due to the cells being normal, and not cancerous.

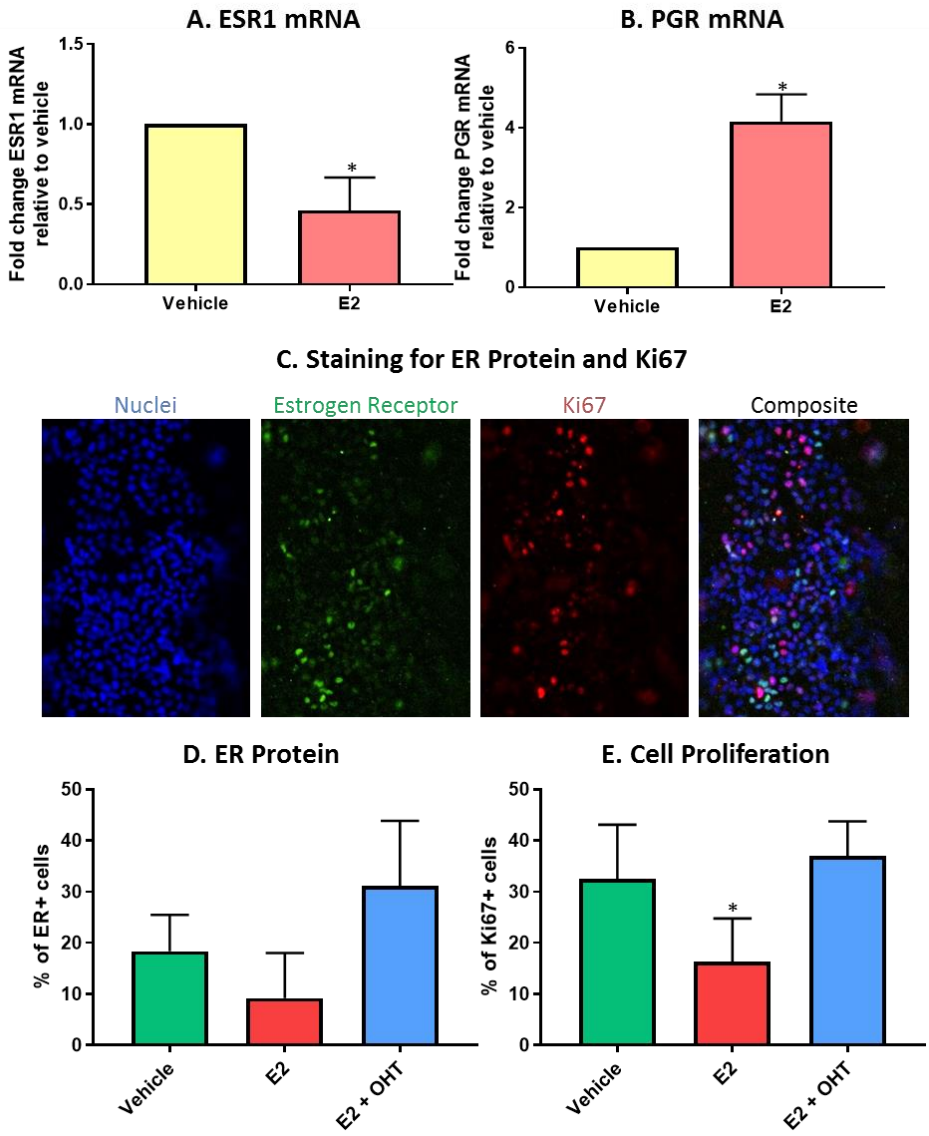



**Figure A.2. Biomimetic ducts lined with HMECs are estrogen responsive.** The expression of ER-driven genes **(A)** ESR1 and **(B)** PGR were evaluated after HMEC-derived ducts were exposed to a vehicle control or estradiol for 24 hours. After a 48 hour exposure to a vehicle control, estradiol, or estradiol and the ER antagonist 4-hydroxytamoxifen (OHT), cultures were stained for nuclei (blue), estrogen receptor protein (green), and the proliferation marker Ki67 (red). **(C)** shows a representative image, while **(D)** and **(E)** show the percentage of Ki67+ cells and ER+ cells, respectively.

Next, we hypothesized that we would observe phenotypic changes in the ductal structures when grown with different ratios of breast cancer cells to normal mammary epithelial cells. Ductal structures were lined with HMEC cells, MCF7 cells, or a 1:10 mixture of MCF7 cells and HMEC cells. The mixed cell cultures developed hyperplastic nodules after only 24 hours of culture, while both the MCF7 and HMEC ducts were lined with a single layer of cells **(Fig. A.3)**.

Altogether, this data demonstrates that primary normal $ER^+$ breast epithelial cells grown as ductal structures are estrogen responsive and phenotypically similar to normal breast tissue. We also showed that HMECs grown as ductal structures differ in breast cancer-related genes compared to HMECs grown in 2D. Future studies could use the model could be used to explore mechanisms of estrogen signaling in normal mammary epithelial cells, which is a poorly understood area as all ER+ breast epithelial cell lines are derived from cancerous tissue. Alternatively, the model could be used to explore the significance of organotypic culture models. The breast cancer field is often in disagreement on which culture model best recapitulates *in vivo* behaviors and primarily relies on either 2D monocultures or spheroid cultures. To provide insight into this controversy, future studies could culture cells in the lumen model, in a 3D spheroid model, or a conventional 2D monoculture at various time points, then compare the gene expression of cells in each model to one another and to the mRNA extracted from cells seeded at day zero. We have done a preliminary experiment to investigate this, where we compared the mRNA expression of HMECs grown in the lumen model, in spheroid culture, or in a conventional 2D monoculture. While the mRNA expression of P63 and CCE1 was similar between HMECs grown in spheroids and lumens, ESR1 mRNA was increased in lumens, but decreased in

spheroids **(Fig. A.4)**. These findings add to existing information that implicates the culture environment as a regulator of cell behavior.

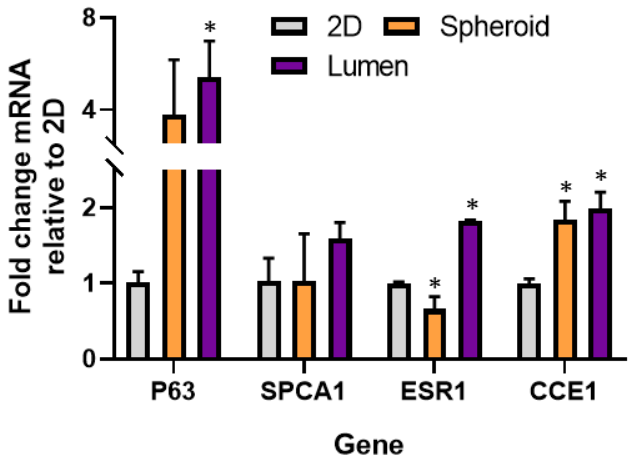



**Fig. A.4. Gene expression of HMECs differs by the culture environment.** HMEC cells were seeded on a 2D plastic surface, in the lumen platform, or in methyl cellulose spheroids. After 3 days in culture, cells were lysed and evaluated for the mRNA expression of P63, SPCA1, ESR1, and CCE1.

## Appendix B: Development of a benign and metastatic organotypic prostate model

Scientists have begun to scrutinize the stroma of the prostate microenvironment as research strongly supports the essential role that the stroma plays in prostate cancer progression. The stroma in cancerous tissues, coined the reactive stroma, has distinct pro-cancerous properties such as altered phenotypes and genotypes of stromal cells[277; 537]. The attention to this relationship has emphasized the growing need for a reliable, time efficient and physiological relevant model that can be used to investigate the interactions between cancer cells and the reactive stroma. To address this need, we have developed an organotypic microscale system that can be used to study stromal-epithelial interactions in an environment that is more physiological relevant than traditional *in vitro* approaches.

