

Assessment of Morphologic and Molecular Effects of Estrogen Exposure Using MCF-7 3D Microtissues

By

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Preface

The work presented in this thesis was performed in Dr. Kim Boekelheide's laboratory. I performed all of the experiments with the following exceptions:

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List of Abbreviations

AHR	Aryl hydrocarbon receptor
AKT1	V-akt murine thymoma viral oncogene homolog 1
ANOVA	Analysis of Variance
APOD	Apolipoprotein D
AR	Androgen receptor
AREG	Amphiregulin
BCAR1	Breast cancer anti-estrogen resistance 1
BAD	BCL2-associated agonist of cell death
BAX	BCL2-associated X protein
BCL2	B-cell CCL/lymphoma 2
BCL2L1	BCL2-like 1
BCL2L2	BCL2-like 2
BDNF	Brain-derived neurotrophic factor
BMPs	Bone morphogenic proteins
BMP4	Bone morphogenic protein 4
BMP7	Bone morphogenic protein 7
BPA	Bisphenol A
BRCA1	Breast cancer 1, early onset

BRCA2	Breast cancer 2, early onset
CASP1	Caspase 1
CASP3	Caspase 3
CLDN1	Claudin 1
CCNA1	Cyclin A1
CCND1	Cyclin D1
CCND2	Cyclin D2
CCNE1	Cyclin E1
CDH1	Cadherin type 1, E-cadherin
CDH2	Cadherin type 2, N-cadherin
CDK2	Cyclin-dependent kinase 2
CDKN1A	Cyclin-dependent kinase inhibitor 1A
CST5	Cystatin D
CST6	Cystatin E/M
CTNNB1	Beta-catenin
CTSD	Cathepsin D
CYP1A1	Cytochrome P450 1A1
CYP19A1	Cytochrome P450 19A1
DES	Diethylstilbestrol

DPN	Diarylpropylnitrile
E ₁	Estrone
E ₂	17 β -Estradiol
E ₃	Estriol
EDCs	Endocrine disrupting chemicals
ENPP2	Ectonucleotide pyrophosphatase/phosphodiesterase 2
ER- α	Estrogen receptor-alpha
ER- β	Estrogen receptor beta
ERBB2	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2
ERE	Estrogen response element
ESR1	Estrogen receptor 1 (alpha)
ESR2	Estrogen receptor 2 (beta)
ESRRA	Estrogen-related receptor alpha
ESRRB	Estrogen-related receptor beta
ESRRG	Estrogen-related receptor gamma
FN1	Fibronectin 1
FOS	FBJ murine osteosarcoma viral oncogene homolog
GATA3	GATA binding protein 3

GEN	Genistein
GJA1	Gap junction protein, alpha 1
GPER	G-protein coupled estrogen receptor
GREB1	Growth regulation by estrogen in breast cancer 1
H&E	Hematoxylin and Eosin
IGF1	Insulin-like growth factor 1
IGF1R	Insulin-like growth factor 1 receptor
IGFBP4	Insulin-like growth factor binding protein 4
ITGA1	Integrin, alpha 1
ITGB1	Integrin beta 1
KRT14	Keratin 14
KRT18	Keratin 18
KRT19	Keratin 19
KRT5	Keratin 5
KRT8	Keratin 8
LALBA	Lactalbumin
LAMA5	Laminin 5
LIMMA	Linear models of analysis
LRRC15	Leucine rich repeat containing 15

LTF	Lactotransferrin
MAPK1	Mitogen-activated protein kinase 1
MAPK3	Mitogen-activated protein kinase 3
MAPK8	Mitogen-activated protein kinase 8
MFGE8	Milk fat globule-EGF factor 8 protein
MMP2	Matrix metalloproteinase 2
MMP9	Matrix metalloproteinase 9
MUC1	Mucin 1
MYBL2	V-myb myeloblastosis viral oncogene homolog-like 2
MYC	V-myc myeloblastosis viral oncogene homolog
NOTCH1	Notch 1
NOTCH2	Notch 2
OCLN	Occludin
PASH	Periodic acid-Schiff's hematoxylin
PDZK1	PDZ containing 1
PGR	Progesterone receptor
PPT	Propylpropionitrile
PTGS2	Prostaglandin-endoperoxide synthase 2
SNAIL1	Snail homolog 1

SNAI2	Snail homolog 2
TFF1	Trefoil factor 1
TGFA	Transforming growth factor alpha
TGFB3	Transforming growth factor beta 3
TJP1	Tight junction protein 1
TJP2	Tight junction protein 2
TWIST1	Twist homolog 1
VEGFA	Vascular endothelial growth factor A
VIM	Vimentin
WISP2	WNT1 inducible signaling pathway protein 2
WNT4	Wingless-type MMTV integration site family member 4
WNT5	Wingless-type MMTV integration site family member 5A
XBP1	X-box binding protein 1

Abstract of “Assessment of Morphologic and Molecular Effects of Estrogen Exposure Using MCF-7 3D Microtissues” by Marguerite M. Vantangoli, Ph.D., Brown University, May 2016

Toxicity testing is undergoing a revolution, as the reliance on animal models has resulted in a large backlog of chemicals that have not been adequately safety tested. Animal models are expensive, time-intensive and do not accurately recapitulate human biology. As an alternative, the use of *in vitro* cell culture models is growing, as they are based on human cells and amenable to high-throughput screening approaches. Many of these *in vitro* models use cells grown in monolayer cultures, which respond differently to toxicants when compared to three-dimensional (3D) culture models.

This thesis explored the use of 3D cultures of human MCF-7 breast carcinoma cells cultured using scaffold-free agarose hydrogels as a platform to assess estrogenic endocrine disrupting chemicals. To address the difficulties of imaging 3D microtissues, both traditional histopathological and optical clearing techniques were adapted to enable the visualization of the microtissues. Following these experiments, MCF-7 microtissues were characterized, and were found to contain a luminal space after 7 days in culture. Based on mRNA expression and localization of epithelial markers, MCF-7 microtissues are more differentiated than monolayer cultures. The response of MCF-7 microtissues and monolayer cultures to estradiol was

explored, and demonstrated that at early time points (4 and 8 hours), MCF-7 monolayers and microtissues have a similar response to estradiol. The responses diverge at 24 hours, with 3D cultures showing dynamic changes in proliferation and cell adhesion.

The effects of estrogenic compounds on MCF-7 microtissue morphology and gene expression were assessed. By using receptor-specific agonists, it was determined that ER α activation led to reduced lumen formation with increased proliferation, while ER β activation did not alter morphology. The effects of two estrogenic endocrine disrupting chemicals bisphenol A (BPA) and genistein (GEN) were assessed. At high concentrations, BPA promoted lumen formation, while GEN did not alter lumen formation. BPA and GEN treatment altered gene expression and morphology in a compound-specific manner.

This model provides evidence that supports the use of 3D MCF-7 microtissues to examine both gene expression and morphologic changes following exposure to estrogenic chemicals. This platform allows for the assessment of traditional histopathological endpoints in an *in vitro* system.

CHAPTER ONE

Introduction

1.1 Thesis Significance and Aims

There is an extensive backlog of compounds for which adequate safety information is lacking, due largely to the time-intensive and expensive nature of animal-based toxicity testing [1]. For decades, regulatory agencies have relied on the use of *in vivo* testing to inform safety regulations for pharmaceutical, industrial and environmental compounds. However, animal testing has many limitations, including the low-throughput capacity, high financial cost of conducting *in vivo* experiments, and the reliance on an excessive number of animals [2-4]. Additionally, there are considerable differences in the biology of humans and laboratory animals, that complicates the interpretation of data obtained from animal studies [5]. Due to the biological and logistical complications associated with animal testing, there is an estimated backlog of 80,000 chemicals for which adequate safety information is lacking, posing a significant risk to human health. To address this large backlog of untested compounds, there is an increasing need to develop new approaches for high-throughput toxicity testing models based on human biology.

While large-scale efforts are underway at several locations to develop high-throughput assays to screen thousands of chemicals for their potential to interfere with human biology, these assays are largely based on cells grown in two-dimensional (2D) monolayer culture. Cells grown in 2D lack the

complex cell-cell and cell-extracellular matrix (ECM) signaling present in *in vivo* systems, and often lose their cell-type specific functions [6-8]. The use of three-dimensional (3D) cultures systems is growing, due to their role as a bridge between 2D *in vitro* and *in vivo* assays. Cells grown using 3D culture models have been shown up-regulate tissue specific markers, regain tissue-specific functions and have different gene expression profiles when compared to cells cultured in traditional 2-dimensional (2D) systems [6-9]. This study uses a scaffold-free culture system, which allows for cells to self-assemble and self-sort into 3D microtissues, free of the influence of extracellular matrix [10, 11].

Many of the assays deployed in both the ToxCast and Tox21 compound screening programs focus on evaluating compounds for their ability to interfere with normal endocrine signaling. A large number of environmental and industrial compounds are believed to alter endogenous hormone signaling pathways, and a large number are suspected to exert their effects by altering the estrogen, androgen and thyroid signaling pathways. Compounds that interfere with the processing, secretion, activity or degradation of normal hormones within the body are called endocrine disrupting chemicals (EDCs). While the assays deployed in these screening programs are laudable, they rely heavily on 2D *in vitro* models, which ignore the complex nature of *in vivo* tissues.

From a molecular viewpoint, the ToxCast and Tox21 screening assays cover

many steps of estrogenic activation, including receptor activation, transcriptional changes and proliferation [12]. While these encompass important steps in estrogenic activation, the use of traditional 2D assays does not allow for assessment of phenotypic alterations, in contrast to *in vivo* endpoints. To address this shortcoming, scaffold-free 3D cultures of MCF-7 human breast carcinoma cells were formed and exposed to estrogenic compounds to characterize the morphologic and molecular changes. This unique model has provided insight into the pathways activated by xenoestrogens and allowed for assessment of phenotypic endpoints through investigation of the following aims:

Specific Aim 1: Characterize the differentiation, phenotypic changes, and molecular profiles of human breast cells grown in a 3D scaffold-free cell culture system.

Specific Aim 2: Determine differential 3D model responses to estradiol and estrogen receptor-specific agonists.

Specific Aim 3: Investigate the response of the MCF-7 cells in the 3D scaffold-free system to estrogenic endocrine disrupting compounds including genistein, bisphenol A, and diethylstilbestrol.

1.2 Toxicity Testing

Since its beginnings, toxicity testing has relied on *in vivo* animal models, largely mice and rats. Traditionally, small mammals are exposed to high doses of test compound for a designated period of time, killed, apical endpoints within target organs examined and doses extrapolated to those likely relevant to human exposures [13]. Studies are designed using acute, sub-chronic and chronic exposures and examine endpoints such as cancer, developmental, reproductive, dermal, hepatic, neuro- and nephrotoxicity. The use of intact organisms allows for the evaluation of all relevant aspects of toxicant exposure, including absorption, distribution, metabolism and excretion (ADME). Additionally, the use of animal models allows for histopathological assessment of many organs from the same experiment.

The use of small animal models is based on general assumptions, including that dose-response curves are monotonic and therefore extrapolation is easily calculated, and that small rodent biology captures all of human biology. While such assumptions were historically sufficient, it is now well understood that these assumptions are not always true. Large species differences exist between humans and both rats and mice, and as an example, have been observed in metabolic pathways that influence cancer development and severity [14]. Animal models are not necessarily predictive of human responses to exposure, and interspecies differences can have profound

impacts on the extrapolation and interpretation of data [5].

In addition to large species-specific differences, animal tests are limited by high cost and exorbitant use of animals. The estimated cost of an initial endocrine disruptor toxicity screen for a given compound is greater than \$750,000 and uses more than 500 animals [15].

To bridge the gap and begin to address the large backlog of chemicals which are lacking adequate safety information, the National Research Council released the report “Toxicity Testing in the 21st Century: A Vision and A Strategy” [16]. The report outlined four objectives with regards to revolutionizing toxicity testing: broad assessment of chemicals; cost-effective, higher-throughput methods; reduce the reliance on animal models; and to accurately reflect both dose and mechanism in risk assessment [16]. To address these goals, large-scale efforts are underway to develop and optimize *in vitro* and *in silico* methods and assays for use in screening programs. *In vitro* high throughput screening (HTS) assays are attractive as they allow for the evaluation of thousands of chemicals across cellular responses in a short amount of time. Additionally, a large range of concentrations can be tested, and the use of human-derived test systems allows for the evaluation of effects within relevant cellular models. *In vitro* systems are also amenable to mechanistic studies, and these assays can examine specific components of toxicity.

By transitioning to the use of human-relevant *in vitro* assays, the paradigm of toxicity testing is shifting from high-dose studies in animal models to the elucidation of cellular and molecular pathway changes. These “toxicity pathways,” are described as normal cellular signaling pathways that can be significantly perturbed by chemical insults to result in an adverse cellular outcome[16, 17]. In recent years, the toxicity pathways approach has evolved and has been expanded in risk assessment approaches as adverse outcome pathways (AOPs). AOPs are defined and begin with an event in which a chemical binds with the biological target, which initiates cellular signaling that may lead to alterations at the subcellular, cellular, tissue, organ and organismal levels [18]. The AOP framework was initially proposed for use in ecotoxicology[18, 19], but has been widely used to describe the effects of a chemical insult in *in vitro* assays[20-22]. The use of toxicity pathways and AOPs has provided insights into the molecular events underlying toxic responses [23-25].

To begin to transition to more-relevant toxicity screening assays based in human biology, the United States Environmental Protection Agency (EPA) launched the ToxCast program in 2007 [26]. The initial goal of ToxCast was to profile the alterations of hundreds of endpoints to hundreds of well-characterized chemicals *in vitro* assays. The results of this initial screen, based on reporter assays, biochemical assays, transcriptomics studies and developmental screens using zebrafish are being used to prioritize chemicals

for further testing. ToxCast relies on the use of high-throughput screening integrated with computational technology to screen large numbers of compounds through many assays. The ToxCast program has been successful in integrating data across many assays, particularly in assessing the estrogenic potential of environmental chemicals [27, 28].

Tox21 is a complimentary program to ToxCast, and is a collaborative effort that includes the National Toxicology Program (NTP), Environmental Protection Agency (EPA), the National Center for Advancing Translational Sciences (NCATS) and the Food and Drug Administration (FDA). The goals of the Tox21 collaborations are to identify, develop and characterize assays for use in HTS, utilizing existing *in vivo* data, computational toxicology, toxicity data, bioinformatics and *in vitro* cell-based assays.

To prioritize compounds for further screening, Tox21 was broken into three phases. For Phase 1, 70 assays were used to test 2,800 compounds using a high-throughput robotic system at NCATS. Phase 1 screening has been completed, and complete results are publicly available[29, 30]. The compounds screened in phase 1 were chosen as a “proof of principle” library, and most were well characterized in animal models. This initial screen included phenotypic cell based assays, nuclear receptor activation assays, immune-response cell-based assays as well specific molecular target assays, which has provided a large data set with concentration response curves for most chemicals in most of the assays[29, 30].

Following the conclusion of the Phase I study, Phase II began in 2011 and was focused on 10,000 chemicals of interest[31]. Phase II included compounds with known chemical structures that ranged from little to no known toxicity data to compounds that were well characterized. Phase II assays included assays that were covered in Phase I, as well as a variety of reporter-based and in vitro toxicity assays. Following the conclusion of Phase II, Phase III is planned to expand the chemical library, as well as the breadth of assays employed. Many of the Phase I and II cell-based assays use cancer-derived cell lines, which do not completely reflect *in vivo* human biology. One of the goals of Phase II is to employ more relevant cell-based assays (using stem cells and/or primary cells), and to assess gene expression endpoints to gain a more comprehensive understanding of the molecular pathways initiated following exposure to chemicals.

Like other assays, the Tox21 testing strategy does not come without limitations. Due to technical restrictions, the current assays in Phase I and Phase II do not cover metabolic activation of compounds [29], a key component in toxicological studies. This limitation does not allow for the assessment in a HTS model of metabolic activation of compounds. Another limitation of the current cell-based assays is inability to use volatile compounds in this model. Additionally, most of the assays cover 1-2 biological endpoints, and this limitation requires that a large number of assays to be used to cover a single biological pathway. Most of the complex signaling

cascades within cells are difficult to identify based on simple endpoints, since this requires that the cross-talk between many cellular pathways be combined to make this identification possible. *In vitro* assays are also limited in that a positive result in an assay does not necessarily translate to an adverse cellular change. Additionally, the use of cancer-derived and transformed cell lines to interrogate biological pathways may not translate to normal *in vivo* human biology. However, despite these limitations, the success of Tox21 has demonstrated that progress is being made in adapting systems biology approaches to toxicity testing.

1.3 In Vitro Cell Culture Systems

1.3.1 Two-Dimensional Culture Systems

Traditionally, cells have been cultured in *in vitro* settings as flat, two-dimensional (2D), monolayer cultures and grown on plastic tissue culture substrates. In this setting, most cells and cell lines will adhere to the plastic and proliferate. In most cases, before cells become confluent, the cells need to be removed from the flask, divided and subcultured into new flasks. Monolayer cultures are beneficial, as they are technically easy to work with and highly amenable to HTS assays. Cells grown in 2D are easy to image, allowing for live-cell imaging, cell tracking, the use of fluorescent reporters, and protein and cell organelle tracking. The development of high-content screening (HCS) platforms has enabled the use of 96-, 384- and 1536-well platforms. The use of multi-well plates has facilitated the ability to analyze large numbers of compounds across time and concentration, and has been beneficial in the pharmaceutical industry and in toxicity testing. To address the large number of chemicals that have not been adequately safety tested, the EPA, NIH, NTP and NIEHS have employed the use of many high-throughput assays, relying on HTS to assess the safety of chemicals. As discussed previously, the cell-based assays employed in these screening batteries have begun to address the large backlog of chemicals, and are dependent upon cells grown as 2D cultures.

It is widely recognized that while 2D are cultures advantageous due to their low cost and ease of imaging, cells grown as monolayers do not accurately recapitulate the *in vivo* environment [32]. When cultured as monolayers, cells lose their apical-basal polarity, a defining feature of epithelial tissues *in vivo* (Figure 1.1). Polarization enables cells to establish directionality, a feature extremely important for cellular function. Polarized epithelial cells will often secrete cellular products on the basal surface, and the establishment of polarity permits the cells to carry out normal functions [33]. Additionally, when *in vivo*, epithelial cells contact other cells and establish cell-cell signaling mechanisms, and most epithelial cells contact other cells with 80-95% of their cell surface. When cultured on plastic as monolayers, epithelial cells are limited in the cell-cell connections they can form, as they are flattened with most of their cell membrane contacting plastic substrate and culture media. This results in a large loss of tissue-specific architecture, cell-cell communication and mechanical and biochemical cues under these conditions [34].

1.3.2 Three-Dimensional Culture Systems

Three-dimensional culture has been developed to bridge the gap between *in vivo* and 2D *in vitro* models. While using *ex vivo* models such as organ explants and embryos allow for study of large sized tissues and organisms, they are difficult to work with in regards to imaging and data collection and do not survive for long periods of time in culture. Also, embryonic and *ex vivo*

models are dependent on animal models, and do not reduce reliance on animals. 3D models lend themselves to being much more simplified and cost-effective than *in vivo* and *ex vivo* models, while allowing for the examination of populations of cells within a more natural environment than 2D culture. Toxicity testing of compounds, including chemotherapeutics and nanoparticles, has demonstrated differential responses of cells cultured in 2D vs. 3D [35-37]. However, the imaging and visualization of 3D structures remains a major challenge in 3D cell culture, as microtissue thickness, light scattering and impaired diffusion of reagents across multiple cell layers are contributing factors [38]. There exists a large need to adapt and refine techniques that can enable optimal visualization of microtissues to enhance imaging acquisition and assessment of morphological and toxicological outcomes that inform disease pathologies.

1.3.2.1 Scaffold-based Systems

A popular type of 3D models are typically scaffolded models, in which cells are grown at low densities within a matrix, usually designed to mimic the *in vivo* extracellular matrix (ECM). The culture of epithelial cells on matrices including laminin, collagen and artificial scaffolds allows for the establishment of epithelial polarity, which allows cells to regain cellular functions including secretion of products. In mammary epithelial cells, expression and secretion of milk protein components β -casein, β lactoglobulin and whey protein are dependent on cell-ECM interactions[39, 40].

Several 3D models have been specifically developed to study breast cancer progression and tumor biology. In one model, normal and tumorigenic epithelial cells are grown in Matrigel. In this 3D system, normal mammary epithelial cells form into polarized hollow spherical monolayers, called acini, that resemble normal breast tissue; while tumorigenic mammary epithelial cells form disorganized clusters [41, 42]. 3D culture of mammary epithelial cells has demonstrated how the extracellular matrix (ECM) affects many aspects of mammary cells, including morphogenesis and homeostasis of the tissue[41, 43]. Additionally, mammary epithelial cells on flat surfaces upregulate the expression of cellular markers such as β -integrins, an observation not seen in 3D culture models [44, 45]. As shown by Bissell et al, primary human mammary cells grown on the commercially available reconstituted basement membrane Matrigel (laminin-rich) form polarized acini-like structure, and these structures are fully capable of organ function such as milk production [41, 43].

These matrix-dependent models have limitations as well, including the disadvantage of a small size, which further restricts diffusion of waste and nutrient transport. Additionally, Matrigel and other matrices provide artificial environments that have variation between lots and labs. This ECM rich environment has the potential to induce changes within the cells that are not normally seen *in vivo*. For example culture of fibroblasts on laminin-rich matrices leads to a normal, nonmalignant phenotype, while the culture of the

same cells on a collagen-rich matrix leads to an aggressive and invasive malignant phenotype [46, 47].

1.3.2.2 Scaffold-free Systems

An alternative method in 3D culture is using a scaffold-free model; such as the hanging drop method and agarose hydrogels. In these methods, cells are seeded at high densities and allowed to self-sort and self-assemble, free from the influence of external matrices [48].

Easily the earliest form of 3D cell culture, the hanging drop culture technique utilizes trypsinized cells in cell culture media, in which the drop is attached to a plate cover or coverslip (Figure 1.1). The plate cover or coverslip is then inverted, so cells collect at the bottom of the drop of media, come into contact with each other and assemble into spheroids [49]. Hanging drop cultures are easy to assemble and don't require any specialized equipment, unlike other 3D culture models, however they have several technical limitations. Hanging drop cultures models are technically difficult to work with over long periods of time, as the cultures are sensitive to mechanical disruption, and media changes are difficult which makes treatments over long periods of time laborious. The low media to cell ratio of hanging drop cultures alters culture viability over long times in culture, as nutrients are quickly depleted and waste accumulates, which can cause necrosis in the cores of the spheroids. The small amount of biological material per sample is too low for most

molecular techniques; meaning large numbers of samples need to be pooled for assessment of these endpoints. Recently, advances have been made in the hanging drop plates to address some of the technical difficulties associated with hanging drop cultures, though these cultures are still limited by media volume-to-cell number ratios [50].

Despite the technical limitations, hanging drop cultures more closely recapitulate human *in vivo* biology than do 2D monolayers. HepaRG hepatocyte cells cultured as spheroids using the hanging drop method demonstrate treatment-related induction of cytochrome P450 enzymes, similar to the response *in vivo* [51]. HepaRG spheroids are also more sensitive to acetaminophen toxicity than 2D cultures, likely due to the higher expression of cytochrome P450 enzymes in the 3D cultures [52]. The greater similarity of hanging drop 3D cultures to *in vivo* biology is not exclusive to HepaRG cells, as increased differentiation has been observed in stem cell [53] and cardiac cultures [54].

In the method developed by Napolitano, et al. [48], trypsinized cells are seeded into non-adhesive agarose hydrogels and allowed to settle into recesses to self-sort and organize free of the influence of external matrices found in scaffolded models (Figure 1.1). This model provides more control over spheroid size by having the capability to seed different amounts of cells. The use of hydrogels is inexpensive and easy to manipulate, providing a system that is amenable to HTS. Agarose hydrogels containing microtissues

can also be embedded in methylacrylate and in optimal cutting temperature medium for histological examination [55]. Agarose hydrogels are versatile and allow for long-term culture of microtissues. Most cell types are amenable to 3D culture using agarose hydrogels, as many cell types have been cultured including human fibroblasts[56], neural cells [57], breast cancer cells [10, 58] and cardiomyocytes[8].

Several recent studies have demonstrated the powerful nature of using agarose hydrogels to generate 3D microtissues. When cultured using agarose hydrogels, MCF-7 human breast carcinoma cells self-organize into microtissues that contain a luminal space, similar to the of the *in vivo* mammary gland [9]. Compared to 2D monolayer cultures, MCF-7 microtissues display increased expression of differentiation markers including e-cadherin and keratin 19 and decreased expression of mesenchymal markers including vimentin and keratin 14 [9]. Similarly, neonatal rat cardiomyocytes will self-assemble into spherical microtissues when cultured using agarose hydrogels. These cardiomyocyte microtissues undergo spontaneous contractions, and can be paced to beat simultaneously [8]. The ability to assess the molecular, structural and functional effects of exposure to drugs and environmental compounds is a useful attribute of 3D cultures and bridges the gap between 2D *in vitro* and *in vivo* models and assays.