To model a benign or cancerous prostate, a biomimetic duct generated using the LumenNEXT method[1] was lined with a non-tumorigenic prostate epithelial cell line (BCaP NT) or a metastatic prostate epithelial cell line (BCaP M1). The BCaP NT and M1 cell lines belong to the BCaP cell line series that models different stages of prostate cancer[538]. After 3 days of culture, the bottom **(Fig. B.1A)** and side **(Fig. B.1B)** planes of the biomimetic ducts were imaged, which revealed that the NT-duct model formed a confluent monolayer while the M1-duct model appeared invasive and hyperplastic. To better characterize ductal morphology, the ducts were stained for F-Actin and nuclei then imaged at different z-sections then reconstructed. While both the non-tumorigenic and prostate epithelial cells formed hollow ductal structures, the cells of the NT-duct model were confined to the ducts while the cells of the M1-ducts left the lumen **(Fig. B.1C )**. Next, the ducts were embedded in agarose then cross-sectioned with a Compresstome as described previously[116].

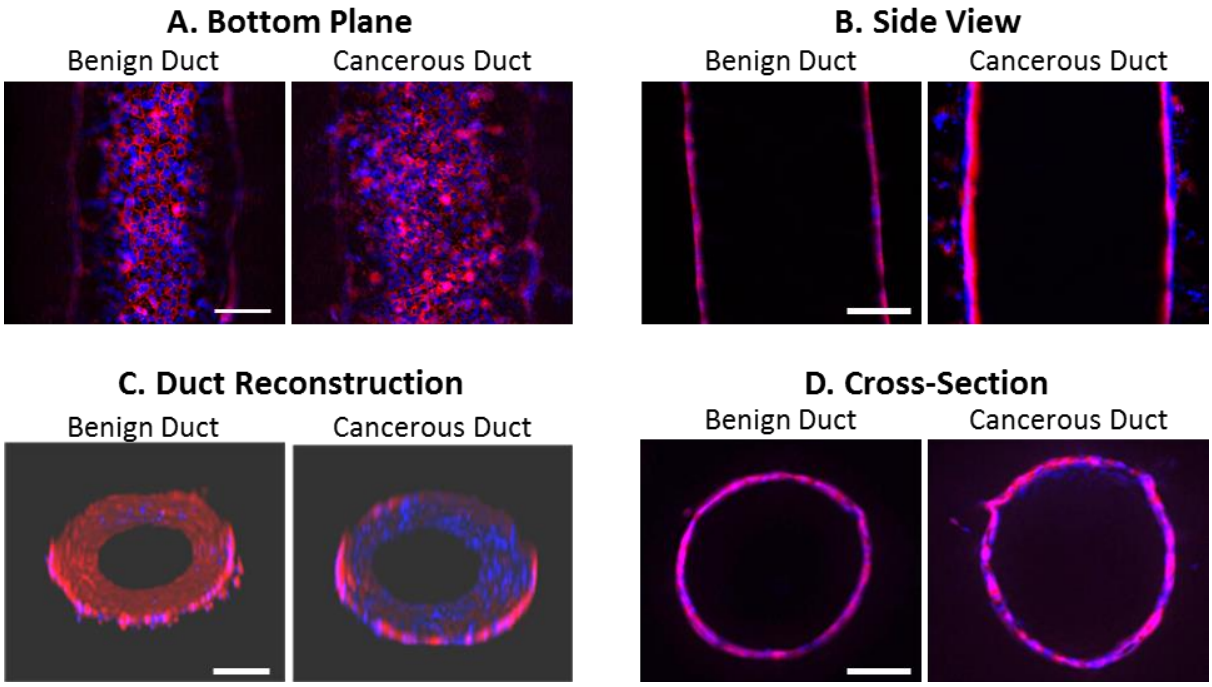



**Figure B.1 BCaP cells grown in biomimetic ducts appear phenotypically similar to benign and cancerous human prostate ducts.** All images shown are stained with Hoescht to evaluate nuclei (blue) and phalloidin to evaluate F-actin (red). **(A)** shows the bottom plane of a lumen, while **(B)** shows the side planes of a lumen. **(C)** Cultures were imaged at different z planes then reconstructed with ImageJ to visualize the ductal structure. **(D)** To cross-section the ducts, cultures were embedded in agarose then cross-sectioned with a Compresstome. Scale bars represent 100 micron.

Quantification of cell invasion confirmed that cells remained confined to the lumen in the NT-duct model, where cells of the metastatic lumen invaded into the surrounding matrix **(Fig B.2A)**. ATP production was measured to evaluate the number of viable cells in each model, which revealed increased ATP production in the M1-duct model relative to the N1-duct model **(Fig. B.2B)**. To assess if cell proliferation could account for the increased cell number, the ducts were stained for the proliferation marker, Ki67. Consistent with the CellTiter Glo data, the M1-derived ducts an increase in the percentage of Ki67+ cells (representative picture in **Fig. B.2C,** quantification shown in **Fig. B.2D**). Interestingly, 7% of epithelial cells lining the prostate duct are Ki67+, which is similar to a normal human prostate duct where approximately 5% of prostate epithelial cells are Ki67+[539].