The generation of microtissues using agarose hydrogels is not restricted to

the use of one cell type. This system can be used to generate co- and tri-cultures of several different cell types, as well as cultures of dissociated *ex vivo* tissues. Tricultures of normal human fibroblasts, human endothelial cells and rat hepatoma cells seeded as dispersed cells will self-assemble and self-sort into organized microtissues [11, 48]. In a recent study by Dingle et al, primary postnatal rat cortical cells were isolated and cultured as microtissues[57]. The isolation of cells from the rat cortex retained the cell-types present in the *in vivo* neonatal brain, and microtissues contained oligodendrocytes, neurons, astrocytes, progenitor cells and microglia. The neural microtissues fired action potentials, an important finding which allows for the assessment of the effects of injury and exogenous compounds on the structure and function of the cerebral cortex in an *in vitro* setting [57]. The presence of other cell types has important implications for understanding the effects of drugs on microtissue responses, as the presence of transporters and gap junctions can alter the effectiveness of drugs, an area of concern particularly for chemotherapeutics [59]. The presence of transporter pumps on the exterior surface of a microtissue can prevent the treatment from reaching the inner layers of the microtissue, making models such as these important for understanding drug kinetics.

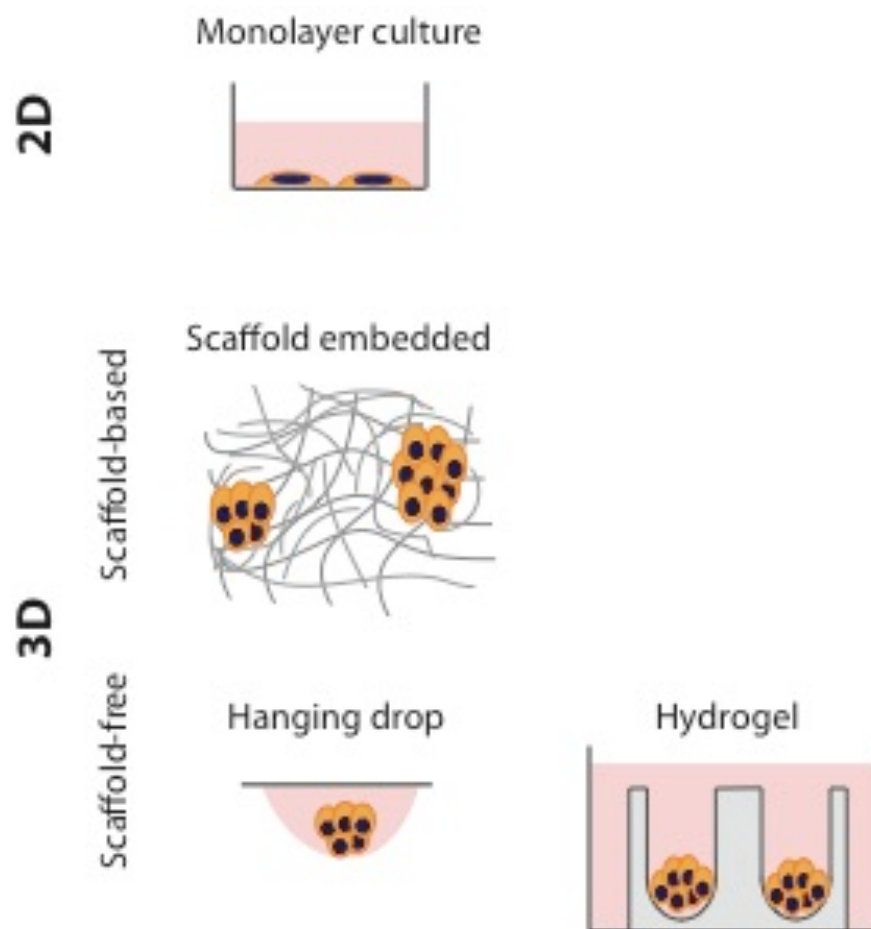


Figure 1.1 Comparison of 2D and 3D cell culture models. In monolayer culture models, cells are grown on plastic substrates (tissue culture plate, flasks, glass) as a single layer (A). Cells grown in 2D are thin, and have very little contact with other cell types. The majority of their membrane is in contact with the plastic substrate and media. Generally, cells grown in 3D regain cell-type specific appearance and functions. In scaffold-based embedded models, trypsinized cells seeded at low density and grow attached to the matrix (B). When grown on insert models, cells attach to the matrix at the basal surface, and will often form a tightly attached, polarized membrane.

1.4 Estrogen Signaling

Estrogen signaling is a complex, highly regulated process that is tissue-specific and dependent on a number of receptors. Like other steroid hormones, estrogens are produced from cholesterol by steroidogenic enzymes. Estrogens are produced in response to levels of endogenous hypothalamic and pituitary hormones, and have a wide range of target tissues. Several classes of estrogen receptors have been characterized, including the classical nuclear receptors ER α and ER β , GPER and orphan nuclear receptors.

1.4.1 Estrogen Production

Steroidal hormones, including estrogens, are derived from cholesterol. The process of hormone synthesis is complex, and is a chain involving many enzymes (Figure 1.2). In the last step of estrogen formation, the cytochrome P450 19A1 (CYP19A1, aromatase) in the smooth endoplasmic reticulum hydroxylates the precursors androstenedione and testosterone. The hydroxylation of the androstenedione forms estrone (E1), while hydroxylation of testosterone forms estradiol (E2) (Figure 1.2) [60].

Hormonal production is highly controlled, and is reliant on several hormones within the hypothalamic-pituitary-gonadal (HPG) axis. Gonadotropin releasing hormone (GnRH) is produced in the hypothalamus and binds to GnRH receptors in the anterior pituitary, which results in the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the

pituitary gland [61, 62]. GnRH release is pulsatile, and the frequency of GnRH release dictates the production and secretion of FSH and LH. LH and FSH regulate reproduction and the levels of peripheral sex hormones, including estrogen, progesterone, and testosterone. In premenopausal females, LH stimulates the secretion of E2 by the thecal cells in the ovary, which is then converted to testosterone by aromatase in the granulosa cells of the ovary. Estradiol regulates the negative feedback of GnRH production. During menstruation, due to decreasing levels of estradiol, FSH is secreted at high levels, which causes the growth of antral ovarian follicles. LH and FSH regulate reproduction and the levels of peripheral sex hormones including progesterone, testosterone and estradiol through feedback loops in the HPG axis.

The ovaries produce E2, the most potent endogenously produced estrogen, from testosterone. Estrone, a metabolite of estradiol (Figure 1.2), is a high affinity ligand that is a weaker agonist on estrogen receptors than estradiol, but has tissue-specific effects [63].

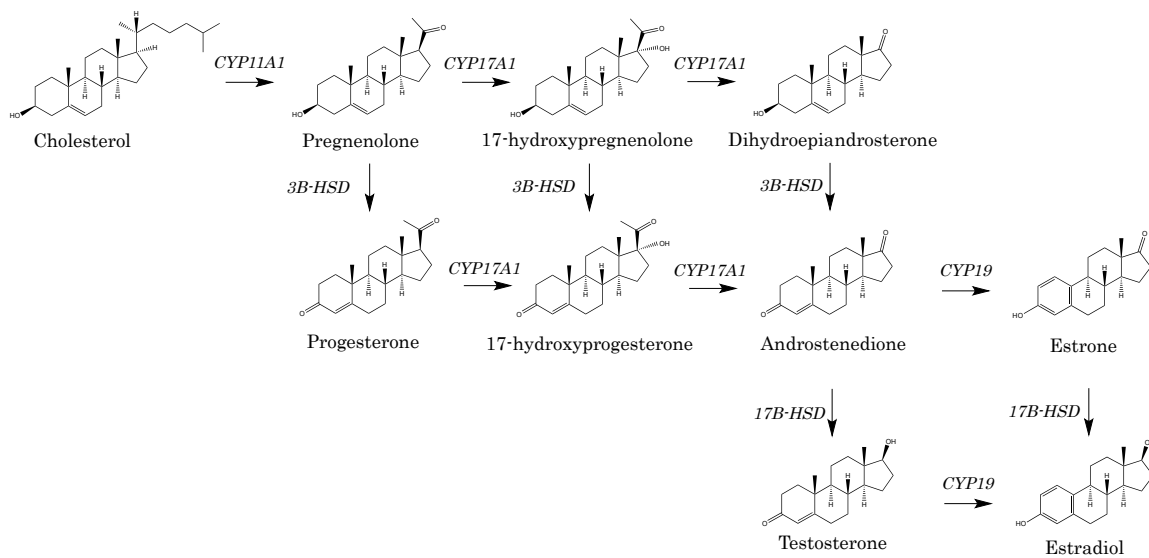


Figure 1.2 Estrogen biosynthetic pathway, The side chain of cholesterol is cleaved by CYP11A, into pregnenolone, which can undergo two hydroxylations by CYP17A1, to become 17-hydroxypregnenolone and dihydroepiandrosterone (DHEA), respectively. Pregnenolone, 17-hydroxypregnenolone and DHEA are converted to progesterone (P4), 17-hydroxyprogesterone and androstenedione, respectively, by 3 β -HSD. Androstenedione can be converted to estrone (E1) by CYP19, or to testosterone (T) by 17 β -HSD. E1 can be converted to estradiol (E2) by 17 β -HSD, and T is converted to E2 by CYP19. Figure made using ChemDraw.

1.4.2 Molecular Mechanisms of Estrogen Signaling

E2 is the most potent estrogen produced in the body, and it is also the most prevalent. E2 and other estrogens exert their effects through estrogen receptors. There exist several receptors for estrogens, including the nuclear receptors estrogen receptor alpha ($ER\alpha$) and estrogen receptor beta ($ER\beta$), the g-protein coupled estrogen receptor (GPER), and several orphan estrogen-related receptors (ERRs).

1.4.2.1 Classical Estrogen Receptors Structure

The first estrogen receptor, now termed $ER\alpha$, was cloned in 1985. The development of knockout mice for estrogen receptor in 1993 demonstrated that although reproductive structure and function were disrupted, the knockout mice were able to survive [64]. This finding indicated that there was a second form of estrogen receptor, which was cloned in rat prostate and ovary in 1996 [65], and $ER\beta$ knockout mice were generated in 1998 [66].

$ER\alpha$ and $ER\beta$ are nuclear receptors, and act as transcription factors. Both $ER\alpha$ and β contain structurally conserved regions. The DNA-binding domain (DBD) is highly conserved, and is involved in DNA recognition and binding following ligand binding at the C-terminal ligand-binding domain (LBD). The most variable, least conserved domain is the N-terminal domain, which is responsible for transcriptional activation. The N-terminus is responsible for

recruitment of co-regulatory protein complexes following DNA binding. Following ligand binding, the cell- and receptor-specific responses to estrogens is dependent on the presence of co-regulatory proteins and the promoters of specific genes.

Both ER α and ER β act as ligand-activated transcription factors, and the classical mechanism for activation of these receptors involves ligand binding to the receptors in the nucleus, causing the receptors to dimerize, bind to specific estrogen response elements (EREs) in the promoters of target genes and initiate transcription [67]. Classical estrogen-responsive genes that have been demonstrated to contain an ERE include b-cell lymphoma 2 (BCL2)[68], cathepsin D (CTSD) [69], progesterone receptor (PGR) [70], and trefoil factor 1 (TFF1) [71]. However, nearly a third of the genes reported to be regulated by ERs do not contain EREs in their promoters, indicating that ERs can also influence signaling through indirect interactions with DNA [72].

Through indirect regulation, ERs can influence gene expression by modulating protein-protein interactions, known as ERE-independent genomic actions (Figure 1.3). Two well-characterized examples of indirect regulation of gene expression by ERs include Fos/Jun through activator protein 1 (AP-1) responsive elements[73] and SP-1 [74]. Genes reported to be regulated by ERE-independent genomic signaling include insulin-like growth factor 1 (IGF1) [75] and cyclin D1 [76, 77].

1.4.2.2 Classical Estrogen Receptors Isoforms

There exist several splice variant isoforms of ER α and ER β , and these isoforms have been demonstrated to play different roles in estrogen signaling. Three ER α variants have been described, and include the full-length ER α 66, as well as the truncated forms ER α 46[78] and ER α 36[79]. ER α 46, which lacks the N-terminus of ER α 66, can act as a strong inhibitor of ER α 66, and has been suggested to play a role in cellular proliferation [78]. The splice variant ER α 36 lacks the ligand-transactivation domains, but retains its DBD and LBD [79]. ER α 36 has previously been demonstrated to be involved in cellular proliferation through induction of the MAPK/ERK pathway[80]. Differences in the structure of these isoforms translate into differences in ligand binding and receptor function, as various estrogens and estrogen modulators have been demonstrated to bind each form with varying affinities [81]. The endogenous ligand E2 binds ER α 66 and ER α 46 with similar affinity at physiologic concentrations, but does not bind ER α 36 [81], likely due to the alterations in the ligand binding domain, indicating that ER α 36 likely plays a complimentary role, possibly mediating GPER-related pre-genomic signaling [82]. Interestingly, while known specific estrogen receptor agonists propylpyrazole triol (PPT), diarylpropyl nitrile (DPN) and G1 had similar affinities for ER α 66 and ER α 46, the affinities for SERM antagonists were markedly different. The antagonists tamoxifen, raloxifene and ICI 182,780 had

significantly lower affinity to ER α 46 than ER α 66, offering the potential to target one isoform to elucidate the physiologic effects of each receptor.

1.4.2.3 G-protein Coupled Estrogen Receptor

GPER is an orphan G-protein coupled membrane-associated protein that contains seven transmembrane domains [83]. Several studies have demonstrated the ability of E2 to bind to GPER in transfected and breast cancer cell lines [84, 85]. GPER is expressed in many tissues, including mammary, endometrial, ovarian, liver, lung and prostate tissues [86].

The data surrounding the importance of GPER with regards to estrogen signaling is contradictory, with some groups reporting that E2 is able to stimulate and activate GPER [84, 87], and others reporting that E2 acts independently of GPER[88-90]. Studies examining GPER knockout mouse models have suggested that GPER is not critical for reproductive development, with female mice showing no significant alterations in mammary gland development, uterine function or reproduction [91]. This is in contrast with *in vitro* results derived in human cell lines that demonstrate a role for GPER in estrogen signaling pathways. [85, 87, 92, 93]. The role of GPER in estrogen signaling remains an open debate, and it is likely that GPER plays a strong role in diseases including breast cancer [94].

1.4.3 Biological Effects of Estrogens

Estrogens are steroid hormones, and are critical for regulating growth, differentiation and function in a wide range of target tissues within the human body. Estrogens are central in regulating the development and maintenance on sexual and reproductive function in both males and females, and are also essential for correct development and function of the cardiovascular, central nervous, immune and musculoskeletal systems[95-99]. Estrogen is morphogenic, and is known to be involved in the morphogenesis of several organs, including the mammary gland [100-103], ovary[101, 102, 104], brain [105] and prostate [106, 107].

ER α is highly expressed in the reproductive tissues, including the breast, uterus and ovary, and also in the testis and epididymis of males. ER α is also detectable in other organs including the pituitary gland, hypothalamus liver, kidney and the adrenal glands. Expression of ER β is found in the prostate, ovarian and breast tissues, and is expressed in many tissues and organs including the skin, bone, brain, lung, bladder and adipose tissue[65, 108].

1.4.4 Estrogens and Mammary Development

The female mammary gland is a well-characterized target of estrogens, and is unique due to its multiple stages of proliferation, elongation and involution[109, 110]. The mammary gland is composed of luminal epithelial, myoepithelial, adipocytes and stromal fibroblast cells. At puberty, the onset of 17- β estradiol production by the ovaries stimulates ductal morphogenesis of the breast. Following the onset of puberty and the increase in estrogen

and progesterone, the female human breast begins to form terminal end buds (TEB), leading to ductal elongation, branching and expansion into the surrounding fat pad [111]. TEB are highly proliferative, and display characteristics of both epithelial and mesenchymal cells, indicating that there is a degree of epithelial to mesenchymal transition (EMT) occurring [112]. The branching and development of the human female mammary gland has distinct embryonic, adolescent and adult phases, all of which are very sensitive to both the internal and external hormonal environments [113]. Branching of the adult epithelium is highly dependent on hormonal signals triggered by estrogen and progesterone, as well as cross-talk between the epithelium and stromal tissues of the breast. This process is tightly controlled by the signals generated from the mesenchyme [114]. Many studies have focused on the dependence of the mammary gland on endocrine hormones including estrogen, insulin-like growth factor 1 (IGF-1) and growth hormone (GH) [115].

During pregnancy, the ducts expand laterally and differentiate. This layer is then filled with secretory cells, which are stimulated after birth to produce milk. Following pregnancy and breast-feeding, the compartment undergoes extensive remodeling and apoptotic cell death, in an effort to involute and restore the mammary gland to its pre-pregnancy architecture [46, 116-118]. Subsequently, throughout its lifetime, the mammary gland continues to undergo significant amounts of remodeling, proliferation and cell death,

leading to the hypothesis that the dysregulation of the pathway governing this process can lead to disease states, including tumorigenesis [113, 119]. In fact, the developing mammary gland displays many of the properties associated with Weinberg and Hanahan's "Hallmarks of Cancer" [120, 121], used to describe tumor progression, including invasion, cell proliferation, resistance to apoptosis and angiogenesis[119]. Based on these hallmarks and the highly proliferative potential of the mammary gland, it is not hard to imagine that an environmental insult such as exposure to an EDC during a critical period of remodeling, or genetic susceptibility may manifest itself as a breast tumor, taking advantage of the growth promoting environment within this tissue [122].

As demonstrated in ER α knockout mice, mammary gland elongation is dependent on ER α signaling. In normal development, In ER α $-/-$ mice, ductal elongation does not occur, and the mammary glands resemble those of a pre-pubertal mouse , demonstrating that mammary gland development begins independent of ER α signaling, but the formation of fully-developed and differentiated glands are dependent on ER α [115, 123]. In contrast, in ER β $-/-$ mice mammary gland development precedes normally, indicating that ER α signaling is largely responsible for the estrogen effects observed in mammary tissue. This correlates with the high levels of expression of ER α in normal mammary tissue, indicating that ER α is largely responsible for the proliferative events in the mammary gland following estrogen stimulation.

1.4.5 Estrogen Agonists and Antagonists

Largely due to the involvement of estrogen receptor-mediated pathways in breast cancer, a large number of estrogen-receptor specific agonists and antagonists have been developed. Selective agonists for ER α , ER β and GPER as well as antagonists for ER α are widely used pharmacologically to modulate estrogen signaling.

1.4.5.1 Selective Estrogen Agonists and Antagonists

1.4.5.1.1 Propylpyrazole Triol

Propylpyrazole triol (PPT) is a selective agonist for ER α , with a 410-fold selectivity for ER α over ER β [124, 125]. Treatment of human endometrial cancer cells with PPT resulted in concentration-dependent increases in expression of PGR, a gene regulated directly by ER α [126]. When treated with PPT, a murine mammary cell line (HC11) displayed significantly increased proliferation coupled with increased expression of the proliferative markers CCND1 and proliferating cell nuclear antigen (PCNA), while treatment with specific agonists for other receptors did not induce proliferation [127]. Through competitive binding assays, the relative binding affinity of PPT was demonstrated to be higher than that of the endogenous ligand E2 [81]. Additionally, PPT bound both the ER α 66 and ER α 46 isoforms with similar affinity, and did not display significant selectivity for either isoform. Like E2, PPT did not bind to ER α 36, likely due to the missing ligand transactivating domain in the isoform [81].

1.4.5.2 Diarylpropionitrile

Diarylpropionitrile (DPN) is a selective agonist of ER β with a 50 to 70-fold higher binding affinity for ER β than ER α [126, 128]. Numerous *in vitro* studies have demonstrated that DPN activation of ER β , and not ER α , results in decreased cell proliferation [126, 129, 130], which correlates with altered expression of cell cycle regulators following induction of ER β [131, 132]. These observations are corroborated by various *in vivo* studies. In ovariectomized Sprague Dawley female rats, DPN was unable to induce mammary gland proliferation, and was able to counteract the proliferative effects of PPT when animals were exposed to both compounds [133]. At low concentrations in *in vitro* and *in vivo* models, DPN selectively binds to and activates ER β , resulting in decreased cell proliferation and increased apoptosis [127].

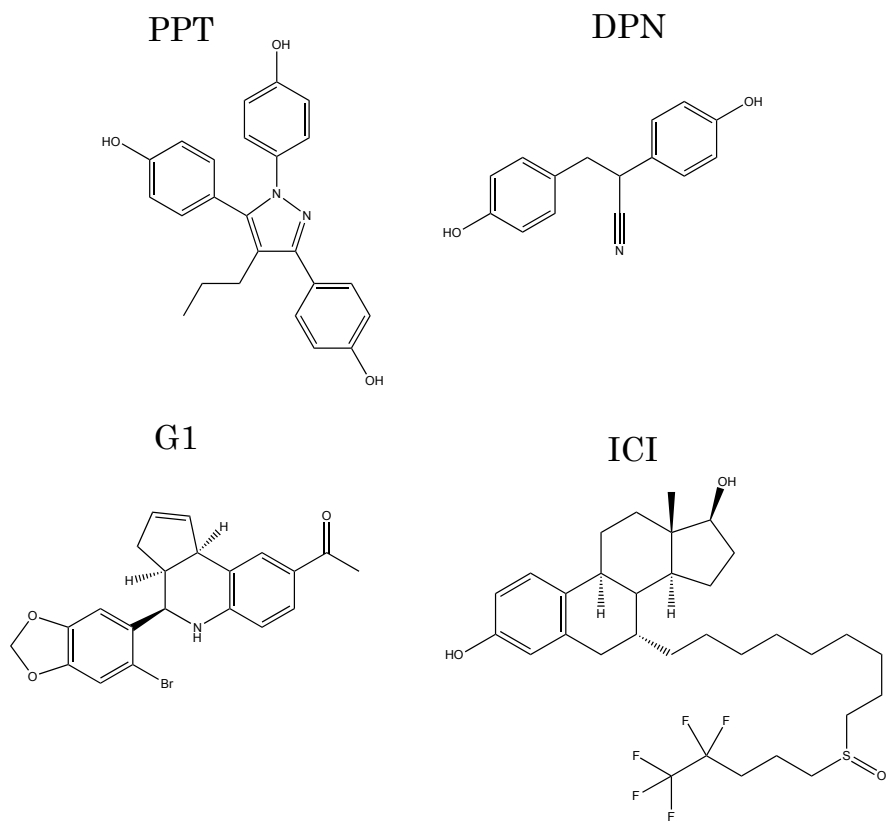
1.4.5.3 G-1

The substituted hydroquinoline 1-[4-(6-bromobenzo[1,3]dioxol-5yl)-3a,4,5,9b-tetrahydro 3Hcyclopenta[c]quinolin-8-yl]-ethanone (G-1) is a nonsteroidal, high affinity, agonist selective for GPER [134]. As demonstrated by competitive binding assays using GPER, G-1 has a K_i at 11nM at GPER, compared with the K_i of E2 of 5.6nM. Additionally, G-1 did not exhibit binding to either ER α or ER β at concentrations up to 1 μ M[134]. In nontumorigenic, immortalized MCF-10A mammary epithelial cells that express GPER, but not ER α or ER β , treatment with both E2 and G-1

increased proliferation, indicating that GPER signaling influences proliferation in breast tissue [135].

1.4.5.4 Fulvestrant

Fulvestrant, commonly known as ICI 182,780 (ICI) is a specific ER α antagonist. Sold under the trade name Falsodex, ICI is used as a treatment for ER-positive breast cancer in postmenopausal women. ICI is a steroidal molecule that contains a large, bulky side chain (Figure 1.3) that selectively binds ER α [136]. Following ICI binding, ER α is detected in the cytoplasm and nucleus and then quickly undergoes proteasomal degradation, resulting in undetectable levels of ER α protein. Interestingly, ICI demonstrates selectivity for the ER α isoforms, with higher affinity for ER α 66 than ER α 46, and no demonstrated binding to ER α 36 [81], potentially allowing for pharmacological targeting of these isoforms.



Figure

Chemical Structures of Specific Agonists and Antagonists. Propylpyrazoletriol (PPT) is an ER α -specific agonist, with a center pyrazole with a propyl chain and 3 phenol groups attached. Diarylpropionitrile (DPN) is an ER β -specific agonist, which is made of two phenol groups attached to a central propionitrile. The GPER agonist G1 is a substituted hydroquinolone. The selective ER α antagonist ICI results in proteasomal degradation of ER α , and contains a steroidal backbone with a large, bulky side chain.

1.3

1.5 Endocrine Disruptors

As defined by the United States Environmental Protection Agency (EPA), an endocrine disrupting compound is “an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process” [137]. There is increasing evidence that these environmental compounds, termed endocrine disrupting compounds (EDCs), have the potential to disrupt the endocrine systems in both humans and wildlife by disrupting many endogenous signaling pathways within an organism, including those that control reproduction, growth and development. In fact, both in *in vitro* and *in vivo* studies [138, 139] have demonstrated the ability of EDCs to disrupt many signaling pathways, including estrogenic signaling pathways.

1.5.1 Estrogenic Chemicals

EDCs such as genistein and diethylstilbestrol (DES) target nuclear hormone receptors, including estrogen receptors (ERs). Some EDCs exert their effects via non-genomic signaling pathways, including membrane bound ERs and GPER [140]. Adding to their complexity, different EDCs can exert their effects through various mechanisms. Exogenous estrogens such as DES can exert their effects through nuclear ER signaling, and bind estrogen receptor alpha with greater affinity than its endogenous hormone, estradiol [141].

Conversely, bisphenol A (BPA) and genistein bind to and activate GPER [140]. Because of the large number of chemicals that have been implicated as EDCs, there is an increasing need to study the safety of compounds and their effects on endocrine signaling.