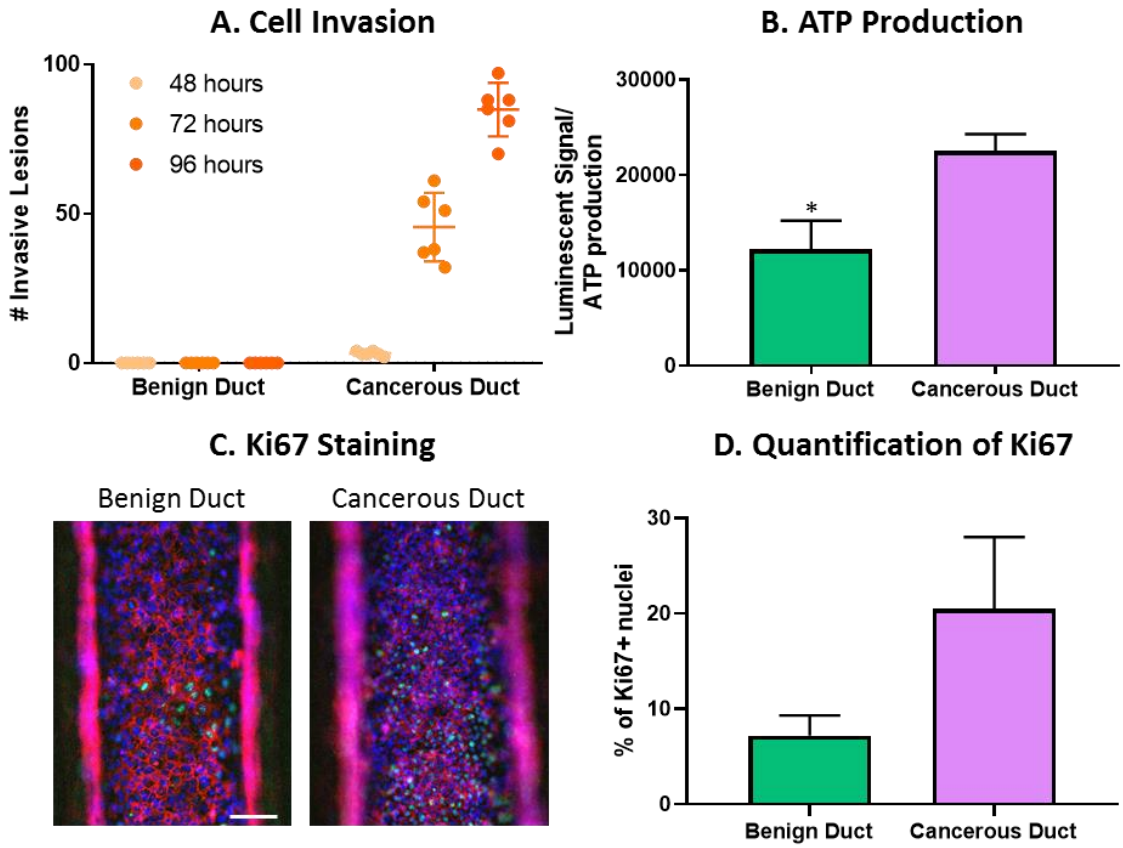



**Figure B.2. Cancerous prostate ducts are more invasive and proliferative than benign prostate ducts**. **(A)** Cell invasion was measured 48, 72, and 96 hours after seeding by counting the number of cells that invaded out of the lumen and into the surrounding. **(B)** Cell number was defined by measuring ATP production using the CellTiter Glo assay. **(C)** After 72 hours of co-culture, ducts were fixed then stained for nuclei (blue), F-Actin (red), and the proliferation marker Ki67 (green). **(D)** The percentage of Ki67+ cells was quantified by dividing the number of Ki67+ cells by the total number of nuclei.

Prostate cancer progression is characterized by a decreased expression of the tight junction marker, E-Cadherin[540]. The NT- and M1-duct models were stained for an anti-E-Cadherin antibody which revealed that cells in the M1-duct model express less E-Cadherin than cells in the NT-duct model **(Fig. B.3A)**. Another hallmark of prostate cancer is the loss of cell polarity[540]. Each model was stained for basal marker laminin V or apical marker golgi. Similar to human prostate tissue, cells in the non-tumorgenic model were polarized while the metastatic model was not polarized **(Fig. B.3B)**. As invasive breast cancer cells have been shown to rearrange the extracellular matrix[541], we hypothesized that the

collagen surrounding invasive cells of the cancerous ducts would be rearranged. We used the imaging

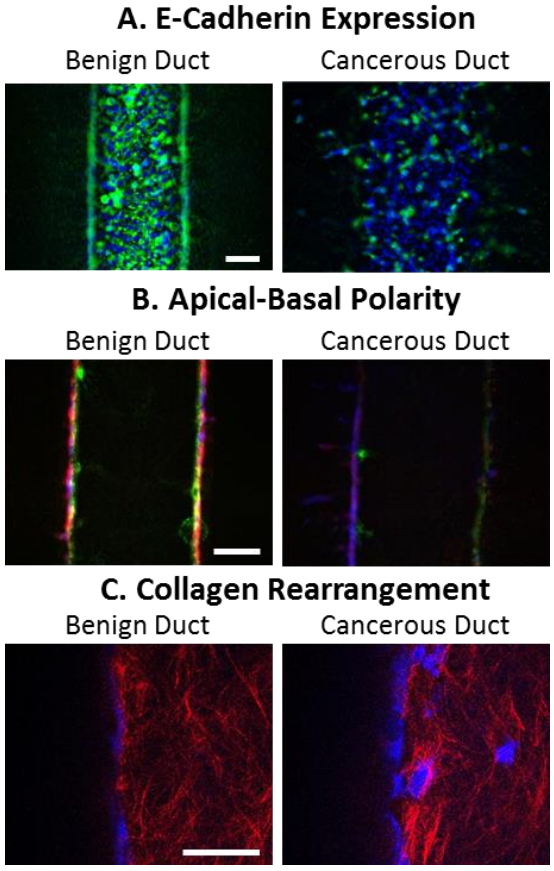



**Figure B.3. BCaP-M1 cells retain their metastatic phenotype in organotypic culture.** Benign and cancerous lumens were stained with an **(A)** E-Cadherin antibody to evaluate tight junctions, and with **(B)** antibodies against laminin-5 and golgi to evaluate basal and apical polarity, respectively. **(C)** Second Harmonic Generation was used to visualize rearrangement of collagen fibers. All cultures were stained with nuclei and scale bars represent 100 micron.

technique, second harmonic generation, to visualize the collagen fibers surrounding the biomimetic ducts. As expected, collagen density was increased in areas near invading cells **(Fig. B.3C)**.

A major advantage of the organotypic prostate duct model is the ability to integrate stromal cells into the matrix surrounding the biomimetic duct (as shown in **Fig. B.4A**), as it enables the study of stromal:epithelial interactions in a physiologically relevant environment. As prostate fibroblasts have been previously shown to regulate the invasive behavior of prostate cancer cells, we hypothesized that cancer-associated fibroblasts would increase the invasive behavior of cells in the biomimetic ducts,

while normal fibroblasts would decrease invasion or have no effect. Cell invasion was evaluated when the NT- and M1-duct models were grown in monoculture, or in co-culture with normal or cancer-associated fibroblasts. While cells were not invasive in the NT-duct model, cells branched out of the lumen when co-cultured with normal fibroblasts as well as cancer-associated fibroblasts. The degree of

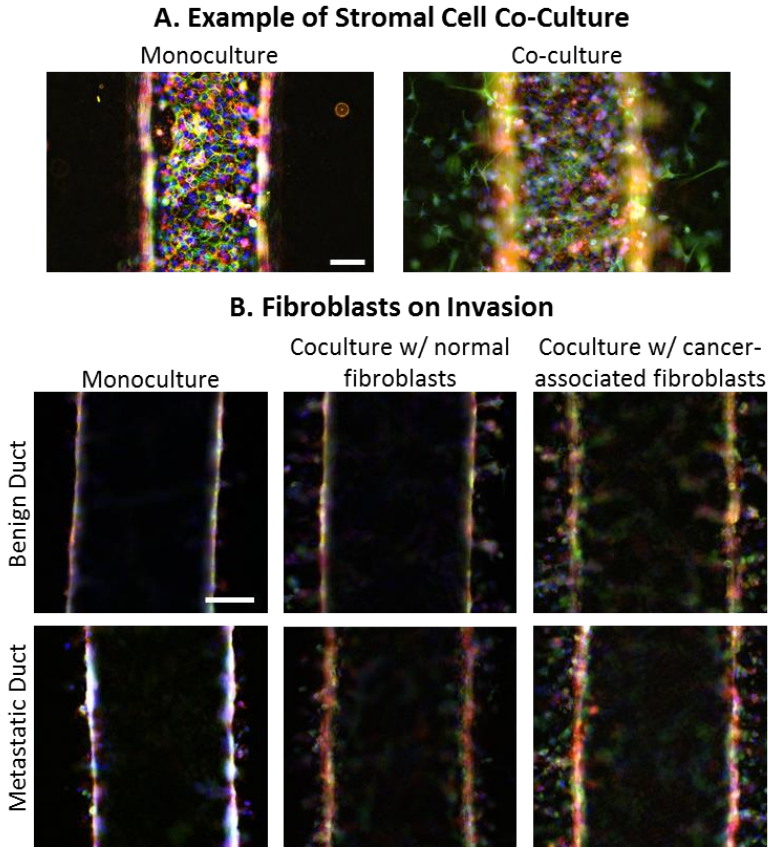



**Figure B.4. Prostate stromal cells induce functional responses in biomimetic prostate ducts. (A)** shows a picture of a benign prostate duct grown in monoculture or co-culture with prostate stromal cells. **(B)** After a 3-day co-culture, cultures were fixed then stained for nuclei (blue), F-Actin (red), and EpCAM (green).

invasiveness appeared more dramatic when co-cultured with cancer-associated fibroblasts compared to normal fibroblasts. While cell invasion was similar in the M1-ducts cultured alone or with normal fibroblasts, co-culture with cancer associated fibroblasts increased cell invasion **(Fig. B.4B)**.

To summarize, the data shown here demonstrates that the BCaP prostate epithelial cell lines NT and M1 grown in a biomimetic duct model appear phenotypically similar to a benign and metastatic

human prostate, respectively. In addition, we demonstrated that the system enables the assessment of tissue level readouts that are not possible in a traditional *in vitro* system, such as invasion, hyperplasia, and rearrangement of the extracellular matrix.

# Appendix C. Miscellaneous Illustrator Figures.

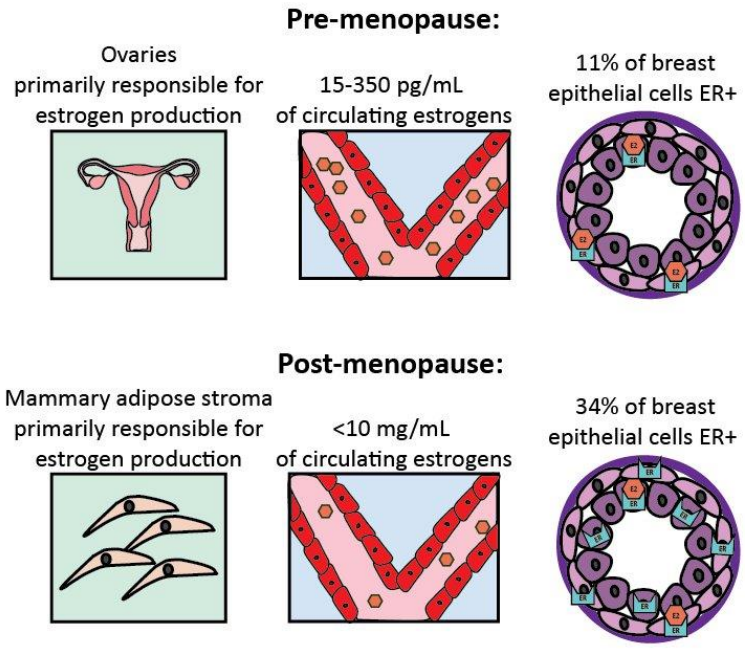



**Figure C.1. Estrogen production and ER expression before and after menopause.**

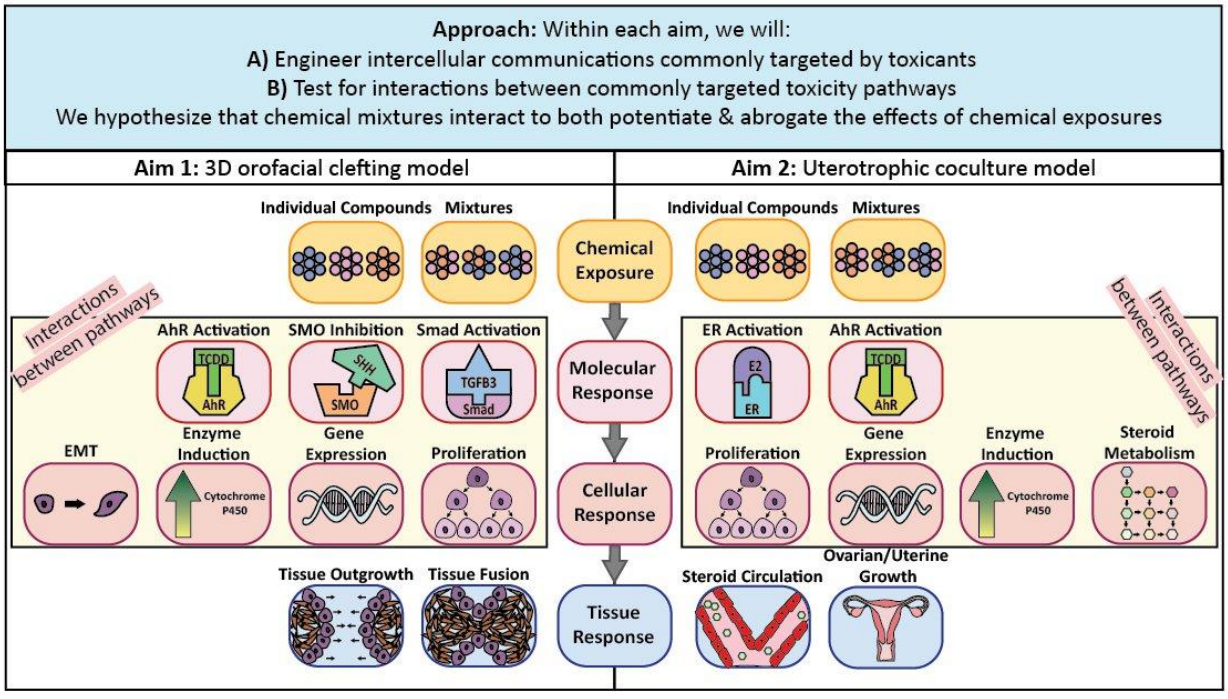



**Figure C.2. Aim overview for Brian Johnson's K99 application.**

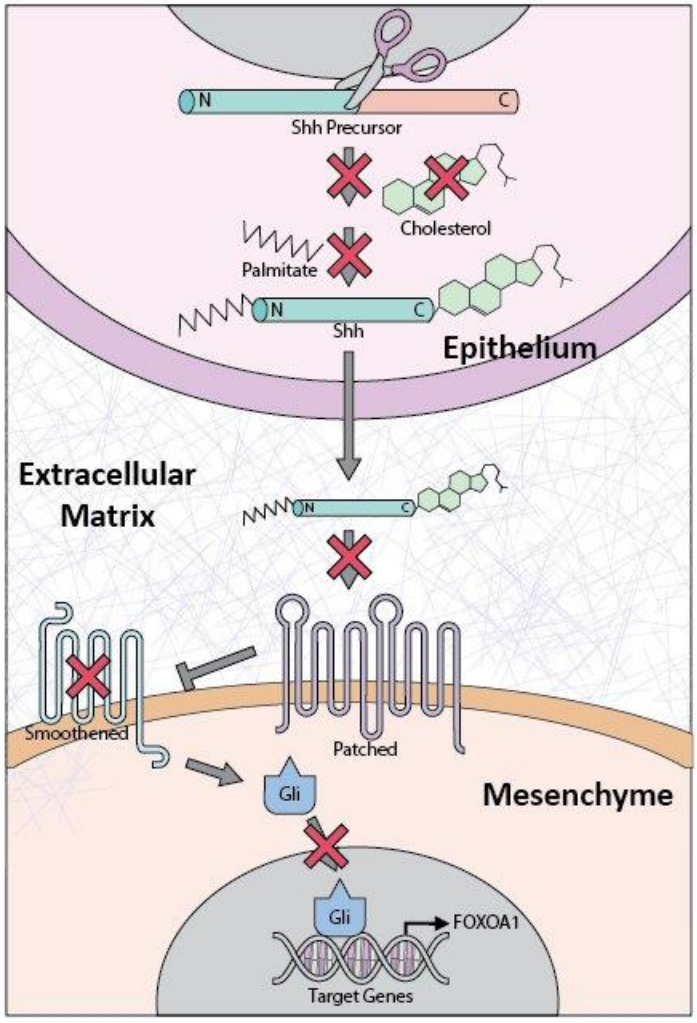



**Figure C.3. An overview of sonic hedgehog signaling in the developing palate.** Made for Brian Johnson.

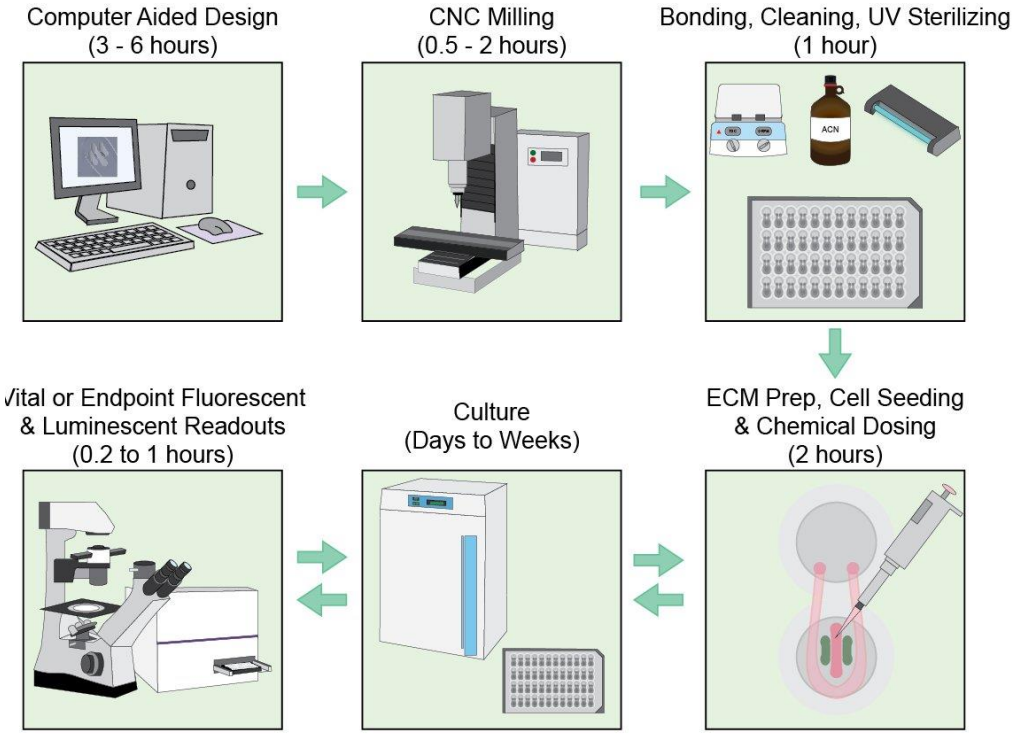