Exposure to EDCs has been associated with adverse outcomes in human health, particularly in the well-known case of exposure to DES. DES is a potent synthetic estrogen that was prescribed to pregnant women between 1947 and 1971 to prevent miscarriages [142]. Exposure to DES was linked to higher risks for infertility in male offspring and reproductive cancers in female offspring of mothers prescribed DES, and has been associated with higher breast cancer rates in women exposed to DES [143, 144]. Many other xenoestrogens have been suggested to have effects in humans, including reproductive tract anomalies and increased risk of cancers of the reproductive tissues.

1.5.1.1 Bisphenol A (BPA)

A number of environmental compounds have been reported to act as EDCs, including dioxins, polycyclic aromatic hydrocarbons, BPA and isoflavones. BPA is an industrial chemical that has been produced for over 100 years, and was widely used in the production of polycarbonate plastics and epoxy resins [145]. BPA is ubiquitous in the environment, but is metabolized quickly in the body to a non-estrogenic BPA-glucuronide. A number of studies have

demonstrated that BPA can act as an endocrine disruptor in *in vitro* and *in vivo* models, though the results are highly contradictory[146-149]. BPA binds both ER α and ER β , though its affinity for each is reported to be 1,000 to 10,000 times lower than that of estradiol [150]. In addition, BPA is also binds GPER and ERR γ [151-155]. Based on these studies, BPA has a distinct mode of action and produces its own effects, independent of the signaling of the classical nuclear ERs.

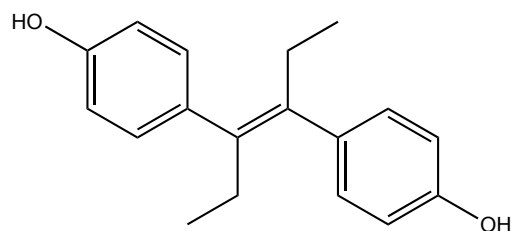
Despite its rapid metabolism and clearance, and low binding to estrogen receptors, BPA research is still contentious. Some researchers claim that there is a low-dose, non-monotonic dose response, well below that of the LOAEL. Several large-scale animal studies have been undertaken to examine a large range of doses and target tissues[156]. These guideline studies demonstrated effects of BPA at only the highest doses, far above the human exposure amounts.

1.5.1.2 Genistein (GEN)

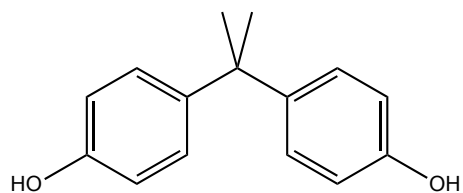
Another widely studied xenoestrogen is the soy isoflavone GEN. Genistein exposure has been linked to decreased risk of breast cancer development in epidemiologic studies[157], but has been associated with increased risk of endometrial hyperplasia in a 5 year prospective study [158]. Genistein has high affinity for ER β [159, 160], but at high concentrations (i.e. 1 μ M) can also activate ER α and initiate proliferation. The overexpression of ER β results in

reduced proliferation and reduced tumor formation of MCF-7 cells *in vivo*[161-164]. Genistein also activates ER β and the observed reduction of proliferation was due to inhibition of the S-phase transition [165] and G1 arrest[166].

Diethylstilbestrol



Bisphenol A



Genistein

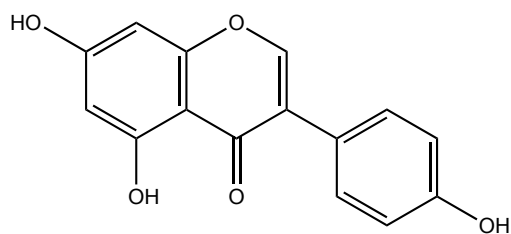


Figure 1.3 Chemical Structures of estrogenic EDCs. DES is a non-steroidal estrogen with high affinity for ER α . BPA contains two phenol groups, and has some reported binding to GPER. Genistein, a phytoestrogen and a soy isoflavone, binds strongly to ER β . Structures generated using ChemDraw Professional 15.0.

1.5.2 Endocrine Disruptor Screening Programs

These findings regarding DES, BPA and GEN, among others, have led to the development of programs to screen compounds for their endocrine disrupting potential. In addition to the ToxCast and Tox21 screening programs, the EPA has established the Endocrine Disruptor Screening Program (EDSP) to evaluate industrial and environmental chemicals for their potential to interfere with the endocrine system, particularly the estrogen, androgen and thyroid hormone pathways. Like the ToxCast and Tox21 programs, the EDSP relies on HTS and computational approaches.

The EDSP is tiered, with the Tier 1 assays focused on the broad identification of chemicals that have the potential to impact the endocrine system. The Tier 1 screening battery includes *in vitro* and *in vivo* assays, which limits the capacity of the system. The *in vitro* assays for estrogenic compounds include assays for ER binding and ER α transcriptional activation, and the *in vivo* assays include the rat uterotrophic and pubertal female assays and a short-term fish reproduction assay. The *in vitro* test systems are limited in that they do not assay directly for ER β , GPER and ERR related mechanisms, and many environmental compounds act through pathways independent of ER α . This limitation is addressed to some extent by the *in vivo* assays; however, the *in vivo* assays are not amenable to high-throughput screening, and are expensive.

1.6 MCF-7 Cells

Since being isolated from a pleural effusion of a female patient with metastatic mammary carcinoma and characterized in 1973 [167, 168], the MCF-7 cell line has been widely used to study breast cancer and estrogen signaling. MCF-7 cells express ER α [167], ER β [169] and GPER [170, 171], as well as many of the orphan estrogen receptors [172, 173]. Additionally, MCF-7 cells are the most commonly used breast epithelial cell line, despite their genomic abnormalities.

Attributed partially to their hyperploidy, stocks of MCF-7 cells can exhibit large intra- and inter-laboratory differences. In the 1980s, several publications described the variability of MCF-7 cells grown in different laboratories, with one study observing large biological differences in responsiveness to estrogen and morphology in MCF-7 cell stocks from different laboratories[174], while another study described the presence of subpopulations with varying rates of growth, DNA content and estrogen receptor expression within stocks from the same laboratory [175]. Often, the Short tandem repeat (STR) analysis employed to verify cell identity does not explain the differences in biology, so more comprehensive techniques have been used to investigate the genomic sources of these differences. Recently, several studies have demonstrated that comparative genomic hybridization (CGH), which allows for direct comparisons of chromosomal structure and specific regions from different sources. A study by Wenger et al. showed that

increased passage number correlated with increased changes in the genomic identity of the cells, with higher passage number MCF-7 and Ishikawa endometrial cells containing greater copy number variations than the original cell samples[176]. Numerous other reports have described large differences in CGH profiles of MCF-7 cells, and the changes are not consistent across labs or within labs[177-182]. While the large intra- and inter-laboratory variability of MCF-7 cells is concerning, MCF-7 cells are not the only cell line with this limitation. Studies using CGH have shown that other commonly used cell lines, including HeLa, also contain large cytogenetic differences[183, 184].

Despite the variability of MCF-7 cells grown in different laboratories, their usefulness and the extensive amount of literature published using them supports their use in *in vitro* studies of estrogen signaling and breast cancer biology. For more than 40 years, MCF-7 cells have been used to study breast cancer and estrogen biology, and there are over 24,000 citations in PubMed that reference this cell line[185]. The expression of estrogen receptors in MCF-7 cells, and their resultant sensitivity to estrogenic stimulation makes this cell line a useful tool to study the effects of estrogens *in vitro* [186-189]. MCF-7 cells have served as the cell platform for several endocrine disruptor screening assays, including the E-SCREEN [190] and the MCF-7 cell proliferation assay test nomination that recently failed validation. In addition to estrogen receptors, MCF-7 cells express progesterone receptor, and express

high levels of differentiation markers including e-cadherin, cytokeratin 18[191] and claudins[192]. The MCF-7 cell line grows easily in monolayer cultures, and also grows in 3D cultures systems [193-195].

For many applications, the benefits of using MCF-7 cells (responsiveness to estrogens and extensive existing literature) outweigh the limitations (high degree of variability). To control for genetic variability, appropriate experimental controls need to be used, and passage numbers limited.

While there exist a large number of breast cell lines in addition to MCF-7 cells (tumorigenic and nontumorigenic), many are not responsive to estrogen, while others are limited by strict growth requirements. The second-most studied breast cancer cell line is the ductal carcinoma cell line T-47D[196]. While T-47D is a cancer-derived cell line, it has fewer issues with genetic drift than the MCF-7 cell line[197]. T-47D cells are used as the cell line platform for the estrogen proliferation assay used in the EDSP battery. T47D cells have been used in 3D culture models, and typically form round structures [195]. Though T-47D cells are reported to have slightly higher expression of ER β [198], they have low levels of expression of GPER[199].

While extensive work has been performed using nontumorigenic, immortalized MCF-10A normal human cells, they do not express ER α or GPER, and express ER β [200]. Due to this limited expression profile, they were not used in the present study. Finally, nontransformed MCF-12A breast

cells are used to model breast cancer progression, though they are not without their limitations. While they express ER α , ER β and GPER[201], it is widely accepted that the expression levels are highly variable from lot to lot, making reproducibility a concern[202]. Finally, while primary human mammary epithelial cells (HMECs) isolated from reduction mammoplasty tissue are easily attainable, they are also highly variable from patient to patient, grow slowly, and are able to survive for only a limited time in 3D culture without being immortalized. Because of the breadth of the literature existing on MCF-7 cells, their expression of ER α , ER β and GPER, and the relative ease of working with them, coupled with the limitations of other available breast cell systems, MCF-7 cells were chosen for this work.

1.7 Relevance and Significance

There is a critical need to develop more relevant, inexpensive, higher-throughput models for toxicity testing. Large efforts are underway to address the backlog of over 80,000 chemicals that are lacking adequate safety data in the United States. While traditional 2D culture systems are easily adaptable to high-throughput screening platforms, they do not completely recapitulate the complexities of *in vivo* biology. In this thesis work, MCF-7 human breast cancer cells were grown in 3D using nonadhesive agarose hydrogels and characterized using a variety of markers to examine the differentiation of these microtissues compared to 2D cultures (**Specific Aim 1**). This system was used to evaluate the effects of specific estrogen receptor activation using

pharmacologic agonists, to establish models of pathway activation. Receptor-specific activation resulted in distinct morphologic and gene expression changes (**Specific Aim 2**). The use of this system to assess the estrogenic endocrine disruptors BPA and GEN provided new insights into the morphologic and molecular changes associated with exposure to EDCs (**Specific Aim 3**). Overall, the evaluation of this model has provided insight into the pathways activated by xenoestrogens, and has allowed for the assessment of both molecular and phenotypic changes in an *in vitro* assay.

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CHAPTER TWO

Into the Depths: Techniques for *In Vitro* Three-Dimensional Microtissue Visualization¹

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Into the Depths: Techniques for *In Vitro* Three-Dimensional Microtissue Visualization

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2.1 Abstract

Three-dimensional (3D) *in vitro* platforms have been shown to closely recapitulate human physiology, compared to conventional two-dimensional (2D) *in vitro* or *in vivo* animal model systems. This confers a substantial advantage in evaluating disease mechanisms, pharmaceutical drug discovery and toxicity testing. Despite the benefits of 3D cell culture, limitations in visualization and imaging of 3D microtissues present significant challenges. In this study, histology and microscopy techniques were refined and optimized to overcome the constraints of 3D imaging. For morphological assessment of 3D microtissues of several cell types, time points and sizes, a two-step glycol methacrylate embedding protocol for evaluating 3D microtissues improved resolution of nuclear and cellular histopathology characteristic of cell death and proliferation. Additional immunohistochemistry, immunofluorescence and *in situ* immunostaining techniques were successfully adapted to microtissues and enhanced by optical clearing. In utilizing the Clear^{T2} protocol, fluorescence signal intensity, imaging depth and clarity were greatly increased, allowing for more complete confocal fluorescence microscopy imaging of 3D microtissues compared to uncleared samples. The refined techniques presented address key challenges associated with imaging, providing new and alternative methods in evaluating disease pathogenesis, delineating toxicity pathways, and

enhancing the versatility of 3D *in vitro* testing systems in pharmacological and toxicological applications.

2.2 Introduction

Current strategies to study disease mechanisms, pharmaceutical drug discovery and toxicity testing rely on *in vivo* animal and two-dimensional (2D) *in vitro* models. Though commonly used to investigate disease pathogenesis and perform toxicity testing, animal models are not necessarily predictive human responses to exposure, and interspecies differences can have profound impacts on extrapolation or interpretation of data [2]. Animal models are limited by time intensive, costly and low throughput experiments, contributing to the large backlog of compounds that have not been adequately safety tested [3-7]. While *in vitro* based models address these limitations, 2D monolayer cultures lack the complex cell-cell interactions, intracellular junctions, cross-talk, and cellular morphology present in the *in vivo* environment [8, 9].

Since standard *in vitro* culture systems and *in vivo* animal models are encumbered in their ability to recapitulate human tissue responses, there exists a need to develop more complex, predictive systems to evaluate disease mechanisms and to serve as platforms for toxicological testing [8, 10, 11]. In 2007, the NRC report “Toxicity Testing in the 21st Century: A Vision and a Strategy” [12] called for a change in the current approach to toxicity testing by emphasizing the development and use of more relevant *in vitro* systems using human cells.

New approaches to three-dimensional (3D) cell culture have shown increased cell-cell interactions, intercellular junctions, and communication, providing a more physiologically relevant microsystem [9, 11, 13]. Toxicity testing of compounds, including chemotherapeutics and nanoparticles, has demonstrated differential responses of cells cultured in 2D vs. 3D [11, 14, 15]. However, the adaptation of 2D fluorescence or imaging based assays for use in 3D models, accentuates a serious caveat of 3D *in vitro* cell culture. The imaging and visualization of 3D structures remains a major challenge in 3D cell culture, as microtissue thickness, light scattering and impaired diffusion of reagents across multiple cell layers are contributing factors [16].

The purpose of the study is to adapt and refine techniques that can enable optimal visualization of microtissues to enhance imaging acquisition and assessment of morphological and toxicological outcomes that inform disease pathologies. To address the challenges associated with visualization, this study describes novel protocols in histopathology, and optical clearing methods for imaging of 3D microtissues to obtain morphological and functional endpoints. Overall, the improved imaging and generation of more intact data sets will lead to better use of 3D microtissues in assessing toxicity.

2.3 Materials and Methods

2.3.1 Culture Methods:

Culture methods and target cells are summarized in Table 2.1. BEAS-2B cells (ATCC, Manassas, VA, USA No. CRL-9609) immortalized human lung epithelial cells were cultured in BEGM media with Single Quot supplement kit (Lonza, Basel, Switzerland No. CC-3170) at 37°C in 5% CO₂. LNCaP (ATCC No. CRL-1740) human prostate adenocarcinoma cells were cultured using phenol-red free RPMI-1640 (Life Technologies, Grand Island, NY, USA, No. 11835) with 10% fetal bovine serum (FBS) and penicillin/streptomycin at 37°C in 5% CO₂. PLHC-1 (ATCC No. CRL-2406) fish hepatoma cells were cultured using EMEM media (ATCC No. 30-2003) with 5% FBS and penicillin/streptomycin, at 30°C in 5% CO₂.

2.3.2 3D Culture Methods:

Hydrogels containing 2% agarose were created from micromolds (Microtissues, Inc., Providence, RI, USA No. 24-9). Hydrogels were equilibrated with appropriate cell culture medium for a minimum of 30 minutes prior to cell seeding [17] and seeded into hydrogels at various densities (Table 2.1). After seeding, cells settled to the bottom of the small recesses and self-assembled into spheroids over the course of 24 hours.

2.3.3 Microtissue Histology:

Microtissues within agarose hydrogels were fixed in 10% neutral buffered formalin (Fisher Scientific, Agawam, MA, USA, No. 225) for a minimum of 24 hours prior to dehydration and processing. Microtissues within the agarose molds were then embedded in Technovit 7100 glycol methacrylate (Electron Microscopy Sciences, Hatfield, PA, USA, Heraeus Kulzer GmbH, No. 14653) per manufacturer's specifications with one modification. Samples were partially submerged in the infiltration solution and hardener 2 mixture to allow the sample to firmly attach to the bottom of the histology mold prior to filling the mold completely and adding a block holder. Samples were sectioned at a thickness of 3 μ m, after which samples were mounted on slides and stained with hematoxylin and eosin (H&E) for histological examination.

2.3.4 Preparation of Frozen Sections:

Microtissues within agarose hydrogels were fixed in 10% formalin overnight at +4°C. Following fixation and two washes with phosphate-buffered saline (PBS), samples were placed in a 15% sucrose/PBS solution for at least 3 hours at room temperature and then in a 30% sucrose/PBS solution overnight at +4°C. Samples were embedded in optimal cutting temperature compound (Fisher Scientific, Tissue-Tek, No. 14-373-65), frozen on dry ice before sectioning, and stored at -80°C until use. Samples were sectioned at 8 μ m and affixed to the slide before storage at -80°C until immunostaining.

2.3.5 Immunohistochemistry:

Frozen sections of microtissues affixed to slides were thawed, dried at room temperature and washed in PBS twice prior to antigen retrieval. For antigen retrieval, samples were placed in pre-heated 0.01M citrate buffer (pH 6.0) for 20 minutes and then allowed to cool for 20 minutes. After two washes in PBS (5 minutes each), slides were treated with a 3% peroxide/methanol solution to quench endogenous peroxidase activity. Sections were then incubated with Avidin/Biotin Block Kit (Vector Laboratories, Burlingame, CA, USA, No. SP-2100) as per manufacturer's specifications, and blocked in a 1% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA, No. A7906) and 15% goat serum (Sigma-Aldrich, No. G9023) solution for 1 hour at room temperature. Slides were incubated overnight with an E-cadherin primary antibody (BD Biosciences, San Jose, CA, USA, No. 610181, 1:6000 dilution) at +4°C. Following washes in PBS, samples were incubated with goat anti-mouse IgG biotinylated secondary antibody (Sigma-Aldrich, No. B0529, 1:1000 dilution) for 1 hour at room temperature and subsequent incubation with Avidin/Biotin Peroxidase Complex Kit (Vector Laboratories, PK-6100) as per manufacturer's recommendation. Detection of antibody expression was determined using the DAB Kit, (Vector Laboratories, No. SK-4100). Sections were then counterstained with hematoxylin and coverslipped with Cytoseal 60 (Thermo Fisher Scientific, Waltham, MA, USA No. 23-244257).

2.3.6 Immunofluorescence of Frozen Sections:

Frozen sections affixed to slides were allowed to warm to room temperature and then fixed for 5 minutes in 4% paraformaldehyde or 10% neutral buffered formalin. Slides were washed with PBS three times at room temperature, and then permeabilized with 0.25% Triton X-100 for 10 minutes at room temperature. Samples were washed in PBS as before, and blocked with 1% goat serum 3% BSA and 0.3 M glycine for 1 hour at room temperature. Slides were incubated overnight at +4°C with anti-E-cadherin antibody (1:500 dilution). Following incubation, slides were washed in PBS as described above and incubated with anti-mouse secondary (Life Technologies, No. A21236, 1:1000 dilution) for 2 hours at room temperature. For F-actin staining to visualize the cytoskeleton, sections were permeabilized as above and incubated with rhodamine phalloidin (Life Technologies, R415, 1:500 dilution) for 25 minutes. Slides were washed as above and stained with Hoechst 33342 (Life Technologies, No. H1399, 1:1000 dilution) for 30 minutes at room temperature. Coverslips were mounted using hard set mounting media (Vector Laboratories, No. H-1400) and stored until imaging in the dark at +4°C.

2.3.7 Immunofluorescence of Whole Spheroids:

Whole microtissues in agarose hydrogels were fixed in 10% formalin overnight at +4°C. Following fixation, gels were washed with PBS three times each (5 minutes each) at room temperature with gentle shaking. Spheroids were permeabilized by incubation in 0.25% Triton X-100 in PBS for

2 hours at room temperature and washed as described above. Samples were blocked for 1 hour at room temperature with 1% goat serum, 3% BSA and 0.3 M glycine, followed by incubation with anti-E-cadherin antibody (1:500 dilution) overnight at +4°C. Samples were then washed in PBS as previously described and incubated with anti-mouse secondary (1:1000 dilution) for 2 hours at room temperature, followed by washes as described above and counterstaining with Hoechst 33342 (1:2000 dilution) for 2 hours at room temperature, followed by additional PBS washes and protected from light during storage at +4°C until clearing. For Hoechst staining alone, previously fixed microtissues stored at +4°C were stained with Hoechst 33342 (1:2000 dilution) in PBS for 2 hours at room temperature.

2.3.8 Staining For Reactive Oxygen Species Generation

Beas-2B microtissues were grown for 4 days, and then treated with 50 μ M Menadione Sodium Bisulfite (Sigma-Aldrich, No. M5750) and counterstained with Hoechst 33342 (1:2000 dilution) for 1 hour. After 1 hour,, 5 μ M CellROX Green reagent (Life Technologies, No. C1044) was added to each sample for an additional hour as per manufacturer's suggestions. Samples were briefly washed in PBS twice for 5 minutes and fixed in 10% neutral buffered formalin for 1 hour before being cleared and imaged using the Zeiss LSM710 laser confocal microscope.

2.3.9 Clear^{T2} Optical Clearing:

Samples were cleared in a chemical fume hood using a modified version of the original Clear^{T2} protocol by Kuwajima *et al.* (2013). The methods were adapted to microtissues and required a 60 minute incubation of sample in the final solution of 50% formamide (Sigma-Aldrich, No. 47670) and 20% polyethylene glycol (Sigma-Aldrich, No. 89510). For imaging of intact microtissues, agarose hydrogels were inverted and placed into 35mm glass bottom dishes (MatTek, Ashland, MA, USA, No. P35G) to allow samples to fall out of hydrogels and onto the coverslips for imaging using laser scanning confocal microscopy and a 20x objective (Zeiss LSM710, Plan-Apochromat, 0.8 NA, 0.55 WD objective). During imaging, plates were sealed with parafilm to prevent evaporation of formamide.

2.4. Results and Discussion

The adaptation of 2D imaging-based assays to 3D culture models has presented significant issues including clear visualization and penetration of reagents into the cores of 3D microtissues. Addressing these challenges required the development of new approaches to evaluate relevant toxicological and morphological endpoints by merging standard *in vivo* histological techniques with faster *in vitro* assays, bridging the gap between *in vivo* and *in vitro* toxicological and pharmacological studies.

2.4.1 Improved Histological Techniques for Imaging of 3D Microtissues

Self-assembly of 3D microtissues created using non-adhesive agarose hydrogels [17] (Figure 2.1A) occurred over the course of the first 20 hours after seeding, a protocol which can be optimized to control spheroid size by regulating seeding density to yield a specific diameter at a given time point (Figure 2.1C). Traditional histological techniques were adapted to enable visualization of microtissue interiors. A two-step amendment to the Technovit 7100 plastic histology protocol enabled *en-bloc* embedding with minimal disruption to whole hydrogels containing an array of 256 microtissues per section (Figure 2.1A and 2.1B). H&E staining of 3 μ m BEAS-2B (Figure 2.1C, top panel) and LNCaP microtissue sections (Figure 2.1C, bottom panel) permitted close examination of nuclear, cellular and overall

tissue morphology. These key changes in cellular structure were easily distinguishable within these histological samples, emphasizing the advantage and clarity of the thin plastic sections in identifying key cellular and morphological features (Figure 2.1C).

Histopathological assessment of tissue-like interactions is important in assessing adverse endpoints as complex cellular interactions and tissue organization are lacking in traditional 2D *in vitro* cultures. Histology of microtissues at 7 and 14 days exhibited mitotic figures and long-term cell viability indicated by intact nuclear and cytoplasmic structures at 21 days in culture (inset image, Figure 2.1C). Although microtissues were viable after 21 days, both BEAS-2B and LNCaP cultures at 14 and 21 days contained areas of cell death and cellular debris at the core (inset image, Figure 2.1C). The observed cell death may be a result of microtissue size, as previous studies have reported a 100 μ m diffusion limit of oxygen and other nutrients within various 3D cell culture models [18, 19]. Exploiting these limitations may be advantageous in mimicking hypoxic tumor environments to study of tumor progression and cancer therapies [20-22]. These techniques may be applicable to other hydrogel and scaffold-free models, however, microtissues must undergo more arduous collection and embedding protocols for histology as displayed with PLHC-1 microtissues (Supplemental Figure 2.1). The ability to manipulate microtissue size, visualize microtissue cores, and assess morphologies using these refined histological techniques accentuates the

strength of 3D *in vitro* models as an alternative method to address a wide variety of pharmaceutical and toxicity testing needs.