**Figure C.4. Illustration of organotypic cleft palate device construction and use.** Made for Brian Johnson.

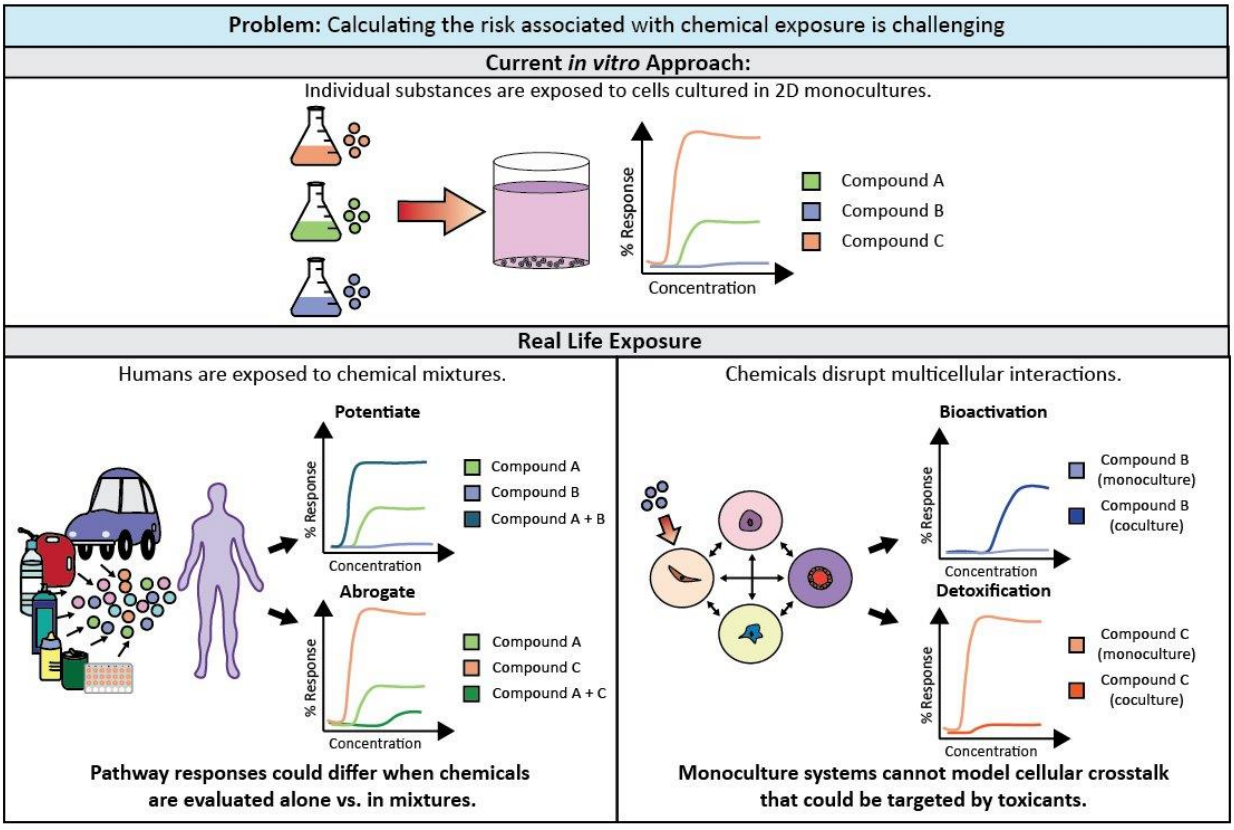


**Figure C.5. Chemical exposures *in vitro* versus in real life.** Made for Brian Johnson.

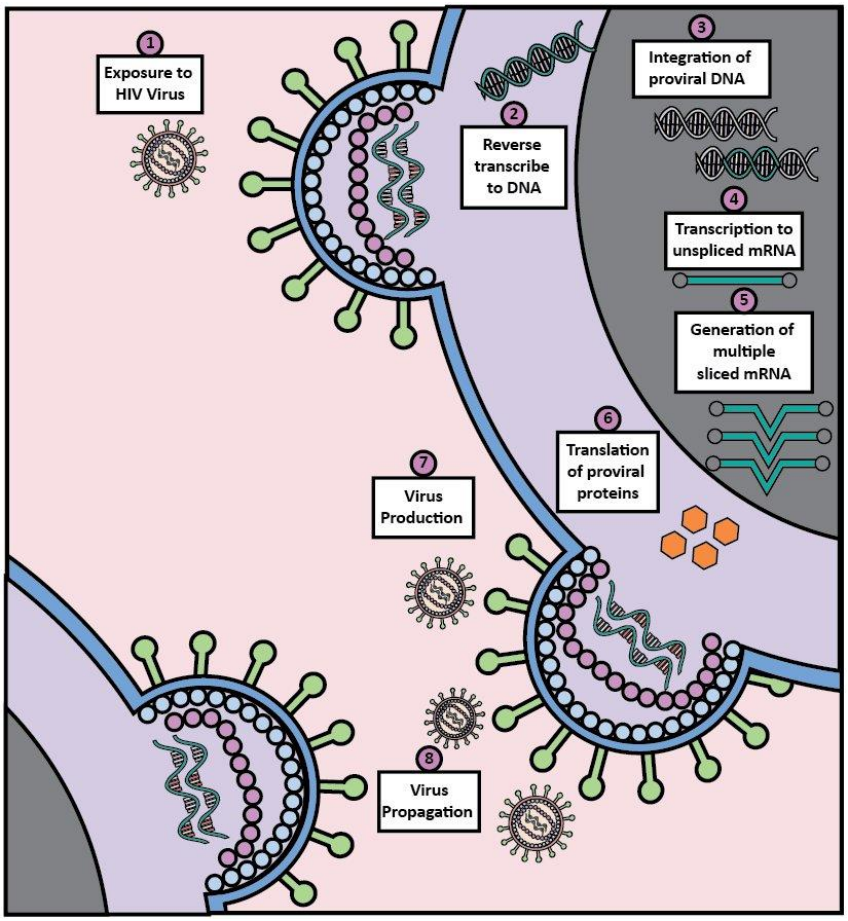



**Figure C.6. HIV virus life cycle.** Made for Hannah Pezzi.

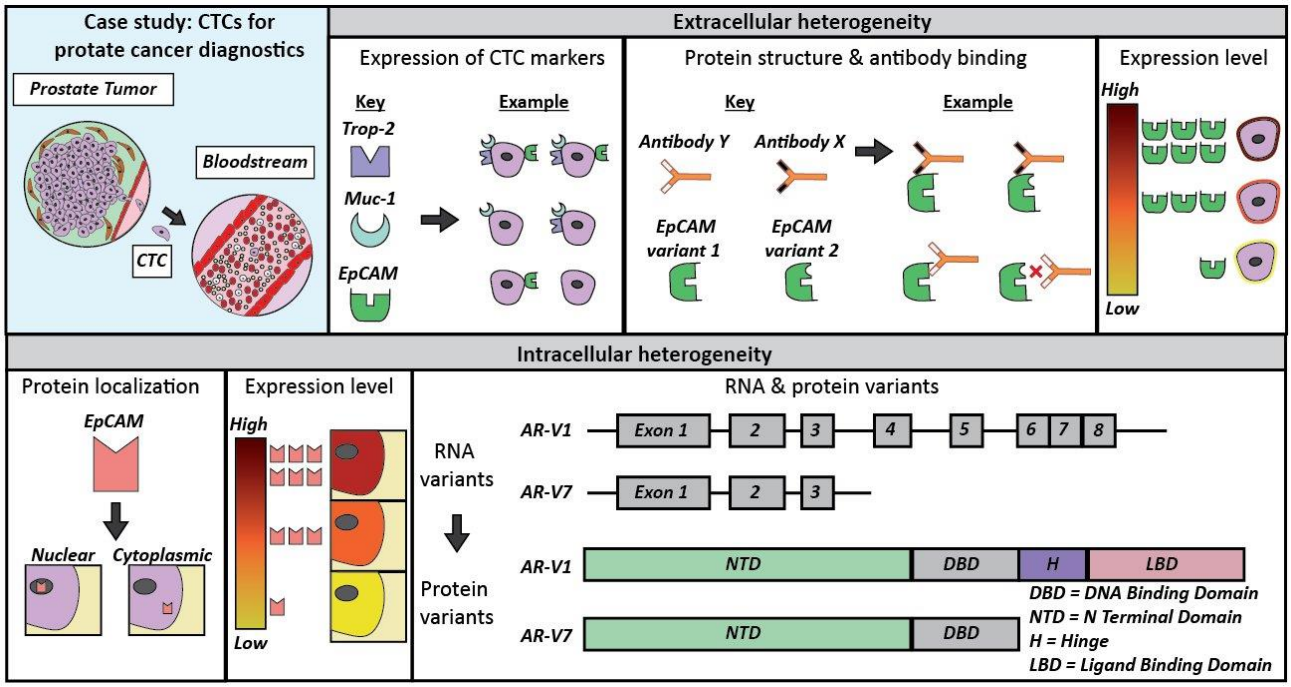



**Figure C.7. Overview of the heterogeneity observed in androgen receptor expression of prostate cancer circulating tumor cells.** Made for Hannah Pezzi.

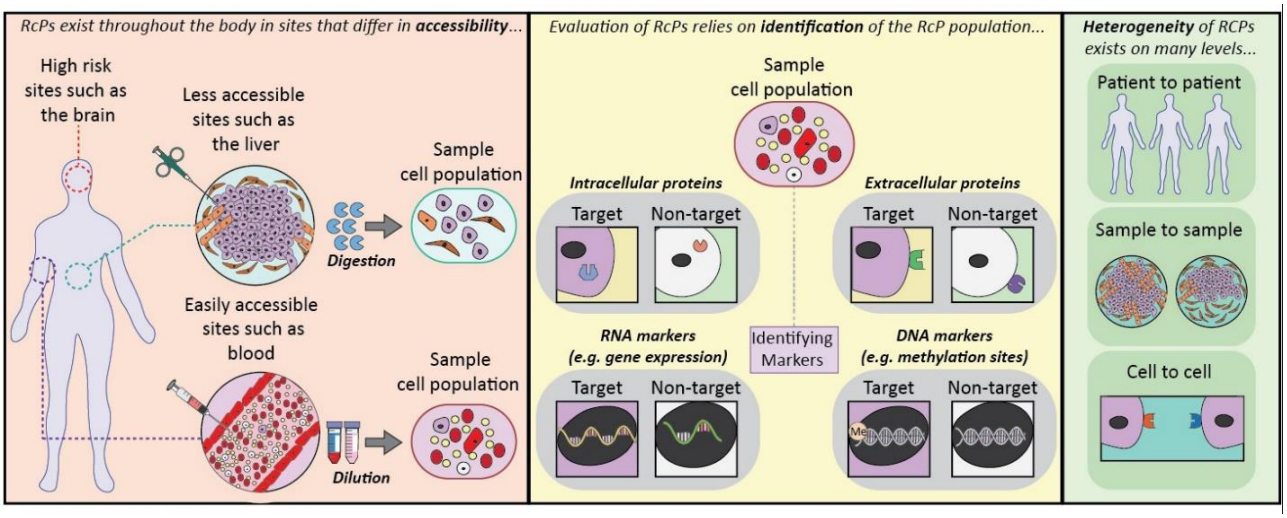



**Figure C.8. Overview of the challenges with studying rare cell populations.** Made for Hannah Pezzi.

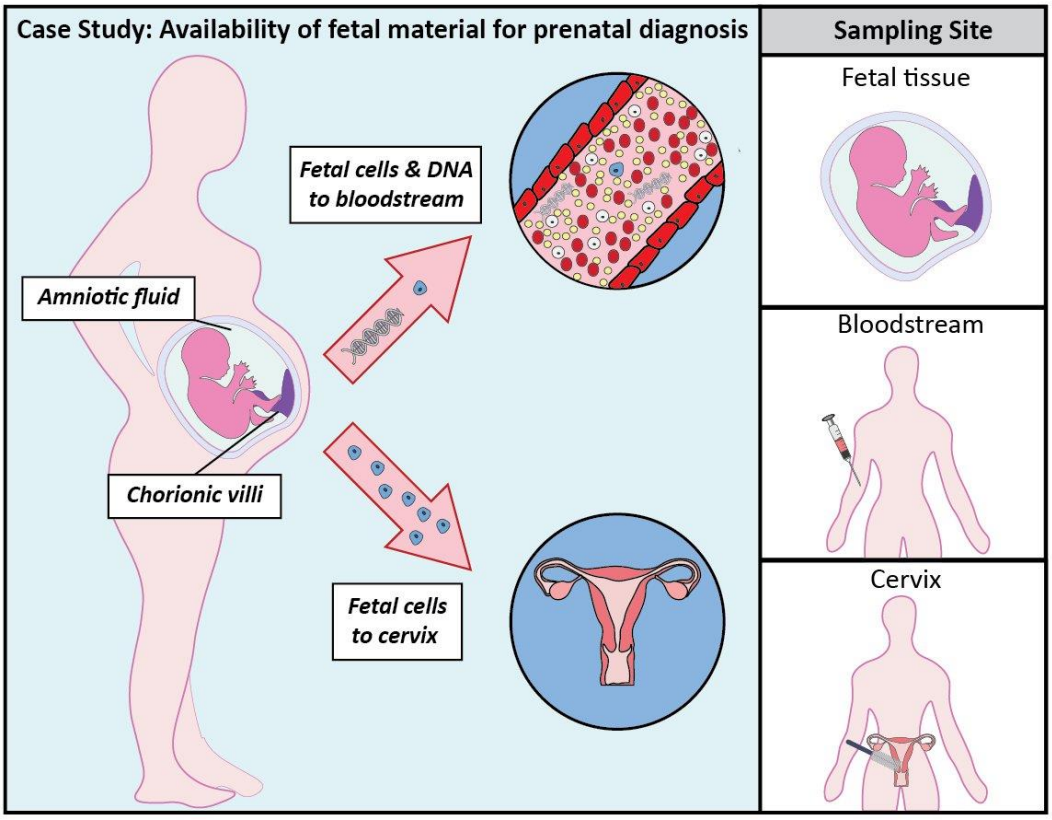



**Figure C.9. Availability of fetal material for prenatal diagnosis.** Made for Hannah Pezzi.

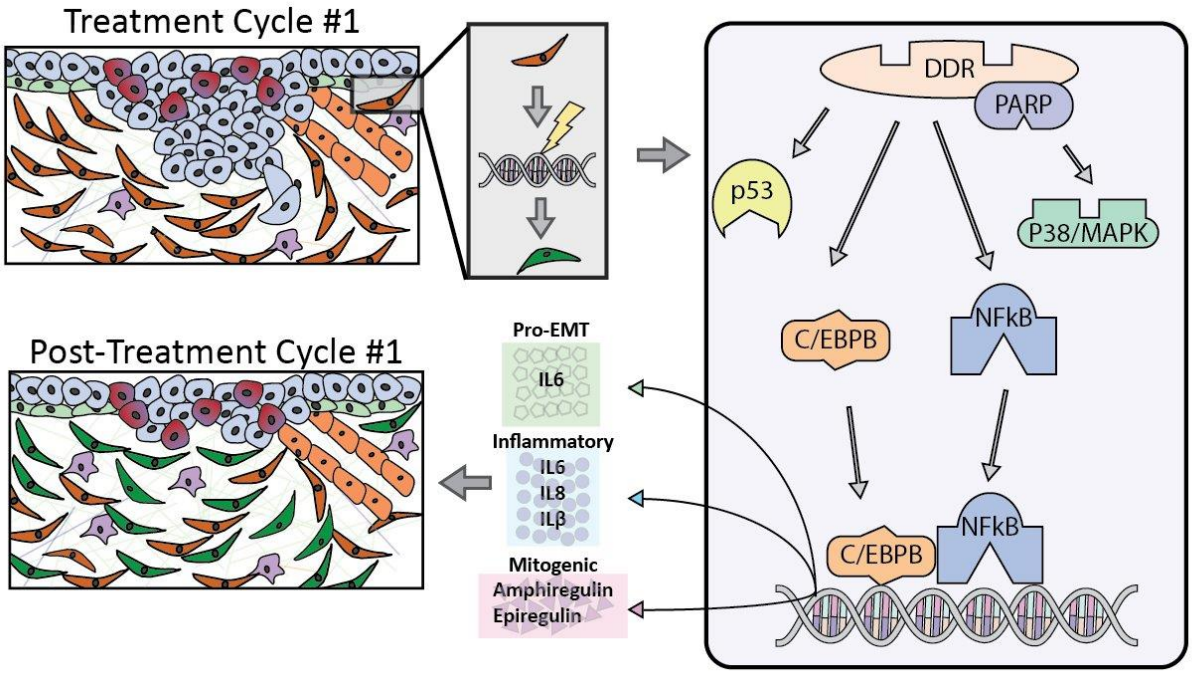



**Figure C.10. Activation of fibroblasts after chemotherapy.** Made for Ross Vitek.

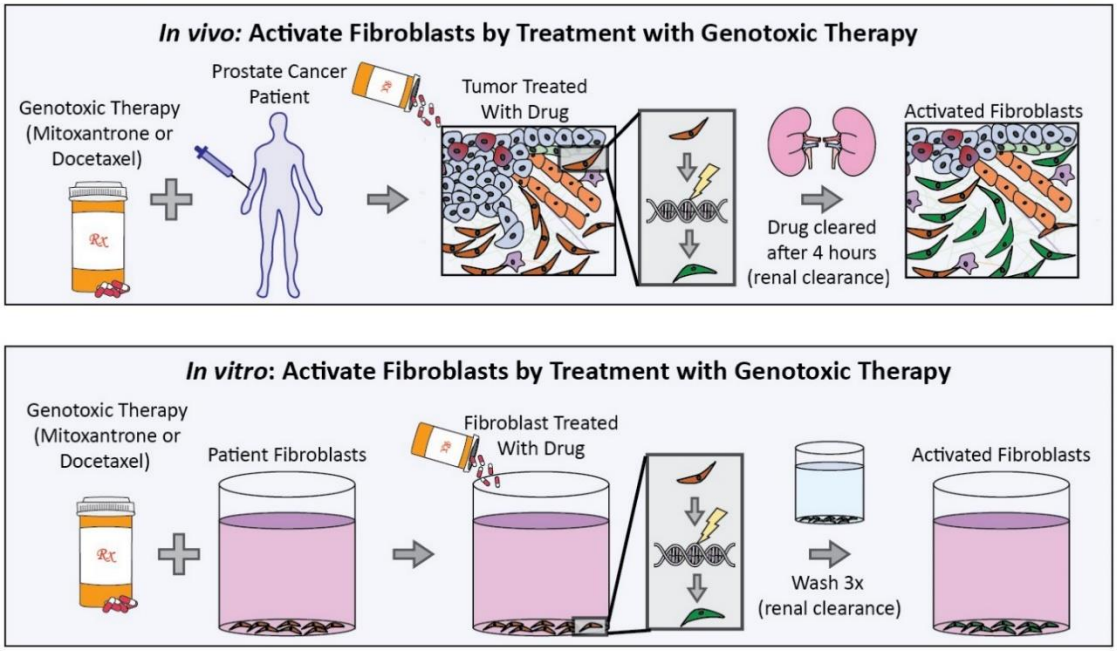



**Figure C.11. Comparison of chemotherapeutic-induced fibroblast activation *in vitro* and *in vivo*.** Made for Ross Vitek.