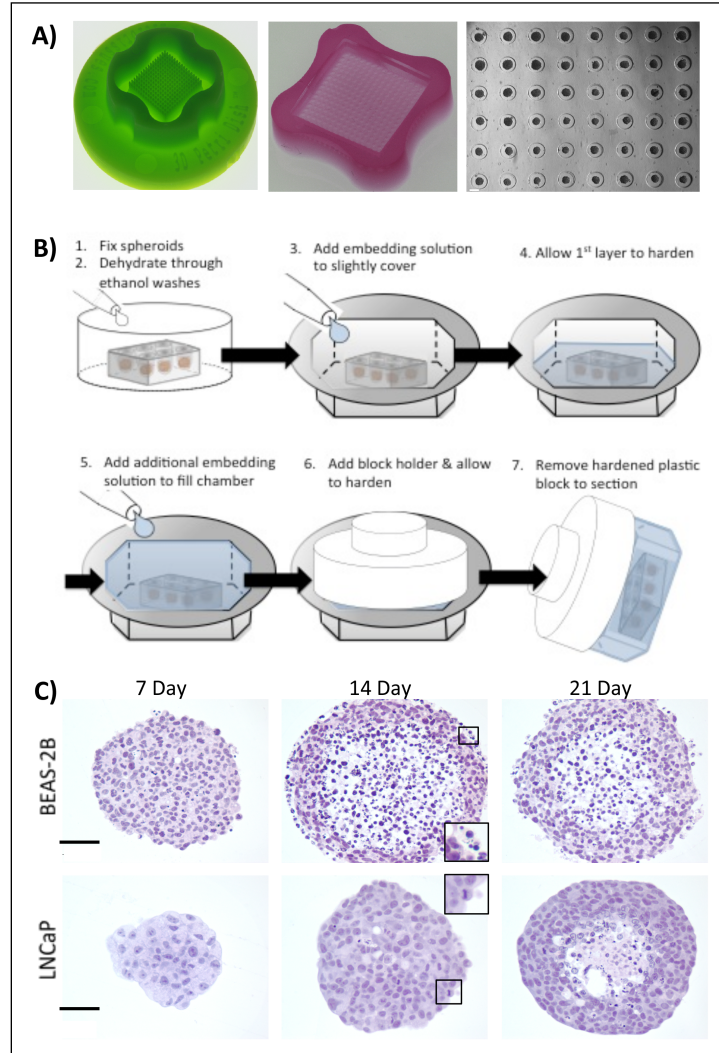


Figure 2.1 3D microtissue embedding and histopathology. (A) Micromolds were used to cast agarose hydrogels containing 256 round bottom recesses for microtissues formation. (B) An array of microtissues can be processed for imaging directly in the hydrogels as shown through the diagram for two step plastic embedding and histology. (C) H&E staining of BEAS-2B (top panel) and LNCaP (bottom panel) microtissues exhibit normal morphology at 7 days and proliferating and apoptotic cells after 14 days (shown in the figure insets). Scale bar = 50 μ m.

2.4.2 Immunostaining of 3D Microtissues

To demonstrate the versatility of 3D microtissues, new approaches to histological and immunostaining techniques were examined. Fixed agarose hydrogels containing microtissues were *en-bloc* embedded in OCT with a two-step embedding protocol similar to the glycol methacrylate embedding procedure. Immunofluorescence and immunohistochemistry of LNCaP microtissue sections stained strongly for epithelial cell marker E-cadherin at cell-cell junctions (Figure 2.2A and 2.2B). This technique was also validated using rhodamine phalloidin to visualize cellular F-actin staining and cytoskeletal organization in BEAS-2B microtissue sections (Figure 2.2C). Like tissue arrays, fixing, embedding and sectioning the whole agarose hydrogel also enables the staining, visualization and assessment of multiple microtissues within a single sample [23-25]. Additional samples fixed in Optimal Fix, an alcohol based fixative, were similarly embedded, but yielded poor results during cryosectioning due to a lack of proper OCT infiltration of the hydrogel (data not shown). The current study exhibits the utility and practicality of protein biomarkers assessed using immunostaining of 3D cultures to visualize specific structures and patterns of expression within microtissues.

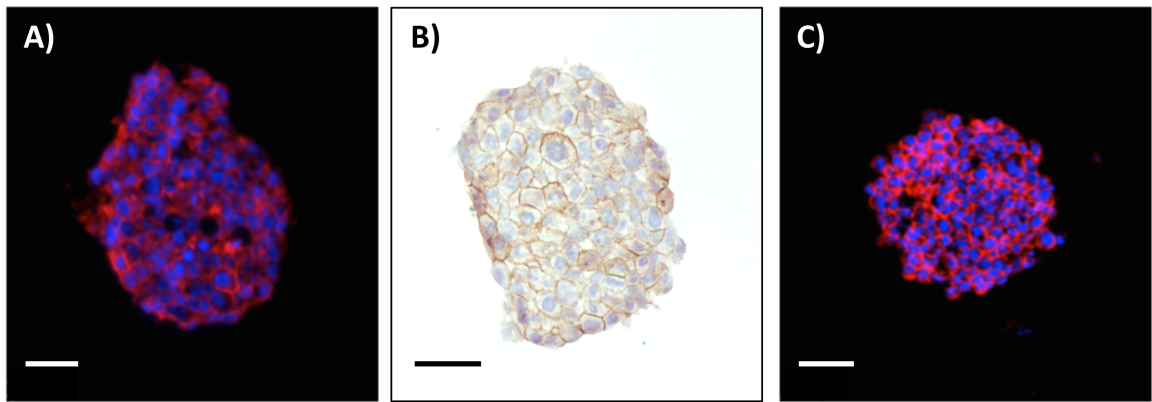


Figure 2.2 Immunostaining of frozen 3D microtissues sections at 3 days. E-cadherin was localized in LNCaP microtissues by immunofluorescence (A); E-cadherin antibody (red) and nuclear counterstain (blue)] or immunohistochemistry (B); E-cadherin staining (dark brown). BEAS-2B cells visualized using immunofluorescence [nuclear staining (blue) and F-actin staining with rhodamine phalloidin (red)]. Scale bar = 50 μ m.

2.4.3 Clearing for Improved Optical Imaging of 3D Microtissues

Imaging of intact 3D samples was greatly improved through the use of optical clearing protocols, allowing for faster and higher throughput fluorescent imaging of spheroids *in situ*. In this study, an organic solvent-free method for optical clearing, Clear^{T2}, improved imaging depth and greatly increased the number of discernable fluorescently labeled nuclei in PLHC-1 microtissues compared to uncleared samples imaged in PBS (Figure 2.3A). The Clear^{T2} protocol enabled sharper visualization of nuclear structure particularly in the center at a depth of more than 75µm along the z-axis, in stark contrast to nuclear staining of uncleared samples, which were obscured or indistinct at depths greater than 30µm (Figure 2.3A, Supplemental Videos 2.1A, 2.1B).

The Clear^{T2} method was also used in conjunction with immunostaining and biochemical staining of microtissues, demonstrating the advantages of this technique in evaluating important functional and toxicity endpoints. LNCaP microtissues, stained and cleared *in-situ*, showed localization of E-cadherin staining at cell-cell contacts (red) and clearly visible nuclear stain (gray) throughout each sequential image in the z-dimension following (Figure 2.3B, Supplemental Videos 2.2A, 2.2B). In addition to evaluating the formation of cell-cell junctions as a functional endpoint, toxicological endpoints such as oxidative stress can also be examined in intact spheroids. To induce the generation of reactive oxygen species, Beas-2B microtissues were treated with menadione sodium bisulfite, a compound known to redox cycle, prior to

staining with CellROX green for reactive oxygen species (ROS) generation and nuclear counterstaining (gray, Figure 2.3B). Cleared samples (Figure 2.3B, bottom panel) depicted increased optical imaging of the microtissue interior with more distinct cellular staining compared with uncleared samples in PBS (Figure 2.3B, top panel), indicating that dye penetration into the microtissue was not a limiting factor. However, optimization of immunostaining protocols was critical in achieving and alleviating the challenges of imaging in 3D. The use of un-optimized protocols did not display adequate immunostaining in microtissues despite the applied clearing method, emphasizing the necessity of refining techniques specifically for 3D *in vitro* assays (Supplemental Figure 2.2, Supplemental Video 2.3A and 2.3B). The staining and clearing of intact microtissues can be applied to other scaffold-free models through a more involved protocol as described in the present study (Supplemental Figure 2.3). The adaptation of the Clear^{T2} method successfully clear human and fish microtissues in less than two hours, validating the efficiency and efficacy of this technique in multiple species and establishing the potential of 3D *in vitro* testing platforms in areas of environmental and aquatic toxicology.

These results establish the first application of the Clear^{T2} method for increased visualization of fluorescence in non-neuronal microtissues and demonstrate enhanced resolution and imaging depth when visualizing intact 3D microtissues. A variety of clearing solutions, including solvent based

methods 3DISCO (3D imaging of solvent cleared organs) and BABB (a 1:2 mix of benzyl alcohol and benzyl benzoate), have previously been developed for the visualization of thick tissues and applied to a wide range of samples derived from brain, skin, mammary gland, immune organs, whole embryos and other tissues from *in vivo* and *in vitro* experiments [21, 26-31]. In contrast to other protocols, Clear^{T2} is a fast, detergent-free, immersion-based method, which are ideal characteristics for 3D *in vitro* applications and toxicity testing [16, 26-29, 32]. A recent study compared multiple clearing methods using neuronal microtissues and determined that Clear^{T2} increased the fluorescence signal throughout the microtissue without altering tissue size unlike other clearing protocols such as SeeDb and Scale. Improved resolution and increased imaging depth provides more complete imaging data sets, allowing for better quantification of morphological and phenotypic outcomes, with the potential to better understand heterogeneous cellular responses within a microtissue.

The use of non-adhesive micromold hydrogels gels to create 3D multicellular *in vitro* models is important platform with the capability to merge the complexities of *in vivo* tissues with the advantages and practicality of 2D *in vitro* models. The ability to clearly assess detailed morphology, evaluate longer-term endpoints with sub-lethal markers and discern adaptive responses from acute toxicity is an invaluable tool. The improved techniques presented in this study have addressed the visualization challenges of 3D

microtissues, highlighting their utility for evaluating classical pathological changes, and laying the foundation for alternative platforms as efficient and effective screening tools, with the potential to contribute to the ongoing revolution in toxicity testing [33].

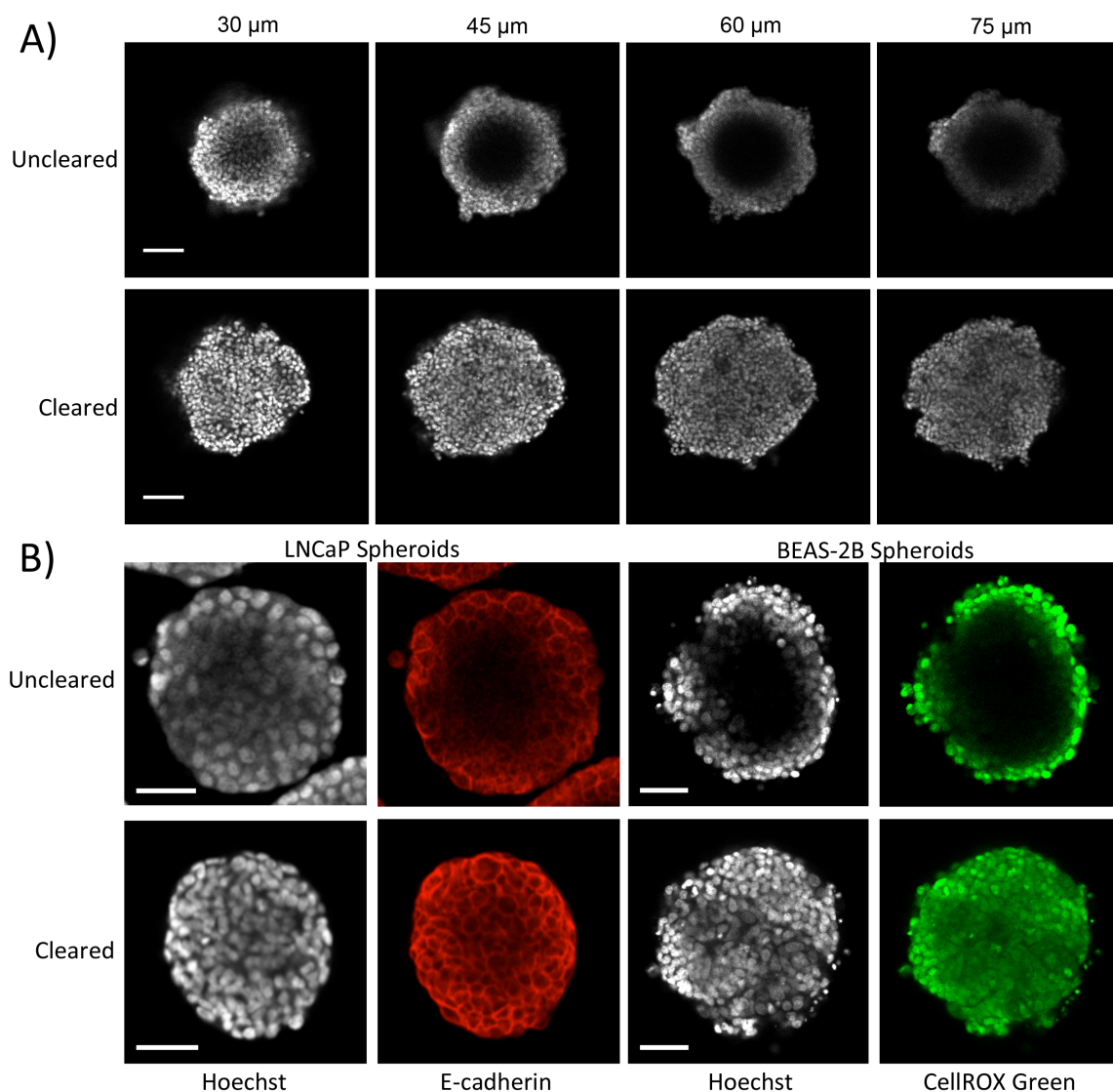


Figure 2.3 Confocal fluorescence imaging of spheroids is improved by clearing. PLHC-1 spheroids (A) were imaged in PBS without clearing (top panel) or formamide and PEG (bottom panel) after nuclear staining (blue). Each confocal cross section image (left-to-right) was taken every 15μm along the z-axis. Confocal cross sections (z-axis) through the middle of LNCaP and BEAS-2B spheroids (B) were imaged at 3 days after staining for E-cadherin (red), and CellROX Green, induced with 50μM Menadione treatment, respectively. Both microtissues were counterstained for nuclei (grey), and visualized in PBS without clearing (top panel) or cleared with formamide and PEG prior to imaging (bottom panel) Scale bar = 50μm.

2.5 Conclusions

The flexibility of the 3D in vitro cell culture hydrogel microtissue platform is a major advantage, allowing the use of various cell types with improved delineation of biological pathways and mechanisms implicated in disease pathogenesis and toxicity responses. The improved histological and visualization techniques described in this study enhance the application, validity and feasibility of 3D in vitro model systems. These models and imaging methods have potential for adaptation to high throughput screening of primary or immortalized cells, thereby increasing the human relevance, efficiency and efficacy of 3D microtissue models for evaluating disease mechanisms, pharmaceutical drug discovery and toxicity testing.

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2.8 Competing Interests

Kim Boekelheide is an occasional expert consultant for chemical and pharmaceutical companies, and owns stock in CytoSolv, an early stage biotechnology company developing a wound healing therapeutic.

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CHAPTER THREE

MCF-7 Human Breast Cancer Cells Form Differentiated Microtissues in Scaffold-Free Hydrogels¹

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MCF-7 Human Breast Cancer Cells Form Differentiated Microtissues in Scaffold-Free Hydrogels

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3.1 Abstract

Three-dimensional (3D) cultures are increasing in use because of their ability to represent *in vivo* human physiology when compared to monolayer two-dimensional (2D) cultures. When grown in 3D using scaffold-free agarose hydrogels, MCF-7 human breast cancer cells self-organize to form directionally-oriented microtissues that contain a luminal space, reminiscent of the *in vivo* structure of the mammary gland. When compared to MCF-7 cells cultured in 2D monolayer culture, MCF-7 microtissues exhibit increased mRNA expression of luminal epithelial markers keratin 8 and keratin 19 and decreased expression of basal marker keratin 14 and the mesenchymal marker vimentin. These 3D MCF-7 microtissues remain responsive to estrogens, as demonstrated by induction of known estrogen target mRNAs following exposure to 17 β -estradiol. Culture of MCF-7 cells in scaffold-free conditions allows for the formation of more differentiated, estrogen-responsive structures that are a more relevant system for evaluation of estrogenic compounds than traditional 2D models.

3.2 Introduction

There is a large backlog of compounds for which adequate safety information is lacking, due largely to the time-intensive and expensive nature of animal-based toxicity testing [2]. Because of issues with reproducibility and predictability of animal models, there is a growing need to develop more differentiated and physiologically relevant *in vitro* test systems. *In vitro* systems have traditionally relied on cells cultured as a monolayer on plastic substrates, in stark contrast to the cell- and extracellular matrix-dense tissues *in vivo*. To address this gap, recent work has focused on the use of human cells and cell lines in physiologically relevant cell culture systems, including microfluidic on-chip models [3-5], 3-dimensional (3D) scaffolded, extracellular matrix-based models [6-9], and scaffold-free models [10, 11]. While on-chip models have been demonstrated to reconstruct *in vivo* biology in a desirable manner, they are labor intensive and difficult to adapt to high-throughput screening systems. Scaffolded models using laminin or collagen have been used for 3D cultures; however, several cell types have been shown to exhibit different phenotypes on each matrix [7, 12]. Overall, 3D cultures are of increasing importance, as they have been demonstrated to up-regulate tissue specific markers, regain tissue-specific functions and have different gene expression profiles when compared to cells cultured in traditional 2-dimensional (2D) systems [13-15].

Many studies have focused on the use of Matrigel and other basement membrane-rich matrices to culture human breast cells in 3D. Both normal and cancerous human breast cells have been grown in matrix-based culture models, with non-malignant MCF-10A cells forming mammary acini containing luminal spaces when cultured in Matrigel, and malignant MDA-MB-231 cells forming disorganized clusters of cells [16, 17]. While matrix-based culture models allow for the growth of cell lines in 3 dimensions, they have several limitations. First, previous work has demonstrated that growth of fibroblasts on a collagen-rich matrix leads to a different phenotype when compared to growth on a laminin-rich matrix [18], which makes the selection of a relevant matrix an extremely important part of study design and interpretation of results. Additionally, Matrigel is derived from Englebreth-Swarm mouse sarcomas [8], calling into question the ability of this system to recapitulate more “normal” environments, and Matrigel exhibits lot-to-lot variability that has the potential to introduce large irregularities in the cell culture system. Finally, when using matrix-based culture models, cells are generally seeded at low densities, which is different from the highly cellular nature of epithelial tissues *in vivo*.

In contrast to the matrix-based 3D culture models, there has been an increase in use of scaffold-free culture models. Scaffold-free culture models such as hanging drop and roller cultures allow cells to maximize cell-cell contacts, and much like matrix-based 3D cultures, cells grown in scaffold-free

conditions generally up-regulate tissue-specific markers and become more differentiated than 2D cultures [10, 11, 15, 19]. However, it is difficult to control the size of aggregates formed using the roller culture method, calling into question the reproducibility of the method for uses including high-throughput screening. Additionally, models like the hanging drop, can be technically challenging to work with, as media changes and exposure to compounds can be difficult and time-consuming to perform, making these methods difficult to scale up for high-throughput experimentation [20].

Scaffold-free culture using non-adhesive agarose hydrogels allows cells to self-assemble and self-sort into 3D microtissues, free of the influence of extracellular matrix [10, 21-23]. Use of non-adhesive hydrogels allows for control of microtissue size, easy maintenance of the cultures, large numbers of microtissues per tissue plate, and the ability to easily culture multiple cell types in controlled ratios to form complex microtissues [10, 21]. Many cell types have been grown in these models, and the system produces large numbers of microtissues that are reproducible and maximize cell-cell contacts [10, 21].

A large majority of published *in vitro* studies focused on breast cancer and/or estrogen receptor biology have used the MCF-7 human breast cancer cell line [24-29]. MCF-7 cells are estrogen responsive, and are often used *in vitro* to study estrogen receptor positive breast cancers [30]. Despite their genomic instability, the sheer amount of existing literature makes MCF-7

cells a useful model to understand estrogen receptor and breast cancer biology. This study demonstrates that MCF-7 cells cultured in a 3D scaffold-free system using non-adhesive agarose hydrogels form microtissues that contain a luminal space. During culture in this system, MCF-7 cells up-regulate breast-specific markers when compared to traditional 2D culture systems. Additionally, 3D MCF-7 microtissues remain responsive to estrogen, an important benefit of using MCF-7 cells in this system. Furthermore, we find that the use of non-adhesive agarose hydrogels to culture breast epithelial cells results in a more differentiated, easy to manipulate cellular system, with potential application to studies in cell and cancer biology, and use as a screening platform.

3.3 Materials and Methods

3.3.1 Chemicals and Reagents

Cell culture media, supplements and SuperScript III First Strand Synthesis kit were purchased from Life Technologies, Inc (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Flowery Branch, GA) and dextran-coated-charcoal (DCC) stripped was purchased from Gemini Bioscience (Sacramento, CA). Estradiol (E2), dimethylsulfoxide (DMSO), Trizol and insulin were purchased from Sigma Aldrich (St. Louis, MO). Agarose was purchased from Fisher Scientific (Agawam, MA). Technovit 7100 was purchased from Heraeus Kulzer GmbH (Wehrheim, Germany).

3.3.2 Cell Culture

MCF-7 cells (HTB-22) [31] were purchased from ATCC (Manassas, VA) and maintained in DMEM-F12 complete media supplemented with 10% fetal bovine serum (FBS), MEM nonessential amino acids, gentamicin and 10 μ g/mL insulin in a 5%CO₂ incubator at 37°C. Media was changed every 2-3 days and cells were passaged when 65-80% confluent. MCF-7 cells were limited to use within the first 10 passages from the original purchased vial from ATCC, to control for genomic drift due to instability. As a comparison, MCF-7 cells purchased from the same lot from ATCC and cultured under the same media conditions were graciously provided by Dr. James Yager of Johns

Hopkins University. For 2D cell samples, MCF-7 cells were plated at a density of 300,000 cells/well in 6-well plates and allowed to grow for 3 days. Cells were scraped into Trizol for RNA analysis and stored at -80°C. For imaging studies, all MCF-7 cells were grown on poly-L-lysine coated coverslips for 3 days at a seeding density of 300,000 cells/well in 6 well plates.

3.3.3 3D Scaffold-Free Cell Culture

Non-adhesive agarose hydrogels were made using molds (Microtissues Inc, Providence RI), equilibrated and seeded with cells as previously described [10]. MCF-7 cells grown in 2D flasks were trypsinized, counted and seeded into agarose hydrogels at a density of 600,000 cells/mL in complete growth media. Cells were allowed to settle into recesses for 30 minutes, with 2mL complete media added for growth. Media was changed every 2-3 days until collection.

3.3.4 Estrogen Treatment of MCF-7 Cultures

For 2D samples, MCF-7 cells were seeded in 6-well plates at a density of 300,00 cells/well and allowed to grow for 3 days in 10% complete media. Cells were then placed in phenol-red free DMEM-F12 treatment media supplemented with 5% dextran-charcoal stripped fetal bovine serum, MEM nonessential amino acids, gentamicin and 6ng/mL bovine insulin for 48 hours. MCF-7 cells were seeded into agarose hydrogels and grown for 7 days in 10% complete media, as described previously, with media changes every 2-3 days. Following 7 days of growth, gels were washed twice with PBS and

transferred to fresh treatment media for 48 hours. Following 48 hours, MCF-7 2D and 3D microtissues were exposed to estradiol or vehicle control (dimethylsulfoxide) for 8 hours, and collected in Trizol for gene expression analysis.

3.3.5 Preparation of Frozen Sections

Microtissues within agarose hydrogels were fixed in 10% formalin overnight at +4°C. For autophagy staining, hydrogels were fixed in ice-cold methanol for 20 minutes. Following fixation and 2 washes with phosphate-buffered saline (PBS), samples were placed in a 15% sucrose/PBS solution for at least 3 hours at room temperature and then moved to a 30% sucrose/PBS solution overnight at +4°C. Samples were then placed in optimal cutting temperature compound (Fisher Scientific, Tissue-Tek, No. 14-373-65) and frozen on dry ice before sectioning. Embedded samples were stored at -80°C until use. Samples were sectioned at 8µm, melted to the slide and refrozen to affix to the slide. Slides were stored at -80°C until immunostaining.

3.3.6 Immunofluorescent Staining of Frozen Sections

Frozen sections affixed to slides were allowed to warm to room temperature and then fixed for 5 minutes in 4% paraformaldehyde. Slides were washed with PBS three times (5 minutes each) at room temperature, and then permeabilized with 0.25% Triton X-100 for 10 minutes at room temperature. Samples were washed in PBS as before, and blocked with 1% goat serum (Sigma, No. G9023), 3% bovine serum albumin (Sigma, No. A7906) and 0.3 M

glycine for 1 hour at room temperature. Slides were incubated overnight at +4°C with primary antibody at the dilutions summarized in Supplemental Table 1. Samples were washed with PBS and incubated with secondary antibody (Supplemental Table 3.1) for 1 hour. For F-actin staining to visualize the cytoskeleton, sections were incubated with rhodamine phalloidin (Invitrogen, R415, 1:500 dilution) for 25 minutes. Slides were washed as above and stained with Hoechst 33342 (Molecular Probes, No. H1399, 1:1000 dilution) for 30 minutes at room temperature. Coverslips were mounted using Prolong Gold Antifade reagent (Life Technologies, P36934) and stored until imaging in the dark at +4°C.

3.3.7 Immunofluorescent Staining of Monolayer Cultures

MCF-7 cells were seeded at low density and grown for 3 days in 10% complete media on poly-l-lysine coated coverslips. Coverslips were fixed in 4% paraformaldehyde for 10 minutes at room temperature and then washed twice with 1x PBS. Following washes, samples were permeabilized for 15 minutes in 0.25% Triton X-100, then washed with PBS. Samples were blocked with 1% goat serum (Sigma, No. G9023), 3% bovine serum albumin (Sigma, No. A7906) and 0.3 M glycine for 1 hour at room temperature. Slides were incubated overnight at +4°C with primary antibody at the dilutions summarized in Supplemental Table 1. Samples were washed with PBS and incubated with secondary antibody (Supplemental Table 3.1) for 1 hour. For

counterstaining, coverslips were incubated with rhodamine phalloidin and Hoechst 33342 as above. Coverslips were mounted as described above.

3.3.8 TUNEL Staining of Frozen Sections

Formaldehyde-fixed frozen sections were allowed to warm to room temperature, washed with PBS three times and then permeabilized for 10 minutes using 0.25.% Triton X-100. Sections were then stained using the Click-iT TUNEL Alexa Flour 647 Imaging Assay Kit (Life Technologies, C10247) and compared to DNase-treated positive controls. Coverslips were mounted using Prolong Gold Antifade reagent (Life Technologies, P36934) and stored until imaging in the dark at +4°C.

3.3.9 Staining and Imaging of 3D Intact Cultures

Samples were fixed *in situ* overnight in 4% paraformaldehyde (PFA) at 4°C. Following fixation, samples were washed 3 times in 1X PBS, permeabilized in 0.25% Triton X-100 for 15 minutes while rocking and washed again with PBS. Samples were incubated with rhodamine phalloidin (1:500 dilution, Invitrogen) for 30 minutes, stained with DAPI (1:1000, Invitrogen) for 15 minutes and stored in the dark at 4°C until imaging on an Olympus FV1000-MPE multiphoton microscope.

3.3.10 Histological Examination

For cells grown in 2D, cells on coverslips were fixed in 4% PFA for 10 minutes and stained with periodic acid-Schiff's reagent followed by hematoxylin counter stain (PASH). For 3D microtissues, samples were fixed in 4% PFA

overnight at 4°C, and then embedded in glycol methacrylate (Technovit 7100; Heraeus Kulzer GmbH, Wehrheim, Germany) for histological examination. Sections were cut at a thickness of 3µm, affixed to slides and stained with PASH. Samples were viewed and images taken on an Aperio ScanScope CS (Aperio Technologies, Vista, CA) at ×40 magnification.

3.3.11 Transmission Electron Microscopy

Microtissues were fixed for 2 hours at room temperature in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, with 0.1 M sucrose, pH 7.4. Samples were then rinsed with buffer and post fixed with 1% osmium tetroxide in buffer for 30 minutes at room temperature. Samples were dehydrated through a series of graded ethyl alcohols for 15 minutes each, with three changes of 100% ethanol for 10 minutes each. The samples were then infiltrated with 1 part Spurr embedding medium (Electron Microscopy Science, Hatfield, PA), and 2 parts ethanol for 3 hours, overnight with a 1:1 Spurr to ethanol, solution, placed in a 3:1 Spurr to ethanol solution for 3 hours and then 100% Spurr for 6-8 hours. Samples were then embedded in molds and polymerized at 60°C. Blocks were sectioned at a thickness of 85 nm on a Reichert Ultramicrotome with diamond knife, placed on copper grids, and stained with uranyl acetate and lead citrate. Sections were viewed with a Phillips 410 transmission electron microscope equipped with an Advantage HR CCD camera. Images were acquired with Advanced Microscopy Techniques imaging software.

2.3.12 Western Blot Analysis

Spheroids were prepared as previously described after 7 days of growth, and MCF-7 cells grown in 2D were scraped into PBS. Samples were collected, centrifuged and washed twice with ice-cold PBS. Samples were then lysed using RIPA buffer with HALT protease and phosphatase inhibitor (Thermo Scientific). Protein concentrations were measured using the DC Protein Assay (BioRad) per manufacturer's instructions. Samples were prepared and 20 µg loaded into SDS-PAGE gels. Following gel electrophoresis, a transfer was performed using PVDF membranes (Thermo Scientific). Membranes were blocked and probed with antibodies as described in Supplemental Table 3.2. Membranes were developed using ECL (Thermo Scientific). The results of 3 individual experiments were quantified using densitometry in ImageJ, as previously described [32].

2.3.13 RNA Isolation, RT-PCR and PCR Array Analysis

Microtissues were collected from hydrogels by centrifugation, pelleted and lysed in Trizol. Samples were then isolated using the RNEasy Mini Kit (Qiagen) per manufacturer's instructions. For use in quantitative real-time PCR (qRT-PCR), cDNA was made using the SuperScript III First Strand Synthesis system per manufacturer's instructions. qRT-PCR was performed to determine expression levels of progesterone receptor (PgR, forward primer (5' to 3'): ACCCGCCCTATCTCAACTACC, reverse primer: AGGACACCATAATGACAGCCT), growth response to estrogen in breast cancer (GREB, forward primer: AATGGGTCCGGCTGTTTTTC, reverse

primer: CCAGTTGTTGGCACTTCGG) and normalized to ribosomal protein, large P0 (RPLP0, forward primer: GTGTTCGACAATGGCAGCAT, reverse primer: GACACCCTCCAGGAAGCGA). The delta-delta-CT method was used to determine changes in mRNA expression compared to untreated control. Data was plotted in GraphPad Prism software and two-way ANOVA used to determine statistical significance between 2D and 3D samples.

A custom SABiosciences PCR Array (Qiagen) was created to include a list of 84 genes generated from literature to evaluate estrogen receptor signaling, breast and ductal morphogenesis, cellular growth and differentiation, proliferation, tumor progression and epithelial to mesenchymal transition as well as 5 housekeeping genes and contamination controls (Supplemental Table 3.3). Samples were prepared per manufacturer's instructions, and were added to 384-well plates using an epMotion 5075 automated pipettor (Eppendorf). Plates were run on an ABI 7900HT machine using cycling conditions recommended by the manufacturer.

3.3.14 ,Statistical Analysis

The raw PCR cycle (Ct) values were imported into the R statistical environment [33]. Raw PCR cycles were normalized (dCt) using the SLqPCR package [34] to optimize selection of house-keeping genes. To compare the expression of 2D cell cultures (day 3) against 3D culture cells (day 7), we used the limma package in R [35] to construct a linear model of the adjusted dCt values. Specifically we used the empirical Bayes statistic (eBayes) of the

linear model (lmfit) and then selected the genes with statistically significant changes between cultures as determined by an adjusted significance p-values for multiple experiments (q-value), $q \leq 0.05$ [36].

3.4 Results

3.4.1 Gross MCF-7 Microtissue Morphology

When grown on coated coverslips, MCF-7 cells exhibited typical 2D morphology, growing in mostly flat sheets with some piles of cells (Figures 3.1A and B). In contrast, MCF-7 cells sourced from Brown University and grown in non-adhesive agarose hydrogels formed 3D microtissues with multiple cellular aggregates per well containing luminal spaces following 7 days of culture (Figures 3.1 C and D). MCF-7 microtissues possessed luminal spaces surrounded by a cell layer 1-2 cells thick (Figure 3.1D). Following 7 days in culture, the luminal spaces contained periodic acid-Schiff's (PAS) positive secretions, indicating that carbohydrate-positive materials were being secreted into the lumens (Figure 3.1D). MCF-7 cells attained from Dr. James Yager were grown in 3D agarose hydrogels in identical culture conditions formed microtissues with cord-like structures and few luminal spaces following 5, 7, and 10 days of growth (Supplemental Figure 3.1A-C). Due to the luminal structure, Brown University MCF-7 cells were selected for further characterization.

Imaging of the intact microtissues stained with rhodamine phalloidin (actin cytoskeleton) and DAPI (nuclear) stains revealed that after 7 days in culture, MCF-7 cells aggregated and organized into microtissues (Figure

3.2A). MCF-7 cells self-assembled into multiple microtissues per well that were 70-150 μ m in diameter, and these microtissues contained luminal spaces (Figure 3.2, B-G).

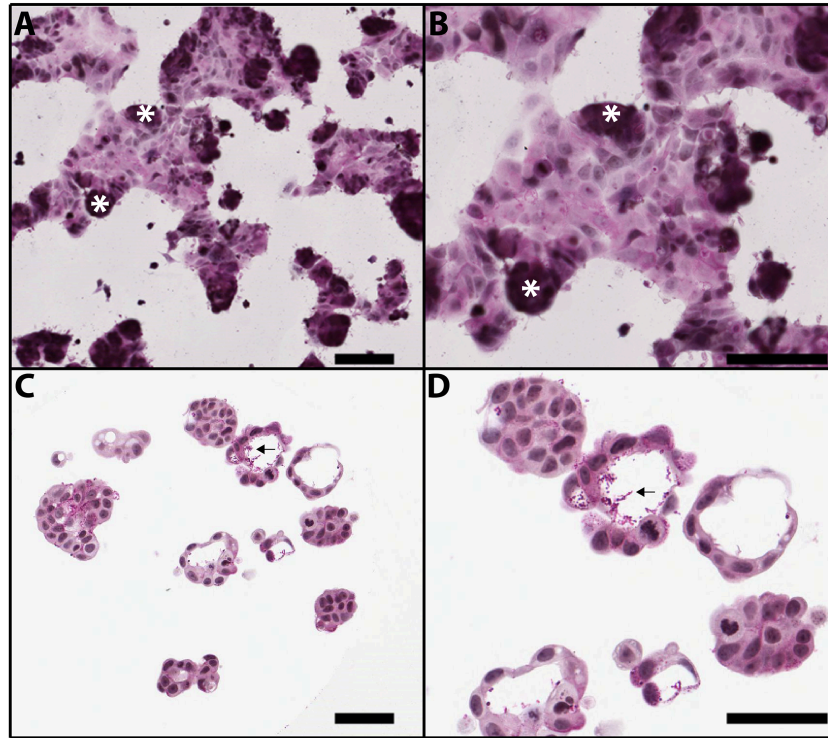


Figure 3.1 Morphology of MCF-7 cells grown in 2D and 3D cultures. MCF-7 human breast cancer cells grown in 2D monolayer culture for 3 days on poly-L-lysine coated coverslips and stained with periodic acid-Schiffs and hematoxylin (PASH) under low (A) and high (B) magnification. Cells exhibit typical cobblestone morphology, with several aggregates of cells (asterisks). MCF-7 human breast cancer cells grown in scaffold free non-adhesive agarose hydrogels for 7 days form microtissues with luminal spaces containing PAS-positive secretions (arrows) under low (C) and high (D) magnification. Scale bar = 50 μ m

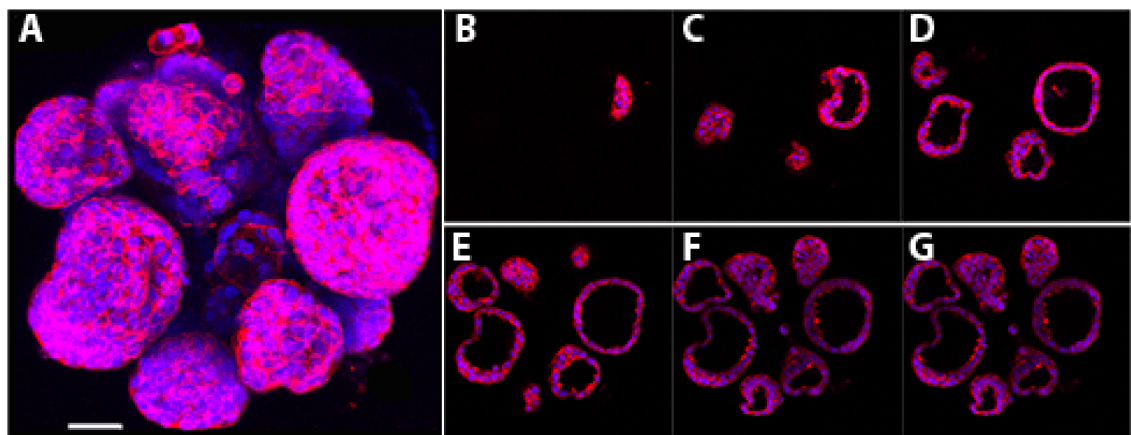


Figure 3.2 3D structure of 7-day MCF-7 microtissues. Intact MCF-7 microtissues were stained with rhodamine phalloidin (red; F-actin) and DAPI (blue; nuclear stain) and imaged on a confocal microscope, with steps taken in the Z direction every $5\mu\text{m}$. Individual images were compiled to make a maximal projection image, demonstrating the morphology of the microtissues (A). Sequential images at intervals of $15\mu\text{m}$ in the Z direction demonstrate that microtissues contain luminal spaces (B- $0\mu\text{m}$, C- $15\mu\text{m}$, D- $30\mu\text{m}$, E- $45\mu\text{m}$, F- $60\mu\text{m}$, G- $75\mu\text{m}$). Scale bar = $80\mu\text{m}$.

3.4.2 MCF-7 microtissue differentiation

MCF-7 cells grown for 3 days as monolayers or 7 days in 3D scaffold-free culture were stained for markers of breast and epithelial cell differentiation (Figure 3.3). Monolayer cultures of MCF-7 cells displayed diffuse staining for milk fat globule-EGF factor 8 protein (MFGES), while 3D cultures showed strong staining along the outside of the microtissues, with select cells staining more strongly. Mucin 1 (MUC1) was localized to the cell membrane of several cells in 2D cultures, while 3D cultures exhibited similar exterior localization of MUC1. Expression of trans-acting T-cell-specific transcription factor (GATA-3) was localized to the nuclei in both 2D and 3D cultures. The basal cell marker keratin 5 (KRT5) was diffusely distributed in MCF-7 monolayers, while in 3D cultures staining was localized close to the cytoskeleton of select cells. In 2D cultures, MCF-7 cells exhibited staining for the luminal epithelial marker keratin 8 (KRT8) at the leading cell edge, while there was high expression of KRT8 throughout MCF-7 microtissues. MCF-7 cells cultured in 2D displayed intense cytoplasmic staining for the epithelial marker E-cadherin (CDH1) while staining was localized to cell-cell contacts in 3D cultures. The mesenchymal marker vimentin (VIM) was present in the nuclear and cytoplasmic compartments of 2D MCF-7 cultures and was not present in 3D cultures. Golgi protein (GM130) showed diffuse staining throughout 2D cultures with small areas of punctate staining, while 3D cultures exhibited punctate staining with varying orientation, with many

cells having staining present on the exterior surface, and others with staining on the apical and lateral sides. Human disc large (hDlg) staining in 2D cultures was mostly localized at cell-cell contacts, but cells did display cytoplasmic staining. 3D cultures stained for hDlg at cell-cell contacts as well as the basolateral surface.

RT-PCR analysis using a custom PCR array (Supplemental Table 3.1) was performed to determine differences in gene expression between MCF-7 cells cultured for 3 days in 2D monolayer culture and MCF-7 cells cultured in 3D for 7 days in agarose hydrogels (Table 3.1 and Supplemental Table 3.4). Gene expression analysis revealed statistically significant up-regulation of keratin 8 (KRT8, $q=0.036$), keratin 19 (KRT19, $q=0.013$), snail family zinc finger 1 (SNAIL1, $q=0.023$), milk fat globule-EGF factor 8 protein (MFGE8, $q=0.033$), g-protein coupler estrogen receptor (GPER, $q=0.036$), leucine rich repeat containing 15 (LRRC15, $q=0.036$), mucin 1 (MUC1, $q=0.039$) and WNT1 inducible signaling pathway protein 2 (WISP2, $q=0.013$) in 3D MCF-7 microtissues compared to cells cultured in monolayer (Table 3.1). Additionally, mRNA expression of bone morphogenic protein 7 (BMP7, $q=0.0019$) cystatin E/M (CST6, $q=0.0065$), amphiregulin (AREG, $q=0.018$), apolipoprotein D (APOD, $q=0.023$), estrogen receptor beta (ESR2, $q=0.025$) and keratin 14 (KRT14, q) was significantly decreased in 3D cultures when compared to 2D cultures (Table 1). Expression of cytochrome P450 19A1 (CYP19A1, $q=0.033$), vimentin (VIM, $q=0.33$), FBJ murine osteosarcoma viral

oncogene homolog (FOS, $q=0.033$) and cyclin D2 (CCND2, $q=0.036$) was decreased in 7 day 3D cultures compared to cells grown in 2D monolayer (Table 3.1). Complete PCR results are summarized in Supplemental Table 3.4.

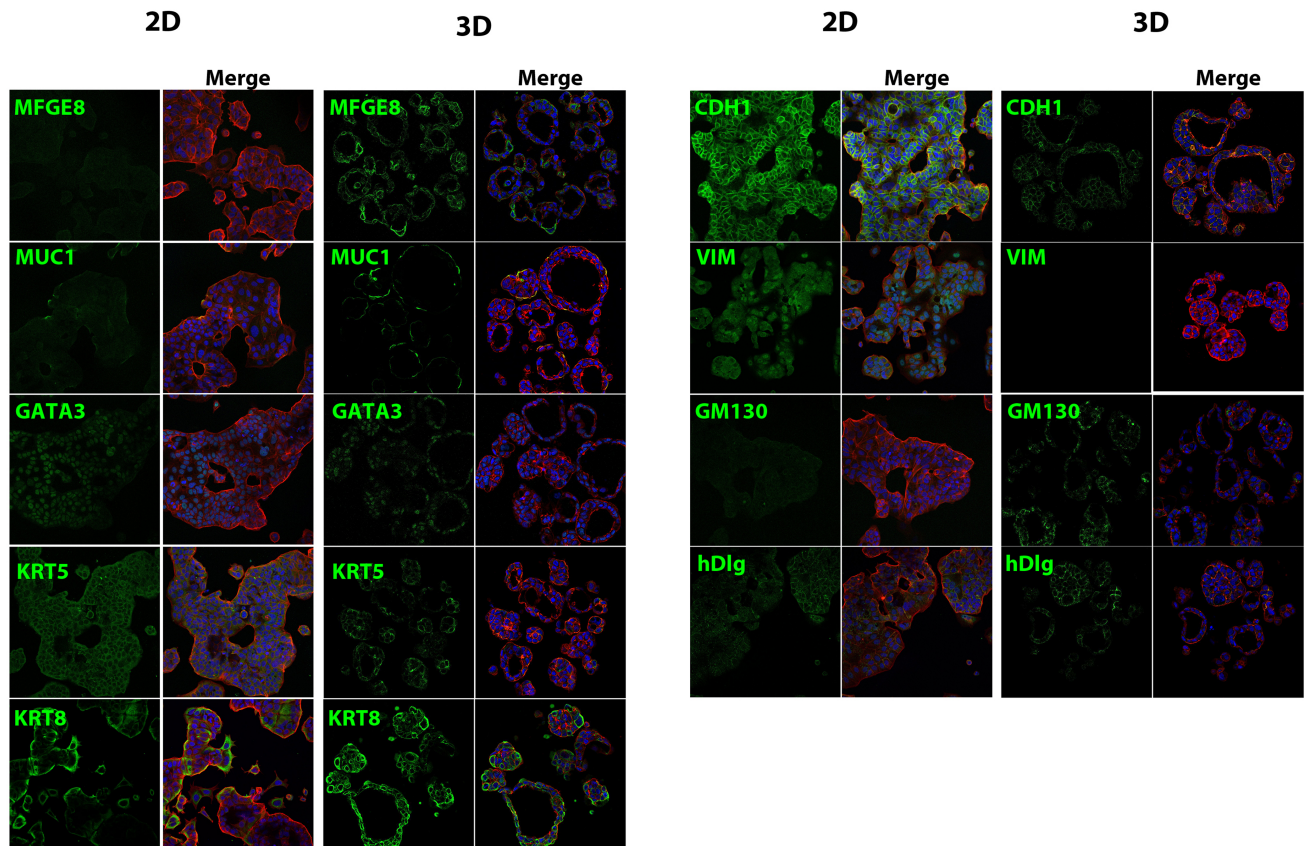


Figure 3.3 Expression of luminal and epithelial markers in 3D MCF-7 microtissues. Three day monolayer cultures and cryosections of MCF-7 cells grown for 7 days in agarose hydrogels were stained for epithelial and luminal markers (green), with f-actin rhodamine phalloidin (red) and Hoechst 33342 nuclear stain (blue) counterstains. Monolayer cultures exhibited diffuse staining for milk fat globule EGF8 (MFGE8) while 3D cultures showed intense, localized staining at the basal surface. Mucin 1 (MUC1) staining was faint and localized to select cells in 2D cultures with more intense basal staining in 3D microtissues. Expression of the luminal transcription factor trans-acting T-cell-specific transcription factor (GATA-3) was localized to the nucleus in both 2D and 3D cultures. Keratin 5 (KRT5) staining was diffuse in 2D cultures and more concentrated in specific cells in 3D cultures. There was high expression of keratin 8 (KRT8) in monolayer cultures, and throughout the microtissues (S, T). MCF-7 cells cultured in 2D demonstrated diffuse staining throughout the cytoplasm for e-cadherin (CDH1), while cells grown

in agarose hydrogels displayed localized at cell-cell contacts. Mesenchymal marker vimentin (VIM) was present in the cytoplasm and nucleus of monolayer cultures and not detectable in 3D cultures. The Golgi marker GM130 displayed diffuse staining in monolayer culture but was punctate in MCF-7 microtissues, with localization at the apical surface in select cells, and in other was basolaterally oriented. Human disc large (hDlg) displayed diffuse staining in 2D cultures, but was expressed at cell-cell contacts and the basal surface in 3D cultures.

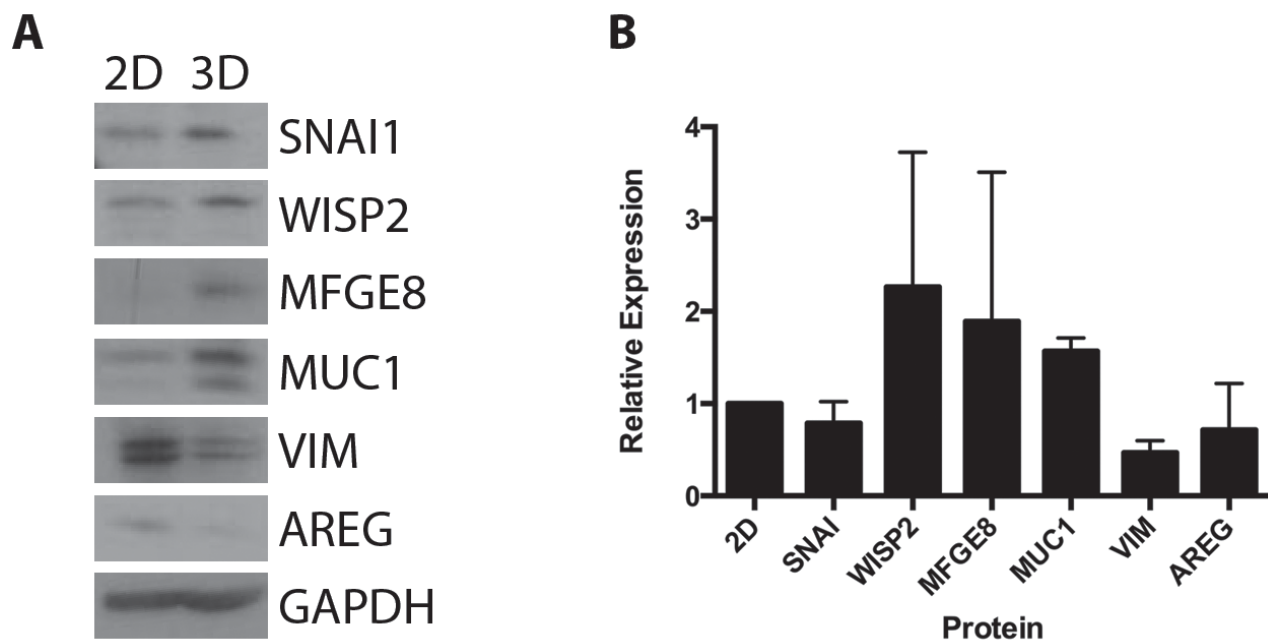


Figure 3.4 Protein expression of significantly increased transcripts in MCF-7 cells grown in 2D and 3D. MCF-7 cells were grown for 3 days in 2D culture or for 7 days in 3D culture. In agreement with increased mRNA expression, MCF-7 cells have increased protein expression of SNAI1, WISP2, MFGE8 and MUC1 and have decreased expression of VIM and AREG (A). Results from three individual experiments were quantified using ImageJ and analyzed using a one-way ANOVA (B), and are represented as relative expression compared to 2D cultures.

3.4.3 MCF-7 Microtissue Formation

To determine the timing of lumen formation in MCF-7 microtissues, samples were embedded, sectioned and stained with PASH after 1, 3, 5, and 7 days of growth in non-adhesive agarose hydrogels. MCF-7 microtissues aggregated into small groups after 24 hours in culture (Figure 3.5A,E) and these microtissues developed luminal spaces by 3 days in culture (Figure 3.5B,F). After 5 days in culture, luminal spaces were well developed, and began to accumulate PAS-positive secretions in the luminal spaces (Figure 3.5C,G), which were increased after 7 days (Figure 3.5D,H). There was no apoptotic or necrotic material in the luminal spaces of MCF-7 microtissues.

TEM was used to assess the ultrastructural morphology of MCF-7 microtissues and the PAS-positive secretions in the luminal spaces. MCF-7 cells within microtissues possessed intracellular secretory vesicles that were released into the luminal space (Figure 3.6A). Microtissues had microvilli on the interior apical surface, along with tight junctions present at the apical surface (Figure 3.6B, C, D, F, H). MCF-7 microtissues also contained lipid droplets (Figure 3.6E) and desmosomal junctions (Figure 3.6 F, G).

Staining for autophagy marker LC3 and TUNEL were used to determine the role of cell death in lumen formation. Following 3 days in culture (Figure 3.7A), MCF-7 microtissues demonstrated diffuse staining for LC3. Over time in culture, MCF-7 cells have increased, diffuse staining for LC3 at 5 (Figure 3.7B) and 7 days of culture (Figure 3.7C). Compared to the DNase-treated

positive control (Figure 3.7A, inset) TUNEL staining revealed no TUNEL-positive nuclei or debris (red) at 3, 5, or 7 days in culture (Figure 3.7D-F).