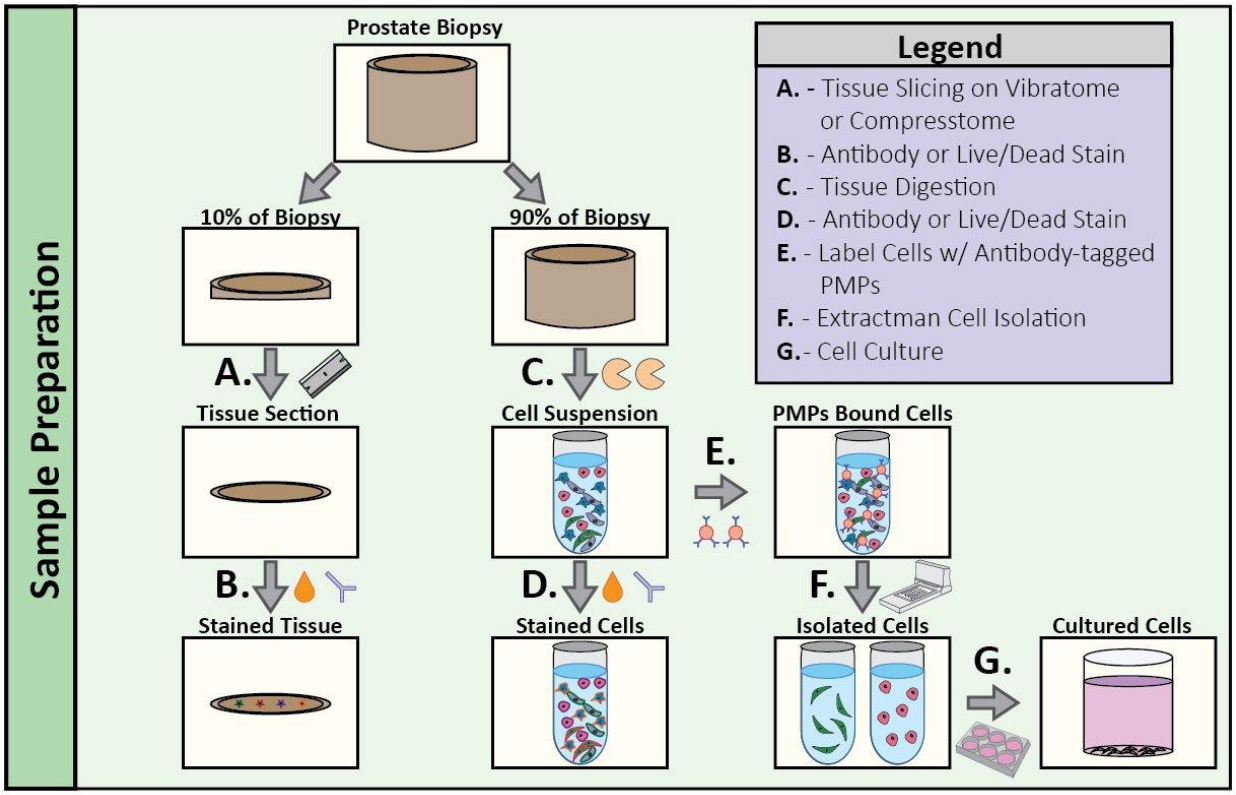
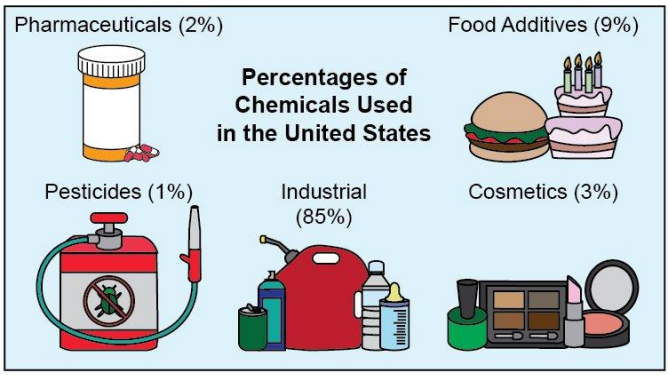


**Figure C.12. Workflow for prostate cancer patient sample processing.** Made for Ross Vitek.




| Regulation of Industrial Chemicals | |
|---|---|
| 1976 - Toxic Substance Control Act | |
| **~60,000 Existing Chemicals:** Deemed safe | **New Chemicals:** No affirmative safety decision required before manufacturing |
| 2016 - Frank Lautenberg Chemical Safety for the 21st Century Act | |
| **~85,000 Existing Chemicals:** Must be assessed | **New Chemicals:** Must be reviewed before manufacturing |

**Figure C.13. Diagram describing the number of untested industrial chemicals in the United States.**

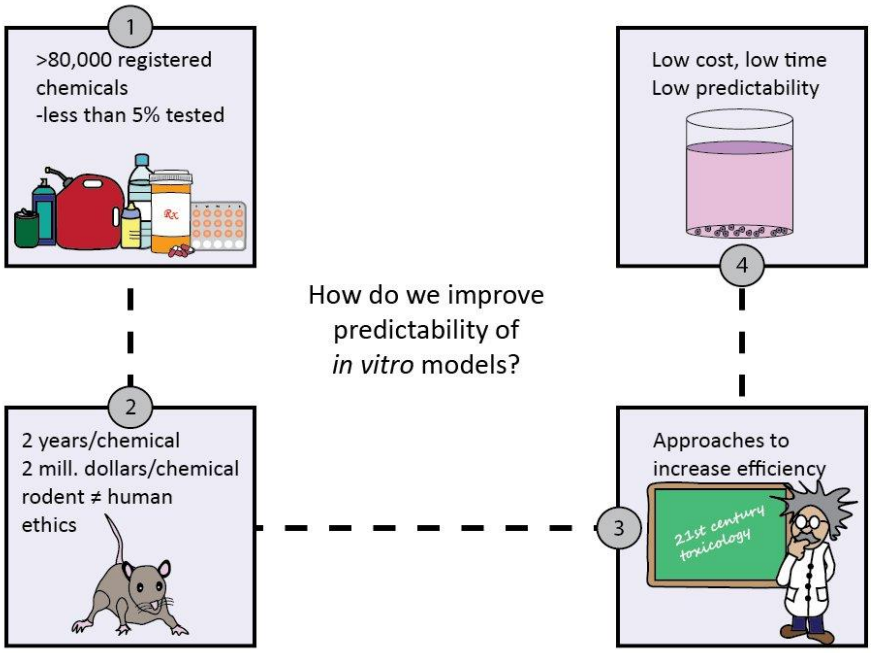


**Figure C.14. Overview of the need for why we need to improve the predictability of *in vitro* models for toxicity testing.**

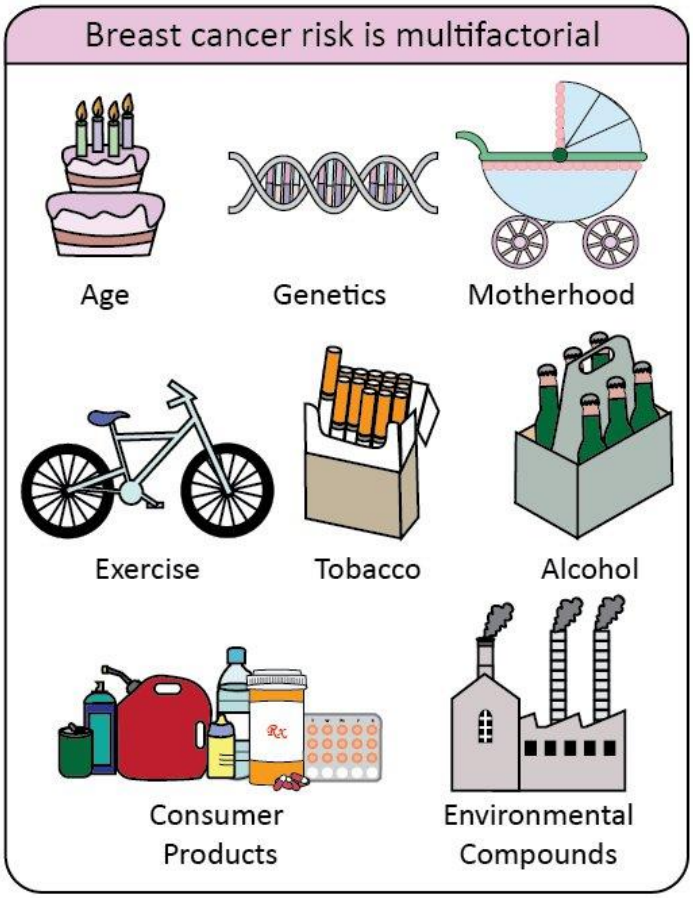



**Figure C.15. Overview of breast cancer risk factors.**

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