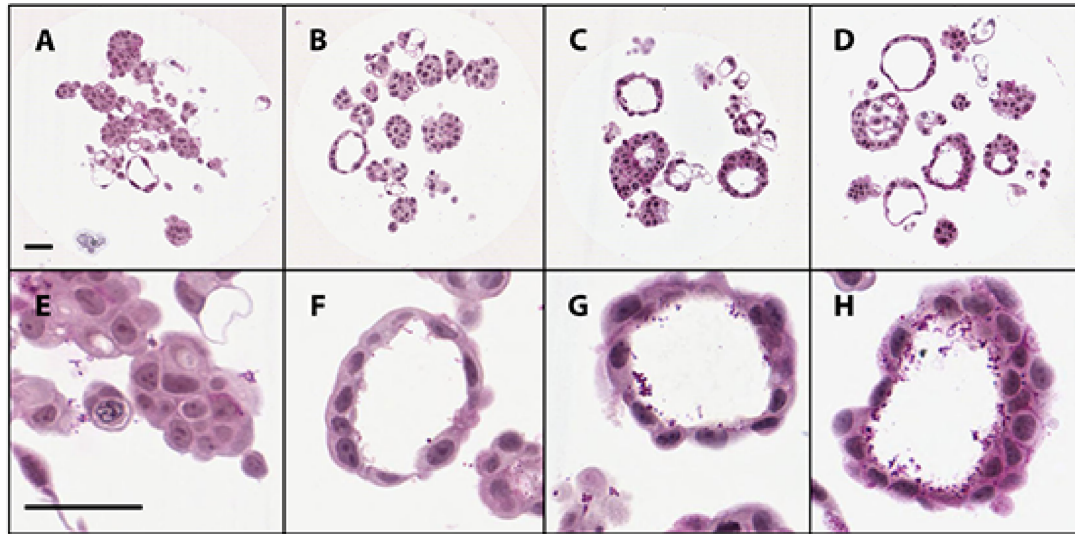


Figure 3.5 Morphology of MCF-7 microtissues over time in culture. MCF-7 cells aggregate into small groups forming microtissues following one day of growth (A, E) and have luminal spaces as early as 3 days in culture (B, F). Following 5 days in culture, luminal spaces are well developed and contain PAS-positive secretions (C, G). PAS-positive secretions continue to collect within the luminal spaces at 7 days (D, H). There is a lack of apoptotic debris in the luminal spaces of microtissues during this period. Scale bar = 50 μ m.

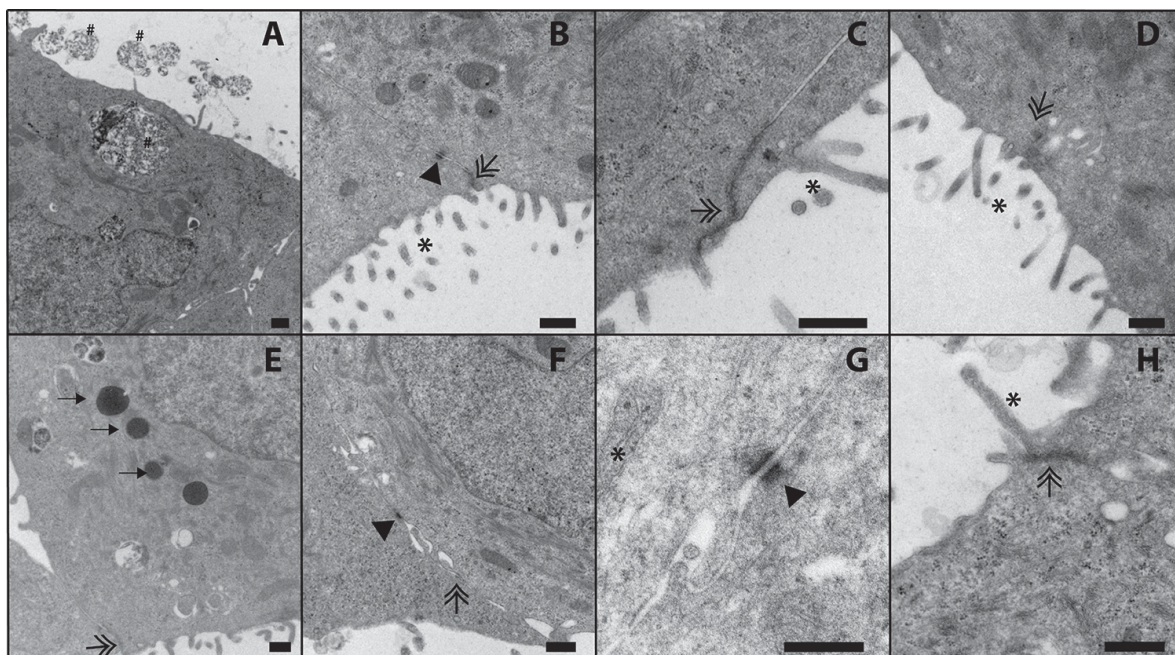


Figure 3.6. Transmission electron microscopy of 7 day MCF-7 microtissues. Following 7 days of growth in non-adhesive agarose hydrogels, 3D MCF-7 microtissues exhibit luminal secretions (A, hashtags), microvilli on the apical surface (B-D, asterisks), tight junctions (B-F, G, double arrows), desmosomes (B, F, G, arrowhead) and lipid droplets (E, arrows). Scale bar = 500nm.

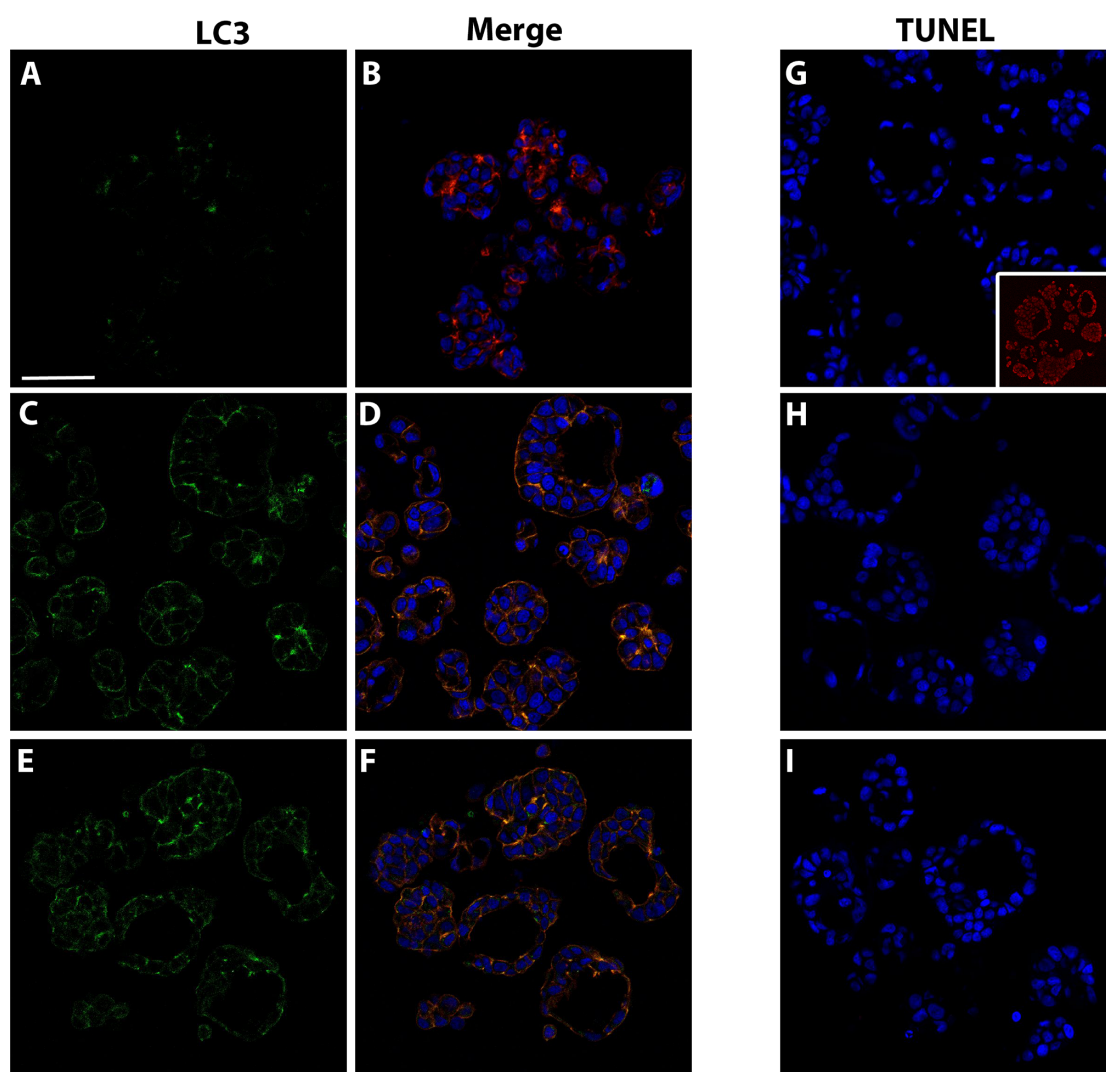


Figure 3.7. Lumen formation in MCF-7 microtissues is not a result of apoptosis or autophagy. Following 3 days in culture (A, B), LC3 staining is diffuse within cells. LC3 staining (green) increases over time in culture at 5 (C, D) and 7 (E, F) days, but is not localized to vacuoles. MCF-7 cells cultured for 3(G), 5 (H) and 7(I) days do not possess any TUNEL positive nuclei (red) when compared to DNase-treated positive control (D, inset). Scale bar = 80 μ m.

3.5 Estrogen responsiveness of MCF-7 microtissues

To determine 3D MCF-7 microtissue responsiveness to estrogen compared to 2D cultures, 2D and 3D samples were treated with 0.01, 0.1 and 1nM concentrations of 17 β -estradiol for 8 hours (Figure 3.8). Estrogen responsiveness was assessed by RT-PCR for known targets of estrogen receptor, including estrogen-induced growth, breast cancer (GREB) and progesterone receptor (PGR). Monolayer and microtissue MCF-7 cultures responded in a concentration dependent manner to 0.01, 0.1 and 1nM estradiol as demonstrated by increased GREB (Figure 3.8A) and PGR (Figure 3.8B) expression compared to untreated, time-matched control samples. Monolayer MCF-7 cells demonstrated significantly higher expression of both PGR and GREB than 3D cultures following exposure to estradiol, particularly at 0.1 and 1 nM concentrations.

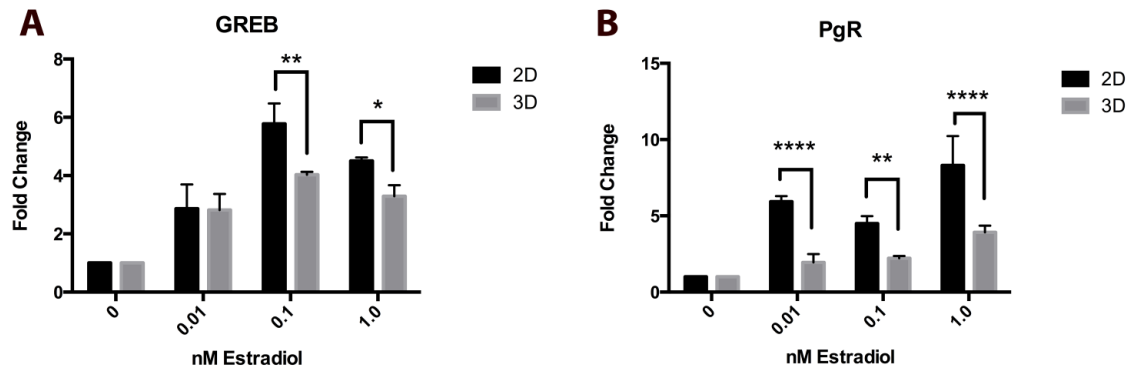


Figure 3.8. Estradiol responsiveness of MCF-7 2D and 3D cultures. 2D and 3D samples are responsive to 17β -estradiol in a concentration-dependent manner, as evidenced by increased expression of GREB (A) and PGR (B) following 8 hours of exposure. MCF-7 monolayer and hydrogel culture respond to estradiol, with monolayer cultures responding more robustly. *= $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$

Full Name	Abbreviation	q-value	fold change
Snail family zinc finger 1	SNAI1	0.02339	47.18
Keratin 19	KRT19	0.01305	3.56
Wnt-induced signaling protein 2	WISP2	0.01305	3.50
Leucine-rich repeat containing 15	LRRC15	0.03640	2.91
Milk fat globule-EGF factor 8	MFGE8	0.03253	2.56
G-protein coupled estrogen receptor	GPER	0.03613	2.48
Keratin 8	KRT8	0.03613	2.29
Mucin 1	MUC1	0.03950	2.21
Cyclin D2	CCND2	0.03613	-2.31
Vimentin	VIM	0.03253	-2.56
FBJ murine osteosarcoma viral oncogene homolog	FOS	0.03253	-2.57
Amphiregulin	AREG	0.01822	-3.20
Apolipoprotein D	APOD	0.02258	-3.98
Estrogen receptor beta	ESR2	0.02545	-4.23
Cytochrome P450 19A1	CYP19A1	0.03269	-4.27
Cystatin E/M	CST6	0.00649	-4.35
Bone morphogenic protein 7	BMP7	0.00190	-6.94
Keratin 14	KRT14	0.03613	-29.19

Table 3.1 Gene expression of 7 day MCF-7 microtissues compared to 3 day monolayer cultures. MCF-7 cells were grown in 3D for 7 days and compared to cells grown in 2D for 3 days; a positive fold-change indicates increased expression in 3D compared to 2D.

3.5 Discussion

When cultured in a scaffold-free, non-adhesive agarose hydrogel system, MCF-7 breast carcinoma cells derived in our lab self-assemble and organize into microtissue structures with luminal spaces surrounded by 1-2 epithelial cell layers, in contrast with the typical 2D morphology which consists of cells growing in a cobblestone-like pattern. The luminal structure of the MCF-7 microtissues shares features with both the *in vivo* morphology of the normal adult breast epithelium that has been previously described [37] and the mammary acini formed by normal MCF-10A cells grown in Matrigel [16]. As a comparison, MCF-7 cells purchased from the same lot from ATCC and derived using identical protocols were obtained from the Yager lab and cultured in this model. Interestingly, the sublines derived at each location exhibit different morphologies when cultured in scaffold-free hydrogels, indicating that the 3D structures formed in this model are due to properties intrinsic to the cells, and are not a result of the culture system.

In agreement with previous work based on MCF-10A cells grown in Matrigel, MCF-7 cell microtissues demonstrate intense staining for MUC1 and MFGE8 localized within selected cells [16], indicating that MCF-7 cells cultured in this model have functions similar to those seen in non-malignant MCF10A cells. However, within MCF-10A 3D cultures, MUC1 and MFGE8 are located at the apical surface, while MCF-7 cells within this model show localization at the basolateral surface. When compared to the staining profile in 2D cells,

MCF-7 cells exhibit stronger, localized staining for both of these markers, correlating with the increased mRNA expression detected in the present study.

Additionally, the staining pattern for GM130 was diffuse in 2D MCF-7 cultures, and in 3D cultures cells displayed punctate, directional staining. When compared to 2D cultures, the staining for these markers is more localized and directional, indicating that 3D culture alters the secretory pathways and allows MCF-7 cells to begin to polarize. GATA3 expression and localization in the nucleus indicates that MCF-7 microtissues are forming luminal-like tissues, as GATA3 has been shown to be a key regulator in luminal fate and differentiation [38]. Coupled with GATA3 expression, MCF-7 cells in 3D stain intensely for luminal cytokeratin 8 [39, 40]. The epithelial phenotype marker CDH1 is more strongly localized to cell-cell contacts and strongly expressed in 3D cultures, compared to the cytoplasmic staining observed in 2D MCF-7 cultures. This is corroborated by the high expression of the mesenchymal marker VIM in 2D cultures. The strong staining for KRT8 is confounded by positive staining for the basal marker KRT5; however, KRT5 staining is decreased and more localized in 3D cultures than in 2D, suggesting that over time in 3D culture, MCF-7 cells are exhibiting a more luminal phenotype.

This finding can also be explained by the heterogeneity observed in MCF-7 cells, such as KRT5-positive MCF-7 sublines that may correspond to therapy-

resistant cell types in breast cancers [41]. Based on the staining of these markers, MCF-7 cells cultured in agarose hydrogels for 7 days differentiate and exhibit several characteristics of luminal-type cells when compared to 2D MCF-7 cultures. It is worth noting that the current study examines cultures of different ages, and this may contribute to some of the differences observed in marker expression. However, based on the histology and organization of the 3D microtissues and expression of markers, it can be concluded that the 3D MCF-7 microtissues are more differentiated than 2D cultures.

As expected, MCF-7 cells cultured as monolayers have different gene expression patterns compared to those cultured in 3D. After 7 days in scaffold-free culture, MCF-7 microtissues show increased expression of mammary epithelial associated genes KRT8, KRT19, MFGE8 and MUC1 and decreased expression of VIM, KRT14 and BMP7. The increased expression of KRT8 in concert with decreased expression of KRT14 in 7 day MCF-7 microtissues suggests that the cells are further differentiating into luminal-like epithelial cells when compared to MCF-7 cells grown in 2D monolayer culture, since breast luminal epithelial cells express high levels of KRT8 and KRT19 while basal cells express KRT14 [39, 42]. Increased expression of MUC1 mRNA and protein indicates that cells cultured in 3D are further differentiated and may be increasing their secretory functions[40]. Previous work examining mammary gland remodeling has indicated that MFGE8, a milk glycoprotein, is highly expressed in the mammary epithelium, and plays

an essential role in mammary gland involution, and the increased expression of this mRNA and protein in 3D MCF-7 microtissues signals that within this model, cells are further differentiating and undergoing a unique morphogenic process that cannot be modeled in 2D systems [43]. The increased expression of MUC1 and MFGE8 proteins coupled with the basolateral, directional staining profiles during culture in 3D implies that cells within this model have secretory function, in contrast to monolayer cultures that display diffuse GM130 and MFGE8 staining. Combined with the presence of secretory vesicles evident by both PASH staining and TEM, this data demonstrates that MCF-7 microtissues are more differentiated in 3D scaffold-free culture than in 2D culture.

The development and remodeling of the mammary epithelia is a dynamic process dependent on many molecular pathways. Coupled with the increased expression of luminal markers is the decreased expression of APOD. APOD is involved in lipid transport, specifically cholesterol transport, and is decreased in 7day 3D cultures. In a recent study of differentiating HC11 mammary epithelial cells, alterations in phospholipid biosynthesis were observed, demonstrated by a decrease in many markers of cholesterol transport including APOD during mammary differentiation [44].

Also indicating that MCF-7 3D cultures are more differentiated than 2D monolayer cultures is the change in expression of several markers of cell invasion and growth, including SNAI1, VIM, BMP7, CCND2, and WISP2.

Compared to 2D cultures, the transcriptional regulator SNAI1 is highly expressed, seemingly confounding the observation above that cells are more differentiated as demonstrated by increased KRT8 and KRT19 mRNA expression, as increased SNAI1 expression typically correlates with increased invasive potential [45]. Increased SNAI1 mRNA expression observed in this study counters the decreased expression of the mesenchymal marker VIM, and the non-significant changes in CDH1 expression. Additionally, despite increased SNAI1 mRNA, there is no significant increase in SNAI1 protein in 3D MCF-7 cultures. We hypothesize that this signaling pathway may be dysregulated in these cells, and this warrants further investigation. However, based on recent studies, it is possible that the increase in SNAI1 mRNA expression observed in 3D MCF-7 microtissues may be linked to the morphogenic changes observed over time in these cultures. Previous work has implicated SNAI1 and other Snail family members in mammary morphogenesis, specifically in the process of tubulogenesis [46]. SNAI1 has also been shown to directly regulate CYP19A1, repressing its expression [47]. Increased CYP19A1 expression has been correlated with poor prognosis in breast cancer [48], and the decreased expression observed in the current study further supports that MCF-7 cells are becoming more differentiated when cultured in scaffold-free conditions. Mesenchymal marker VIM is also down-regulated in 3D microtissues when compared to cells cultured in 2D, indicating that MCF-7 cells cultured in 3D are more epithelial-like. In this

model, MCF-7 cells decrease expression of the cell cycle regulator CCND2, indicating that cells are changing their rate of cell division, compared to cells in 2D [49], corroborating previous work using MCF-7 cells in a scaffold-based 3D model [50]. Additionally, increased expression of WISP2 in 7 day 3D cultures indicates that the cultures are altering their growth patterns, perhaps by slowing cell cycle progression [51]. Previous research has demonstrated that a loss of WISP2 expression promotes epithelial-to-mesenchymal transition in MCF-7 breast cancer cells, leading to invasive growth, while overexpression in invasive MDA-MB-231 cells inhibits proliferation and reduces cell migration [52]. The increased expression of WISP2 in 3D culture coupled with the altered gene expression of classical cell cycle regulator CCND2, further supports the notion that MCF-7 microtissues do not become more invasive in this model, and instead are slowing proliferation and becoming more epithelial-like, similar to previous work in 3D models [51]. The significantly decreased expression of BMP7 supports this interpretation, as BMP7 expression has been correlated with mesenchymal-to-epithelial transition and increased cell growth [53]. It appears that despite being cancer cells, MCF-7 microtissues slow their proliferation to reach a more quiescent state, in agreement with previous work in normal breast cells cultured in 3D in basement membrane [54] when compared to 2D monolayer culture.

MCF-7 microtissues contained PAS positive secretions in their luminal spaces, suggesting that these cells had secretory function following 7 days in culture. The quantity of PAS positive foci in the luminal space increased as culture time increased, and the preferential secretion into the luminal space demonstrated that MCF-7 cells within this model were organized with distinct directionality, a feature strongly lacking in most monolayer cultures. To better characterize the polarization of MCF-7 cells cultured within this model, microtissues were analyzed using TEM. The presence of microvilli on the interior, luminal cell surface in 7 day MCF-7 microtissues indicates that the cells were undergoing polarization, with the apical surface facing the luminal space and the basal-like surface on the exterior of the microtissue. As revealed by TEM, MCF-7 cells also contained membrane-bound secretions in the luminal space. Madin-Darby canine kidney (MDCK) epithelial cells grown in 3D are polarized, and have been used to demonstrate the important role of cell polarity on formation of functional epithelial barriers, proper cellular transport [55] and cell-type specific functions [56, 57]. This previous work has also demonstrated that loss of cell polarity can lead to disease pathogenesis, calling into question the use of non-polarized 2D cell culture methods to study disease states, and lending credibility to the use of polarized 3D models to study both disease progression and potential therapeutics. Unlike MCF-10A cells, MCF-7 cells within this model appear to be hemi-polarized, with microvilli and PAS-positive secretions present on the interior, apical surface.

GM130, MFGE8 and MUC1 staining appear to reveal inverted polarity of MCF-7 microtissues compared to MCF-10A acini in Matrigel, however, this is not surprising due to the cancerous origins of the MCF-7 cell line. The ability of MCF-7 cells to polarize in this model without added extracellular matrix is an important finding, as the use of a scaffold-free culture system eliminates the difficulty of choosing an appropriate matrix material. Previous studies have demonstrated that MCF-7 cells cultured on collagen I have reduced viability compared to those cultured on reconstituted basement membrane [58], indicating that the selection of matrix is of importance and can influence downstream results including gene expression [59]. The use of a scaffold-free culture model allows the focus to be placed on cell-cell interactions, in contrast to focus on cell-matrix interactions in many 3D models.

Interestingly, MCF-7 microtissues displayed no apoptotic cells in the luminal space, in contrast to previous work using the nonmalignant human breast cell line MCF-10A. When grown in 3D in Matrigel, MCF-10A cells organize into cellular aggregates and then undergo apoptosis to form acini containing luminal spaces [16, 60]. The lack of TUNEL-positive nuclei and apoptotic debris in the lumens of MCF-7 microtissues suggests that the luminal spaces may form in a process similar to cyst formation seen with MDCK cells cultured in 3D with Matrigel [61]. The closer resemblance of MCF-7 microtissues to 3D MDCK cultures than MCF-10A cultures may be due to the lack of caspase-3 expression in MCF-7 cells [62]. In the present study, pro-

apoptotic transcripts BAD, BAX and CASP3 and anti-apoptotic transcripts BCL2 and BCL2L2 were not significantly changed between 2D and 3D samples, further indicating that MCF-7 microtissues do not undergo lumen formation in an apoptosis-dependent manner. Staining for the autophagy marker LC3 increased over time from days 3 to 7 in culture, but this staining was diffuse throughout the cells, and did not stain any material in the luminal spaces of microtissues. In contrast to previous work using MCF-7 cells to form spheroids in non-adherent plates [63], MCF-7 microtissues in the present study did not display large autophagic vacuoles or autolysosomes as detectable by TEM. Past studies have demonstrated that combined inhibition of both autophagy and apoptosis is not sufficient to inhibit luminal clearing in MCF-10A acini [60], indicating that other pathways may play a role in lumen formation in epithelial tissues [64, 65]. While this observation warrants further investigation, this system may be useful in studying pathways of lumen formation in the mammary gland.

The use of estrogen receptor positive MCF-7 cells [66] in this model provides a unique 3D system for assessing estrogen signaling and the effects of estrogen on mammary microtissues. To determine if MCF-7 cells cultured in 3D maintain their estrogen responsiveness, microtissues were treated with 17 β -estradiol for 8 hours, and gene expression of known estrogen targets was examined. When compared to untreated controls, MCF-7 cells demonstrate a robust response to estrogen stimulation, indicated by significantly increased

expression of PGR and GREB. However, 2D cultures exhibited a stronger induction of GREB and PGR, targets of estrogen receptors. This correlates with the observation that within this model, MCF-7 cells cultured in agarose hydrogels significantly decrease expression of ESR2. Despite this, MCF-7 microtissues remain responsive to estrogens. The use of more physiologically relevant MCF-7 3D cultures in place of 2D cultures to assess estrogenic activity of compounds has the potential to provide more complete information for screening purposes, and may allow for the evaluation of morphological endpoints in addition to molecular changes.

The current study presents the first characterization of MCF-7 microtissues grown in 3D using non-adhesive agarose hydrogels. Microtissues formed in this model are more differentiated than 2D cultures as demonstrated by both their structural and molecular profiles. The more differentiated state of MCF7 cells grown as 3D microtissues allows for their protracted use as breast cell models in the investigation of estrogen receptor biology, therapeutics, and the toxicological effects of estrogens and xenoestrogens. The formation of differentiated structures free of the influence of external matrices demonstrates the utility and simplicity of this system, a system amenable to high-throughput analysis.

3.6 Conclusions

The present paper reports the successful development of a xenotransplant model for prostate proliferation and maturation that maximizes the benefits of using a rodent host in laboratory-based research while ensuring that the data is not skewed by species-specific variations in prostatic tissue. The use of human fetal tissue maintains the clinical relevance of the data and makes it possible to monitor the whole-system development of the prostate from the fetal stages into adulthood. The description of this model, using various cell type and differentiation specific markers, demonstrated that these human fetal xenografts are capable of progressive differentiation and growth, production of secretions, and expression of estrogen and androgen receptors. DNA methylation revealed an increase in epithelial gene expression and stromal methylation in 30 and 90-day xenografts, thus providing new information into prostatic development. With confirmation that this model recapitulates the normal processes of growth and maturation of the human prostate, future research will explore various hormonal manipulations to identify the potential etiology of prostate cancer and evaluate different therapies for prevention and treatment that are stage-specific and targeted.

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3.8 Competing Interests

Kim Boekelheide is an occasional expert consultant for chemical and pharmaceutical companies, and owns stock in CytoSolv, an early stage biotechnology company developing a wound healing therapeutic.

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CHAPTER FOUR

Three-Dimensional MCF-7 Human Breast Carcinoma Microtissues Respond Differently to Estradiol Exposure Compared to Monolayer Cultures

Three-Dimensional MCF-7 Human Breast Carcinoma Microtissues Respond Differently to Estradiol Exposure Compared to Monolayer Cultures

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4.1 Abstract

The development of three-dimensional (3D) cultures is increasing, as they are able to provide the utility of *in vitro* models and the strength of testing in physiologically relevant systems. When cultured in a scaffold-free agarose hydrogel system, MCF-7 human breast carcinoma cells organize and develop into microtissues that contain a luminal space, in stark contrast to the flat morphology of MCF-7 two-dimensional (2D) monolayer cultures. Following exposure to 1nM E2, expression of typical estrogen-responsive genes, including progesterone receptor (PGR), PDZ containing domain 1 (PDZK1) and amphiregulin (AREG) is increased in both 2D and 3D cultures. When examining expression of other genes, particularly those involved in cell adhesion, there were large changes in 3D MCF-7 microtissues, with little to no change observed in the MCF-7 monolayer cultures. Together, these results indicate that while the initial estrogen-regulated transcriptional targets respond similarly in 2D and 3D cultures, there are large differences in activation of other pathways related to cell-cell interactions.

4.2 Introduction

As a result of the National Research Council report “Toxicity Testing in the 21st Century: A Vision and a Strategy,” [1] there is a large need to develop more efficient and physiologically relevant models for evaluation of compound safety and toxicity. Due to the cost- and time-intensive nature of animal testing, in vitro based screening models are growing in favor. Additionally, in vitro cell culture models allow for the use of human cell lines, enabling researchers to better evaluate human health and disease states when compared to in vivo animal models.

Traditionally, human primary cells and cell lines have been cultured in 2-dimensional (2D) monolayer on plastic substrates. While 2D cultures are technically easy to use and inexpensive, they do not recapitulate the biology of cells in vivo, where tissues are highly cell dense and cells are in contact with other cell types and extracellular matrix (ECM). Previous work has demonstrated that this contact with ECM and cells can alter gene expression profiles and cellular morphology [2-5], potentially leading to differences in experimental results between 2D in vitro and intact in vivo systems.

As an alternative strategy to address several of the shortcomings of both in vivo and 2D in vitro experimental models, 3-dimensional (3D) culture models have been developed. A variety of models exist, with two of the most popular models being scaffold-based and scaffold-free systems. In scaffold-based 3D

culture, cells of interest are seeded at low density within natural or synthetic matrices. When cultured in Matrigel, a laminin-rich matrix, both normal and malignant human breast cells form 3D cultures with morphologies dependent on their gene expression profiles [3]. Nonmalignant MCF-10A cells cultured in Matrigel have been demonstrated to form lumen-containing mammary acini through apoptosis, a structure reminiscent of the *in vivo* morphology of the adult mammary gland [6]. When transformed into malignant cells, MCF-10A cells become invasive, distorting the structure and morphology of the *in vitro* mammary acini in a process that promotes oncogenesis [6-8]. This and similar systems have provided important insight into the process of both morphogenesis and oncogenesis; however, these models are not without limitations. Typically, cells are seeded embedded within matrix at low densities that do not mimic the highly cellular nature of epithelial tissues *in vivo*. Scaffold-based models are highly dependent on the nature of the scaffold material, as previous work has shown that different matrices including collagen I and laminin can have distinct effects on cells, including altering their invasive potential and gene expression profiles [9-12]. Many scaffold materials do not accurately reconstruct the *in vivo* ECM content, and often rely on only one component such as collagen, while ignoring the heterogeneous nature of native ECM.

Scaffold-free culture models including hanging drop and non-adhesive agarose hydrogels allow for cells to assemble into 3D microtissues free from

the influence of outside matrix. While the hanging drop method requires no specialized equipment to form 3D cultures, it is technically difficult to change media and the low media to cell ratio increases the rate of waste accumulation, making long-term cultures difficult to assess. Additionally, hanging drop cultures are susceptible to being physically disturbed, and this can lead to a loss of samples through handling. The use of nonadhesive agarose hydrogels to culture cells in a scaffold-free environment circumvents these problems, as the system allows for easy media changes and handling of cultures. In this system, cells are trypsinized and seeded into non-adhesive hydrogels, where they settle into recesses and come into contact with other cells [13]. Within 24 hours after seeding, cells self-assemble into microtissues free of the influence of outside matrix and substrates [14]. Previous work using MCF-7 cells has demonstrated that when cultured in this model, MCF-7 cells form 3D microtissues that are more differentiated than 2D cultures, have apical-basal polarity and contain a luminal space, reminiscent of the in vivo morphology of the adult mammary gland [15].

Despite cells stocks being highly variable from lab to lab [16, 17] and tumor-derived[18], MCF-7 human breast carcinoma cells are the most widely used breast cell line. MCF-7 cells are particularly attractive when studying estrogenic pathways due to their expression of estrogen receptors alpha[19, 20], beta [19, 21] and the g-protein coupled estrogen receptor[22]. MCF-7 cells have served as the cell platform for several endocrine disruptor screening

assays, including the E-SCREEN [23] and the MCF-7 cell proliferation assay test nomination that recently failed validation. In addition to estrogen receptors, MCF-7 cells express progesterone receptor, and express high levels of differentiation markers including e-cadherin, cytokeratin 18[24] and claudins[25].

In the present study, using a custom PCR array designed to examine a range of targets involved in cell adhesion, estrogen signaling, cell death and cell cycle progression[15, 26], we describe differences in gene expression between 2D monolayer and 3D MCF-7 microtissue culture gene expression following exposure to estradiol (E2). The present study examines the use of MCF-7 cells as a simple, differentiated cell system to detect and measure estrogenic responses. Our results indicate that E2 treatment alters expression of classical estrogen responsive genes in a similar manner in both 2D and 3D cultures. However, with longer exposure to E2, the similarities of the response in both culture systems subside and the responses diverge.

4.3 Materials and Methods

4.3.1 Chemicals and Reagents

Cell culture media and supplements were purchased from Life Technologies, Inc (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Flowery Branch, GA) and dextran-coated-charcoal (DCC) stripped was purchased from Gemini Bioscience (Sacramento, CA). Estradiol (E2), tamoxifen (TAM), dimethylsulfoxide (DMSO), Trizol and insulin were purchased from Sigma Aldrich (St. Louis, MO). Agarose was purchased from Fisher Scientific (Agawam, MA).

4.3.2 Cell Culture

MCF-7 cells (HTB-22) [16] were purchased from ATCC (Manassas, VA) and maintained in DMEM-F12 complete media supplemented with 10% fetal bovine serum (FBS), MEM nonessential amino acids, gentamicin and 10 μ g/mL insulin in a 5%CO₂ incubator at 37°C as previously described [15]. Media was changed every 2-3 days and cells were passaged when 65-80% confluent. MCF-7 cells were limited to use within the first 10 passages from the original purchased vial from ATCC, to control for genomic drift due to instability.

4.3.3 3D Scaffold-Free Cell Culture

MCF-7 cells were seeded into agarose hydrogels prepared as previously described [15, 17] at a density of 600,000 cells/mL. Cells were allowed to settle into wells for 30 minutes and then placed in 2mL complete growth media, with media changes every 2-3 days until estrogen treatment.

4.3.4 Estrogen Treatment of MCF-7 Cultures

For monolayer cultures, MCF-7 cells were seeded at a density of 300,000 cells/mL in 6-well plates, and allowed to grow for 3 days in complete media. Cells were then washed twice with PBS and placed in phenol red-free DMEMF12 media supplemented with 5% dextran-charcoal stripped fetal bovine serum, MEM nonessential amino acids, gentamicin and 6ng/mL bovine insulin for 48 hours. For 3D cultures, cells were seeded into agarose hydrogels as above and grown for 7 days in 10% complete media, with media changed every 2-3 days. After 7 days of growth in complete media, 3D MCF-7 cultures were washed twice with PBS and transferred to fresh treatment media for 48 hours. Following 48 hours, 2D and 3D cultures were exposed to estradiol or vehicle control (dimethylsulfoxide) for 8 hours and collected in Trizol for gene expression analysis.

4.3.5 RNA Isolation, RT-PCR and PCR Array Analysis

Monolayer cultures were collected by scraping into Trizol. Microtissues were collected from hydrogels by centrifugation, pelleted and lysed in Trizol.

Samples were then isolated using the RNEasy Mini Kit (Qiagen) per manufacturer's instructions. Data was plotted in GraphPad Prism software and two-way ANOVA used to determine statistical significance between 2D and 3D samples.

A previously described [15] custom SABiosciences PCR Array (Qiagen) was created to include a list of 84 genes generated from literature to evaluate estrogen receptor signaling, breast and ductal morphogenesis, cellular growth and differentiation, proliferation, tumor progression and epithelial to mesenchymal transition as well as 5 housekeeping genes and contamination controls (Supplemental Table 3). Samples were prepared per manufacturer's instructions, and were added to 384-well plates using an epMotion 5075 automated pipettor (Eppendorf). Plates were run on an ABI 7900HT machine using cycling conditions recommended by the manufacturer.

4.3.6 Statistical Analysis

The raw PCR cycle (Ct) values were imported into the R statistical environment [18]. Raw PCR cycles were normalized (dCt) using the SLqPCR package [19] to optimize selection of house-keeping genes. To compare 2D and 3D vehicle-treated controls, dCt values were plotted in GraphPad Prism software and two-way ANOVA used to determine statistical significance ($p > 0.05$), with complete ANOVA results reported in Supplemental Table 2.

For comparison of E2 treated samples to respective vehicle controls and comparison of normalized E2-treated 2D and 3D samples, dCt were used to construct a linear model of adjusted Ct values using the LIMMA package in R [20]. The empirical Bayes statistic was used and genes with an adjusted p-value significance for multiple comparisons (q-value) or less than 0.05 and absolute fold change of greater than 1.5 were selected.

4.3.7 Cluster Analysis

Fold change values for all significantly changed genes across time and culture method were log transformed and imported into Cluster [21], and clustered using Euclidean distance and complete linkage analysis. Values were imported into Treeview and visualized.

4.4 Results

When compared to vehicle-treated controls, MCF-7 monolayer cultures exposed to 1nM estradiol (E2) for 4 hours exhibit significant changes in expression of 19 transcripts as summarized in Table 1. E2 increased expression of insulin-like growth factor binding protein 4 (IGFBP4), progesterone receptor (PGR), V-Myc avian myelocytomatosis viral oncogene homolog (MYC), transforming growth factor alpha (TGFA) and FBJ murine osteosarcoma viral oncogene homolog (FOS). Treatment of MCF-7 monolayer cultures with E2 for 4 hours also increased expression of growth response to estrogen breast cancer (GREB1), PDZ-domain containing 1 (PDZK1), cyclin D1 (CCND1), snail family zinc finger 1 (SNAI1), transforming growth factor beta 3 (TGFB3) and xbox-binding protein 1 (XBP1). E2 treatment also increased expression of vascular endothelial growth factor alpha (VEGFA), amphiregulin (AREG), and wingless-type MMTV integration site family member 4 (WNT4). Expression of BCL2-like 1 (BCL2L1), Erb-B2 receptor tyrosine kinase 2 (ERBB2), twist family transcription factor 1 (TWIST1), g-protein coupled estrogen receptor (GPER) and cytochrome P450 1A1, (CYP1A1) were significantly decreased following exposure to estradiol. Complete gene expression results are reported in S2 Table.

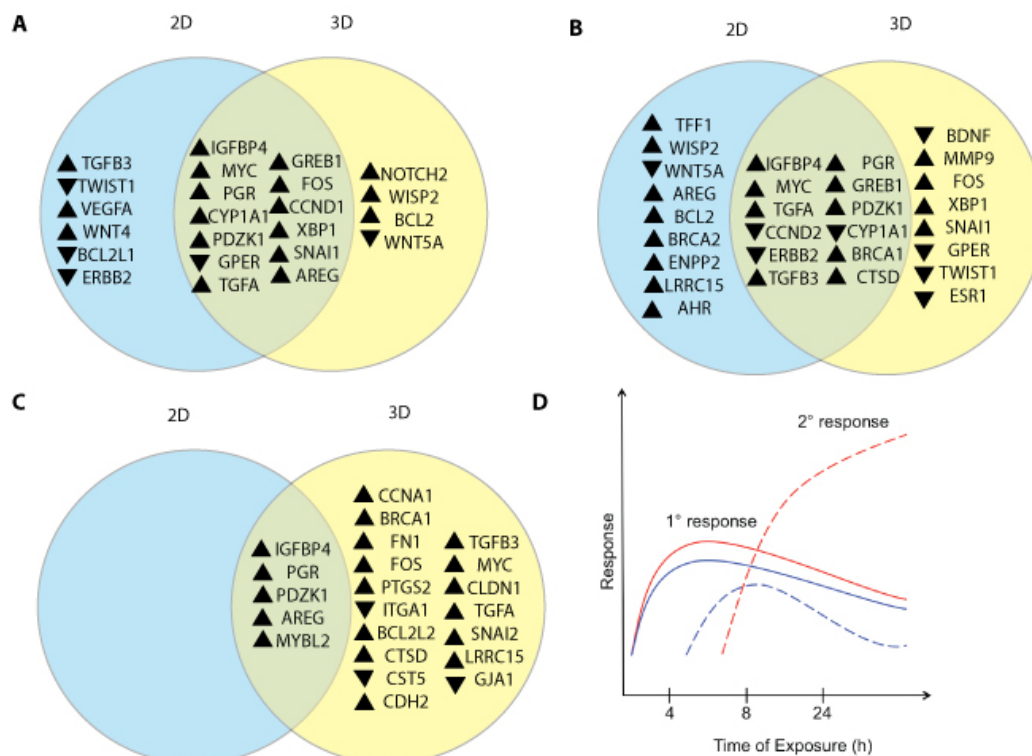


Figure 4.1. Gene expression of 2D and 3D cultures exposed to 1nM E2 for 4, 8 and 24 hours. MCF-7 2D and 3D cultures were treated with 1nM E2 for 4 hours and E2 treatment of 2D cultures altered expression of 19 transcripts (blue), 14 of which were also altered in 3D cultures (A). Four transcripts were significantly altered in 3D microtissues and not in monolayer cultures (yellow) (A). Monolayer MCF-7 cultures treated with E2 for 8 hours exhibited significant gene expression changes in 21 transcripts (blue), while 3D microtissues treated with E2 display altered expression of 20 transcripts (yellow) (B). Twelve transcripts were significantly altered in both 2D and 3D cultures (B). E2 treatment altered expression of 5 transcripts in 2D cultures (blue), while 3D cultures exhibited altered expression of 22 transcripts (yellow) following 24 hours of estradiol exposure (C). Five transcripts were significantly altered in both 2D and 3D cultures (C). A representative model of the divergent responses of 2D and 3D mRNA expression changes following exposure to estradiol. Following 4 hours of exposure, the primary responses of both 2D (blue) and 3D (red) are similar (D). Following 4 hours of exposure, the primary responses of both 2D (blue) and 3D (red) are similar (D). At 8 hours of exposure, secondary responses diverge in 2D and 3D systems, with differences persisting at 24 hours (D).

Table 4.1: Significantly altered transcripts in MCF-7 monolayer cultures following exposure to 1nM estradiol for 4 hours. Fold change relative to vehicle-treated controls, with positive fold indicating increased expression.

Gene Name	Abbreviation	Fold Change	q value
Insulin-Like Growth Factor Binding Protein 4	IGFBP4	5.78	<0.0001
Progesterone Receptor	PGR	4.59	<0.0001
V-Myc Avian Myelocytomatosis Viral Oncogene Homolog	MYC	3.72	<0.0001
Transforming growth factor, alpha	TGFA	3.61	0.0001
FBJ Murine Osteosarcoma Viral Oncogene Homolog	FOS	3.35	0.0001
Growth response to estrogen, breast cancer 1	GREB1	3.23	0.0001
PDZ domain containing 1	PDZK1	3.17	<0.0001
Cyclin D1	CCND1	2.75	0.0002
Snail Family Zinc Finger 1,	SNAI1	2.60	0.0052
Tranforming growth factor beta 3	TGFB3	2.50	0.0004
Xbox binding protein 1	XPB1	2.10	0.0011
vascular endothelial growth	VEGFA	2.01	0.0089

factor alpha					
Amphiregulin			AREG	1.84	0.0207
Wingless-Type		MMTV			
Integration	Site	Family,	WNT4	1.68	0.0123
Member 4					
BCL2-like 1			BCL2L1	-1.58	0.0306
Erb-B2	Receptor	Tyrosine			
Kinase 2			ERBB2	-1.78	0.0454
Twist	Family	BHLH			
Transcription Factor 1			TWIST1	-2.10	0.0023
G-protein	coupled	estrogen			
receptor			GPER	-3.06	<0.0001
cytochrome P450 1A1			CYP1A1	-3.27	<0.0001

Exposure of 3D MCF-7 microtissues to estradiol for 4 hours altered expression of 17 transcripts (Table 2), resulting in an increase in expression of PGR, WNT1-inducible signaling protein 2 (WISP2), IGFBP4 and GREB1. E2 exposure also increased expression of PDZK1, MYC, CCND1, TGFA and AREG. E2-treated MCF-7 microtissues demonstrated increased expression of SNAI1, XBP1, FOS, notch 2 (NOTCH2) and B-cell lymphoma 2 (BCL2). MCF-7 microtissues treated with 1nM E2 for 8 hours displayed decreased expression of GPER, CYP1A1 and wingless-type MMTV integration site family member 5A (WNT5A). All gene expression results are reported in S3 Table. Overlapping and unique expression changes in 2D and 3D cultures exposed to estradiol are reported in Fig. 1A.

Table 2: Significantly altered transcripts in MCF-7 microtissue cultures following exposure to 1nM estradiol for 4 hours. Fold change relative to vehicle-treated controls, with positive fold indicating increased expression.

Gene Name	Abbreviation	Fold Change	q value
Progesterone receptor	PGR	10.49	<0.0001
WNT1 Inducible Signaling Pathway Protein	WISP2	8.37	0.0126
insulin-like growth factor binding protein 4	IGFBP4	5.33	<0.0001
growth response to estrogen, breast cancer 1	GREB1	5.18	<0.0001
PDZ containing domain 1	PDZK1	5.00	<0.0001
V-Myc Avian Myelocytomatosis Viral Oncogene Homolog	MYC	3.39	<0.0001
cyclin D1	CCND1	3.31	<0.0001
transforming growth	TGFA	2.95	0.0002

factor alpha			
amphiregulin	AREG	2.57	0.0012
Snail Family Zinc Finger 1,	SNAI1	2.40	0.0061
xbox binding protein 1	XBP1	2.07	0.0017
FBJ Murine Osteosarcoma Viral	FOS	2.02	0.0049
Oncogene Homolog Notch 2	NOTCH2	1.97	0.0063
B-cell lymphoma 2	BCL2	1.57	0.0431
g-protein coupled estrogen receptor	GPER	-2.14	0.0009
Cytochrome P450 1A1	CYP1A1	-3.78	<0.0001
Wingless-Type MMTV Integration Site Family, Member 5A	WNT5A	-4.26	0.0457

Monolayer MCF-7 cultures treated with 1nM E2 for 8 hours display altered expression of 21 transcripts (Table 3), with increased expression of ectonucleotide pyrophosphatase/phosphodiesterase 2 (ENPP2), PGR, AREG, GREB1, IGFBP4, PDZK1 and LRCC15 compared to vehicle-treated controls. Expression of breast cancer 1 (BRCA1), breast cancer 2 (BRCA2), cathepsin D (CTSD), BCL2, and WISP2 were significantly increased. TFF1, MYC, Aryl hydrocarbon receptor (AHR), TGFA and TGFB3 expression were significantly increased. E2 treatment of monolayer cultures for 8 hours resulted in statistically significant decreased expression of ERBB2, CYP1A1, CCND2 and WNT5A compared to vehicle-treated controls. All gene expression results following E2 exposure are included in S4 Table.

Table 3 Exposure to 1nM estradiol for 8 hours significantly altered mRNA expression in MCF-7 monolayer cultures. Fold change relative to vehicle-treated controls, with positive fold indicating increased expression.

Gene Name	Abbreviation	Fold Change	q value
Ectonucleotide Pyrophosphatase/ Phosphodiesterase 2	ENPP2	15.49	0.0063
Progesterone receptor	PGR	5.63	<0.0001
Amphiregulin	AREG	3.99	0.0026
Growth response to estrogen, breast cancer 1	GREB1	3.83	<0.0001
insulin-like growth factor binding protein 4	IGFBP4	3.33	0.0004
PDZ containing domain 1	PDZK1	3.31	<0.0001
Leucine-rich repeat containing 15	LRRC15	2.71	0.0106
Breast cancer 1	BRCA1	2.37	0.0001
Breast cancer 2	BRCA2	2.13	0.0047
Cathepsin D	CTSD	2.05	0.0002
B-cell lymphoma 2	BCL2	1.93	0.0034
WNT1 Inducible Signaling Pathway Protein	WISP2	1.92	0.0001
Trefoil factor 1	TFF1	1.80	<0.0001

V-Myc	Avian			
Myelocytomatosis	Viral	MYC	1.72	0.0016
Oncogene Homolog				
Aryl-hydrocarbon receptor		AHR	1.67	0.0498
Transforming growth factor alpha	growth	TGFA	1.55	0.0052
Transforming growth factor beta 3	growth	TGFB3	1.53	0.0108
Notch 2		NOTCH2	1.40	0.0059
Erb-B2 Receptor Tyrosine Kinase 2		ERBB2	-1.75	0.0095
Cytochrome P450 1A1		CYP1A1	-2.10	0.0001
Cyclin D2		CCND2	-2.32	0.0090
Wingless-Type	MMTV			
Integration Site Family, Member 5A		WNT5A	-5.29	0.0001

Exposure of MCF-7 microtissues to 1nM E2 for 8 hours resulted in significantly altered expression of 20 transcripts, compared to vehicle-treated controls (Table 4). Estradiol induced expression of PGR, GREB1, IGFBP4, PDZK1, TGFB3 and MYC. Expression of TGFA, SNAI1, CTSD, FOS, BRCA1 and XBP1 were significantly increased by E2 treatment. Complete gene expression results can be found in S5 Table. Differences and similarities in expression are summarized in Fig 1B.

Table 4 Significantly altered transcripts in MCF-7 microtissue cultures following exposure to 1nM estradiol for 8 hours. Fold change relative to vehicle-treated controls, with positive fold indicating increased expression.

Gene Name	Abbreviation	Fold Change	q value
Progesterone receptor	PGR	8.52	<0.0001
growth response to estrogen, breast cancer 1	GREB1	7.05	<0.0001
insulin-like growth factor binding protein 4	IGFBP4	6.10	<0.0001
PDZ containing domain 1	PDZK1	5.43	<0.0001
Transforming growth factor beta 3	TGFB3	3.09	<0.0001
V-Myc Avian Myelocytomatosis Viral Oncogene Homolog	MYC	2.99	<0.0001
transforming growth factor alpha	TGFA	2.38	<0.0001
Snail Family Zinc Finger 1,	SNAI1	1.99	0.0006
Cathepsin D	CTSD	1.88	0.0004
FBJ Murine Osteosarcoma Viral Oncogene Homolog	FOS	1.88	0.0002

Breast cancer 1	BRCA1	1.72	0.0031
xbox binding protein 1	XBP1	1.62	0.0003
Estrogen receptor alpha	ESR1	-1.50	0.0031
Twist Family BHLH Transcription Factor 1	TWIST1	-1.70	0.0021
g-protein coupled receptor 1	GPER	-1.71	0.0006
Matrix metalloproteinase 9	MMP9	-1.75	0.0001
Erb-B2 Receptor Tyrosine Kinase 2	ERBB2	-2.05	0.0020
Cytochrome P450 1A1	CYP1A1	-2.22	<0.0001
Cyclin D2	CCND2	-2.86	0.0026
Brain-derived neurotrophic factor	BDNF	-4.34	0.0001

MCF-7 monolayers exposed to 1nM E2 for 24 hours displayed increased expression of 5 transcripts (Table 5), including IGFBP4, PGR, PDZK1, AREG and MYBL2. Gene expression results for all transcripts are contained in S6 Table.

Table 5: mRNAs significantly changed in monolayer cultures following 24 hours of exposure to 1nM E2. Positive fold change values are representative of increased expression compared to vehicle-treated controls.

Gene Name	Abbreviation	Fold Change	q value
PDZ domain containing 1	PDZK1	7.27	0.0030
Progesterone Receptor	PGR	6.98	0.0017
Amphiregulin	AREG	3.84	0.0184
Insulin-Like Growth Factor Binding Protein 4	IGFBP4	3.68	0.0002
V-Myb Avian Myeloblastosis Viral Oncogene Homolog-Like 2	MYBL2	2.47	0.0468

Exposure to E2 for 24 hours significantly altered expression of 22 transcripts in MCF-7 microtissues (Table 6). Expression of BCL2-like 2 (BCL2L2), cystatin D (CST5), PDZK1, AREG, leucine rich repeat containing 15 (LRRC15) and IGFBP4 was significantly increased following estradiol exposure. CTSD, PGR, prostaglandin synthase 2 (PTGS2), snail family zinc finger 2 (SNAI2) and claudin 1 (CLDN1) expression was significantly increased in MCF-7 microtissues treated with E2 for 24 hours. Increases in expression of TGFB3, MYC, cyclin A1 (CCNA1), MYBL2, TGFA, N-cadherin (CDH2), fibronectin (FN1), BRCA1 and FOS were observed in E2-treated microtissues. Expression of gap junction 1 (GJA1) and integrin alpha 1 (ITGA1) was significantly decreased in E2-treated microtissues. Complete gene expression results are reported in S7 Table. Monolayer and microtissue cultures treated with E2 for 24 hours displayed large differences in gene expression (Fig 1C).

Table 6: Genes significantly altered in MCF-7 3D microtissues following 24 hours of exposure to 1nM estradiol. Positive fold change values indicate increased expression of transcripts compared to vehicle-treated controls.

Gene Name	Abbreviation	Fold Change	q value
BCL-like 2	BCL2L2	34.16	0.0192
Cystatin D	CST5	6.95	0.0197
PDZ domain containing 1	PDZK1	4.49	0.0004
Integrin, alpha 1	ITGA1	4.07	0.0147
Amphiregulin	AREG	4.01	0.0006
Leucine rich repeat containing 15	LRRC15	3.66	0.0073
Insulin-Like Growth Factor Binding Protein 4	IGFBP4	3.35	0.0002
Cathepsin D	CTSD	3.25	0.0194
Progesterone Receptor	PGR	3.08	0.0010
Prostaglandin synthase 2	PTGS2	2.97	0.0117
Snail Family Zinc Finger 2	SNAI2	2.85	0.0072
Claudin 1	CLDN1	2.54	0.0042

Transforming growth factor beta 3	TGFB3	2.35	0.0015
V-Myc Avian Myelocytomatosis Viral Oncogene Homolog	MYC	2.05	0.0026
Cyclin A1	CCNA1	2.02	0.0081
V-Myb Avian Myeloblastosis Viral Oncogene Homolog-Like 2	MYBL2	1.87	0.0049
transforming growth factor alpha	TGFA	1.84	0.0070
N-cadherin	CDH2	1.82	0.0311
Fibronectin 1	FN1	1.66	0.0103
Breast cancer 1	BRCA1	1.65	0.0084
FBJ Murine Osteosarcoma Viral Oncogene Homolog	FOS	1.55	0.0105
Gap Junction 1	GJA1	-2.18	0.0079

4.5 Discussion

Previous work has demonstrated that scaffold-free culture of MCF-7 cells using agarose hydrogels promotes differentiation when compared to monolayer cultures [15]. When compared to 2D monolayer MCF-7 cultures, MCF-7 microtissues display differences in gene expression following exposure to estradiol. While MCF-7 cells are a highly transformed, genetically unstable cell line, they are an important tool to study estrogen signaling. Since being isolated in 1973, MCF-7 breast cancer cells have been used to study estrogen receptor biology [33, 34] and estrogenic endocrine disrupting compounds[23, 35]. The current study indicates that in both 2D and 3D cultures exposed to estradiol, genes that are typically used as indicators of estrogenic response are upregulated by estradiol. These genes include PGR, PDZK1 [36], and IGFBP4[37, 38], which are all increased at 4, 8, and 24 hours of exposure in 2D and 3D cultures, though the magnitude of fold change is greater in 3D microtissues. The similar induction of transcription of other well-known estrogen responsive genes including GREB1[36], AREG [39], MYC and inhibition of CYP1A1 demonstrates that in terms of classical estrogenic activity, MCF-7 monolayers and 3D microtissues respond similarly. Following hierarchical clustering analysis, these genes cluster together and exhibit similar patterns in 2D and 3D cultures over time (Fig 1D).

Interestingly, transcription of other genes in 2D and 3D MCF-7 cultures is not similar following E2 exposure. As early as 4 hours of E2 exposure there are differences in expression of many markers between 2D and 3D cultures, though the majority of significantly altered genes are changed in both 2D and 3D cultures exposed to E2. Over time, the number of genes that are significantly altered in 2D or 3D cultures increases, while the number of genes shared between the two decreases, indicating a divergence of the response in each system. Hierarchical clustering demonstrated that genes expressed in 3D microtissues and not in 2D cluster separately from typical estrogen-responsive genes (Fig 1D). This difference is most striking at 24 hours of estradiol exposure, where 2D cultures display changes in only 5 genes, while 3D cultures have 22 transcripts significantly changed. A number of the 22 genes altered by estradiol exposure in 3D cultures are involved with cell adhesion (FN1, ITGA1, CST5, CDH2, CLDN1, SNAI2 and GJA1). These changes in cell adhesion molecules were not detected in E2-treated monolayers in the present study. *In vitro* and *in vivo* studies have demonstrated that exposure to E2 increases epithelial-mesenchymal transition (EMT), increasing the invasive potential and mobility of human MCF-10F breast epithelial cells [40], MCF-7 cells [41] and human ovarian carcinoma BG-1 and SKOV-3 cells[42]. In the present study, increases in expression of the EMT transcriptional regulators TWIST1 [43] and SNAI1 [44] are detected as early as 8 hours. Both SNAI1 and TWIST initiate EMT,

which results in degradation of cell-cell contacts, loss of cellular polarity and increased invasive potential [45]. Following those increases in expression at 8 hours, at 24 hours increased expression of several classical EMT markers was detected, including FN1, CLDN1 and SNAI2. Previous work has demonstrated that CLDN1 expression is capable of inducing EMT [46], while FN1 and SNAI2 are highly expressed in mesenchymal-like cells [47, 48]. Coupled with the increased expression of mesenchymal markers was the decreased expression of epithelial markers ITGA1 and GJA1 in MCF-7 microtissues treated with E2 for 24 hours, indicating that MCF-7 cells cultured in 3D begin to undergo EMT. Interestingly, in this study, MCF-7 monolayers did not demonstrate any significant changes in expression of EMT-related genes following E2 exposure. It is clear from the current study that 2D and 3D MCF-7 cultures respond differently to E2, underlining the importance of selecting a relevant cell system. The large changes in gene expression of mRNAs involved in cell adhesion and cell structure observed in 3D cultures following exposure to estradiol is likely due to their differentiation within the system, which has been previously described [15]. The ability to assess secondary effects (i.e. changes in proliferation, cell-cell contact and cell adhesion) is powerful, as it may allow for the delineation of adverse and adaptive responses within an *in vitro* 3D culture system. Similar changes in expression of cell adhesion-related transcripts have been detected

in vivo studies following exposure to estradiol[49], which further supports the need to detect complex, secondary changes *in vitro*.

MCF-7 cells are the cell platform for the E-SCREEN assay, which measures cellular proliferation of monolayer MCF-7 cultures following exposure to xenobiotics [23]. In the current study, the 3D system exhibits a stronger response to estradiol with regards to changes in expression of genes involved in cell cycle progression than monolayer cultures. The increased expression of CCNA1 in 3D cultures suggests that MCF-7 microtissues are beginning to proliferate after 24 hours of exposure, as CCNA1 expression is increased in S phase and the G2/M transition[50]. This is further supported by the observation that 3D microtissues display increased expression of BRCA1, a critical component of the DNA repair machinery that interacts with cell cycle regulators[51]. The increase in markers of proliferation in 3D microtissues, not in monolayer cultures, is an interesting finding that suggests that 3D MCF-7 cultures are more sensitive to the effects of estradiol exposure. Differences in gene expression observed in this study may be influenced by cell-cell interactions within the 3D cultures, as many studies have demonstrated that the cellular microenvironment can greatly influence mRNA and protein expression within breast epithelial cells [24, 52-55]. These striking differences in gene expression in the present study may be due to the high amount of cell-cell contact, and therefore cell communication, in the scaffold-free culture system, and warrants further investigation.

Overall, the current study indicates that when examining 2D and 3D cultures for estrogenic responses by assessing the expression of target genes, levels of expression of classical estrogen-induced genes are similar at early time points. However, at later time points, the estrogenic responses diverge and result in activation of different targets in each culture system (Fig 2). In this analysis, both 2D and 3D cultures treated for 4 hours clustered together, with the 8 hour 2D, 8 hour 3D and 24 hour 2D samples clustering together. The 24 hour 3D sample clustered separately from all treatments, further emphasizing the large differences observed in 2D and 3D culture systems. Despite the limited number of mRNA transcripts examined in the present study, there are clear differences in estradiol responsiveness between 2D and 3D cultures of MCF-7 cells. This observation further supports the need to develop more physiologically relevant, human cell based models for toxicity testing, as intricate differences in gene and protein expression in various systems can lead to alternate results.

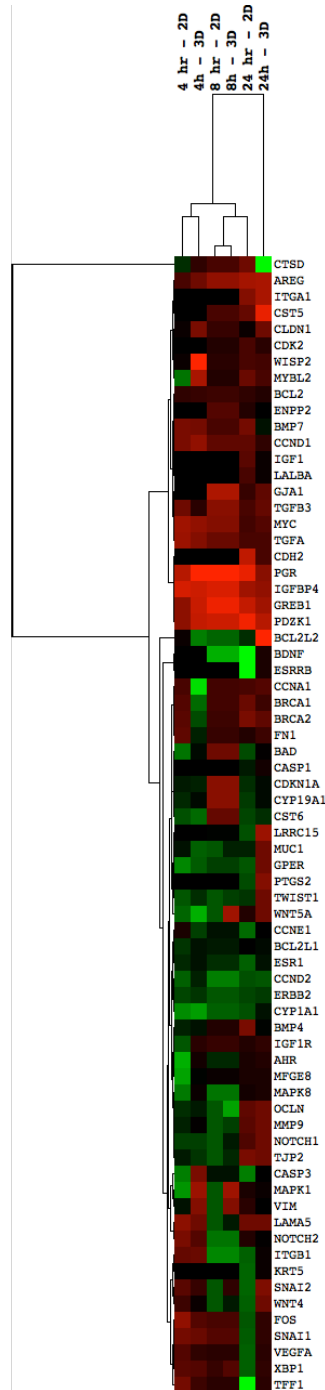


Figure 2. Euclidean hierarchical clustering of all significantly altered genes over time. All fold change values for significantly changed transcripts were analyzed using hierarchical clustering. Transcripts clustered into 2 large groups, aligning with the observation that classical estrogen responsive genes (ex PGR, GREB, AREG) are similarly expressed in 2D and 3D cultures, but secondary responses are different (ex VIM, OCLN). Green is decreased expression, red indicates increased expression.

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CHAPTER FIVE

Morphologic Effects of Estrogen Stimulation on 3D MCF-7 Microtissues¹

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Morphologic Effects of Estrogen Stimulation on 3D MCF-7 Microtissues

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Declaration of Authors' Roles:

Designed Research: M.M.V., K.B.

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Contributed to Data or Statistical Analysis: M.M.V., S.W., S.M.H.

Wrote Manuscript: M.M.V., K.B.

5.1 Abstract

In the development of human cell-based assays, 3-dimensional (3D) cell culture models are intriguing as they are able to bridge the gap between animal models and traditional two-dimensional (2D) cell culture. Previous work has demonstrated that MCF-7 human breast carcinoma cells cultured in a 3D scaffold-free culture system self-assemble and develop into differentiated microtissues that possess a luminal space. Exposure to estradiol for 7 days decreased lumen formation in MCF-7 microtissues, altered microtissue morphology and altered expression of genes involved in estrogen signaling, cell adhesion and cell cycle regulation. Exposure to receptor-specific agonists for estrogen receptor alpha, estrogen receptor beta and g-protein coupled estrogen receptor resulted in unique, receptor-specific phenotypes and gene expression signatures. The use of a differentiated scaffold-free 3D culture system offers a unique opportunity to study the phenotypic and molecular changes associated with exposure to estrogenic compounds.

5.2 Introduction

As summarized in the National Research Council report “Toxicity Testing in the 21st Century: A Vision and a Strategy,” [1] there is a need to develop more efficient and physiologically relevant models for evaluation of chemical safety and toxicity. Due to the cost- and time-intensive nature of animal testing, *in vitro* screening models are needed. Additionally, *in vitro* cell culture models allow for the use of human cell lines, enabling researchers to better evaluate human health and disease states when compared to *in vivo* animal models.

Traditionally, human primary cells and cell lines have been cultured as 2-dimensional (2D) monolayers on plastic substrates. While 2D cultures are technically easy and inexpensive, they do not recapitulate the biology of cells *in vivo*, where tissues have high cell density and cells are in contact with other cell types and extracellular matrix (ECM). It has been demonstrated that contact between cells and with ECM can alter gene expression profiles and cellular morphology [2-5], potentially leading to differences in experimental results between 2D *in vitro* and intact *in vivo* systems.

As an alternative strategy to address several of the shortcomings of both *in vivo* and 2D *in vitro* experimental models, 3-dimensional (3D) culture models have been developed. The use of nonadhesive agarose hydrogels to culture cells in a scaffold-free environment allows for easy media changes and handling of cultures. In this system, cells are trypsinized and seeded into non-adhesive hydrogels, where they settle into recesses and come into contact

with other cells [6]. Within 24 hours after seeding, cells self-assemble into microtissues free of the influence of added matrix and substrates [7]. Previous work using MCF-7 cells has demonstrated that when cultured in scaffold-free agarose hydrogels, MCF-7 cells form 3D microtissues that are more differentiated than 2D cultures and contain a luminal space, reminiscent of the *in vivo* morphology of the adult mammary gland [8].

Both normal and malignant mammary cell lines have been used extensively to study estrogen receptors and their respective signaling pathways, due to their expression of these receptors. Estrogen signaling occurs through both genomic and nongenomic pathways. Genomic signaling is mediated by nuclear receptors estrogen receptor alpha (ER α) and estrogen receptor beta (ER β) that interact with DNA response elements to regulate gene expression following estrogen exposure, while nongenomic signaling is believed to proceed mainly through G-protein coupled estrogen receptor (GPER).

In the present study, the effects of estrogens on gene expression and morphology of 3D MCF-7 microtissues were assessed. Following exposure to selective estrogen receptor agonists, MCF-7 microtissues exhibited altered morphology, with a distortion of the luminal space that yielded compound-specific phenotypic changes. These changes were also associated with compound-specific gene expression changes. Here, we describe a novel system and methodology for assessing the phenotypic effects of estrogens on 3D MCF-7 microtissues. This platform offers an innovative method to assess

tissue structural changes in a human cell line that cannot be assessed in many traditional monolayer *in vitro* systems.

5.3 Materials and Methods

5.3.1 Chemicals and Reagents

Cell culture media and supplements were purchased from Life Technologies, Inc (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Flowery Branch, GA) and dextran-coated-charcoal (DCC) stripped fetal bovine serum was purchased from Gemini Bioscience (Sacramento, CA). Estradiol (E2), dimethylsulfoxide (DMSO) and insulin were purchased from Sigma Aldrich (St. Louis, MO). Propylpyrazoletriol (PPT), diarylpropyl nitrile (DPN), and G1 were purchased from Tocris Bioscience (Minneapolis, MN). Agarose was purchased from Fisher Scientific (Agawam, MA). Technovit 7100 was purchased from Heraeus Kulzer GmbH (Wehrheim, Germany).

5.3.2 Cell Culture

MCF-7 (ATCC No. HTB-22) human breast carcinoma cells [9] were maintained in growth medium made of phenol-red free DMEM-F12 medium containing 10% FBS, MEM nonessential amino acids , gentamicin and 10µg/mL insulin in a 5% CO₂ incubator at 37°C. Media was changed every 2-3 days and cells were passaged when 60-85% confluency was reached, as previously described [8]. To control for genetic drift and genomic instability,

cells were limited to use within 10 passages of the original vial obtained from ATCC.

5.3.3 3D Scaffold-Free Cell Culture

We used 3D Petri Dish molds (Microtissues Inc., Providence RI) to make nonadhesive agarose hydrogels, which were seeded with cells as previously described [8] MCF-7 cells grown in monolayer in tissue culture flasks were trypsinized, counted and seeded into agarose hydrogels at a density of 600,000 cells/mL. MCF-7 cells were allowed to settle into recesses for 30 minutes, and 2mL of treatment media was added.

5.3.4 Compound Exposure

Solutions of E2, PPT, DPN, G1 or vehicle control (DMSO) were made in treatment media made of phenol-red free DMEM-F12 medium containing 5% DCC FBS, MEM nonessential amino acids, gentamicin and 6ng/mL insulin. Following seeding of MCF-7 cells into agarose hydrogels, 2mL of treatment media containing E2, PPT or DMSO were added to agarose hydrogels. Plates were returned to a 5% CO₂ incubator and allowed to grow for 7 days. Treatment media was changed every 2-3 days.

5.3.5 Histological Examination

For 3D microtissues, samples were fixed in 4% PFA overnight at 4°C, and then embedded in glycol methacrylate (Technovit 7100; Heraeus Kulzer GmbH, Wehrheim, Germany) as previously described [10] for histological examination. Sections were cut at a thickness of 3µm, affixed to slides and stained with periodic acid, Schiff's reagent and counterstained with hematoxylin. Samples were viewed and images taken on an Aperio ScanScope CS (Aperio Technologies, Vista, CA) at 40x magnification.

5.3.6 Histological Quantification using Image J

Images of individual microtissues were collected and blinded before quantification in ImageJ. Images were prepared first by determining a pixel/micron ratio using embedded scale markers present in 16-bit images. Background (noncellular debris) was removed in ImageJ. Brightness and contrast were adjusted to allow for generation of binary images through the default automatic thresholding procedure, and this was followed by morphological dilation (d=3). Using an automated count, the area of cellular objects was quantified. Particles of less than 500µm² were excluded as cellular debris, while the area of cellular aggregates with and without luminal spaces were quantified (Supplemental Figure 5.1). Luminal area and total aggregate area were measured directly in ImageJ. Luminal ratio was calculated as the ratio of measured luminal area to measured total aggregate

area. Cellular area was calculated as total aggregate area minus luminal area.

5.3.7 RNA Isolation and PCR Array Analysis

Microtissues were collected from hydrogels by centrifugation, pelleted and lysed in Trizol. Samples were then isolated using the RNEasy Mini Kit (Qiagen) per manufacturer's instructions. A custom SABiosciences PCR Array (Qiagen) was created to include a list of 84 genes generated from literature to evaluate estrogen receptor signaling, breast and ductal morphogenesis, cellular growth and differentiation, proliferation, tumor progression and epithelial to mesenchymal transition as well as 5 housekeeping genes and contamination controls [8](Supplemental table 5.1) . Samples were prepared per manufacturers instructions, and were added to 384-well plates using and epMotion 5075 automated pipettor (Eppendorf). Plates were run on an ABI 7900HT machine using cycling conditions recommended by the manufacturer.

5.3.8 Statistical Analysis

The raw PCR cycle (Ct) values were imported into the R statistical environment [11]. Raw PCR cycles were normalized (dCt) using the SLqPCR package [12] to optimize selection of house-keeping genes. To compare the expression of agonist-treated samples with vehicle-treated, the LIMMA

package in R [13] was used to construct a linear model of adjusted dCt values. The empirical Bayes statistic (eBayes) was used and genes with an adjusted p-value significance for multiple comparisons (q value) of less than 0.05 were selected.

5.4 Results

5.4.1 Effects of Estradiol on 3D MCF-7 Microtissues

MCF-7 microtissues treated for 7 days with varying concentrations of E2 exhibited decreased luminal spaces (Figure 5.1A, B, C) and increased cellular area (Figure 5.1A, D) in a concentration dependent manner. MCF-7 cells in 3D microtissues treated with estradiol lost polarity, became disorganized and lost the luminal morphology as E2 concentration increased.

To determine changes in gene expression, a custom PCR array containing estrogen pathway-related targets was used. When treated for 7 days with estradiol, MCF-7 cells have altered expression of 22 transcripts (q value < 0.05) including genes involved in cell cycle regulation, apoptosis, cell adhesion and estrogen signaling (Table 5.1). Significantly increased transcripts with a fold change greater than 1.5 included PDZ containing domain 1 (PDZK1, q value=0.00078), v-myc avian myelocytomatosis viral oncogene (MYC, q=0.0036) and transforming growth factor beta 3 (TGFB3, q=0.0036). MCF-7 microtissues treated with E2 decreased expression of cyclin D kinase inhibitor 1A (CDKN1A, q=0.0049), cyclin E1 (CCNE1, q=0.0049), BCL2-associated agonist of cell death (BAD, q=0.0043), mitogen activated protein kinase 8 (MAPK8, q=0.0045), occludin (OCLN, q=0.0037), v-AKT murine thymoma viral oncogene homolog 1 (AKT1, q=0.00423) and caspase 3(CASP3, q=0.0013). Transcripts with decreased expression included

mitogen activated protein kinase 1 (MAPK1, $q=0.0036$), tight junction protein 2 (TJP2, $q=0.0040$), beta catenin (CTNNB1, $q=0.0013$), tight junction protein 1 (TJP1, $q=0.0013$), androgen receptor (AR, $q=0.0036$), BCL2-like 1 (BCL2L1, $q=0.0017$) and bone morphogenic protein 4 (BMP4, $q=0.00011$). E2 treated MCF-7 microtissues also had decreased expression of v-erb-b2 avian erythroblastic leukemia viral oncogene homolog (ERBB2, $q=0.00031$), cytochrome P450 19A1 (CYP19A1, $q=0.0037$), estrogen receptor alpha (ESR1, $q=0.0037$), cytochrome P450 1A1 (CYP1A1, $q=0.00011$) and apolipoprotein D (APOD, $q=3.8E-07$). Complete gene expression results are summarized in Supplemental Table 5.2.

Table 5.1: Significantly Altered Genes Following 7 Days of Exposure to 1nM E2			
Gene name	Abbreviation	Fold change	q value
PDZ containing domain 1	PDZK1	3.39	0.000785
Transforming growth factor beta 3	TGFB3	2.95	0.003665
v-myc avian myelocytomatosis viral oncogene homolog	MYC	1.64	0.004019
Cyclin d kinase inhibitor 1A	CDKN1A	-1.50	0.004917
Cyclin E1	CCNE1	-1.68	0.003413
BCL2-associated agonist of cell death	BAD	-1.71	0.004335
Mitogen activated protein kinase 8	MAPK8	-1.71	0.004486
Occludin	OCLN	-1.72	0.003665
V-akt murine thymoma viral oncogene homolog 1	AKT1	-1.93	0.004232
Caspase 3	CASP3	-1.99	0.001280
Mitogen activated protein kinase 1	MAPK1	-2.04	0.003665
Tight junction protein 2	TJP2	-2.16	0.004019
Beta catenin	CTNNB1	-2.20	0.001280
Tight junction protein 1	TJP1	-2.28	0.001280
Androgen receptor	AR	-2.52	0.003665
BCL2-like 1	BCL2L1	-2.56	0.001627
Bone morphogenic protein 4	BMP4	-3.69	0.000113
V-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2	ERBB2	-3.86	0.000310
Cytochrome P450 19A1	CYP19A1	-4.22	0.003665
Estrogen receptor alpha	ESR1	-4.77	0.003665
Cytochrome P450 1A1	CYP1A1	-5.54	0.000113
Apolipoprotein D	APOD	-25.20	0.00000038

Table 5.1: Significantly altered genes following 7 days of exposure to 1nM E2. MCF-7 microtissues were grown for 7 days in the presence of 1nM E2 or vehicle control. Q-significant genes with an absolute fold change value of greater than or equal to 1.5 were included, and are expressed relative to vehicle-treated control.

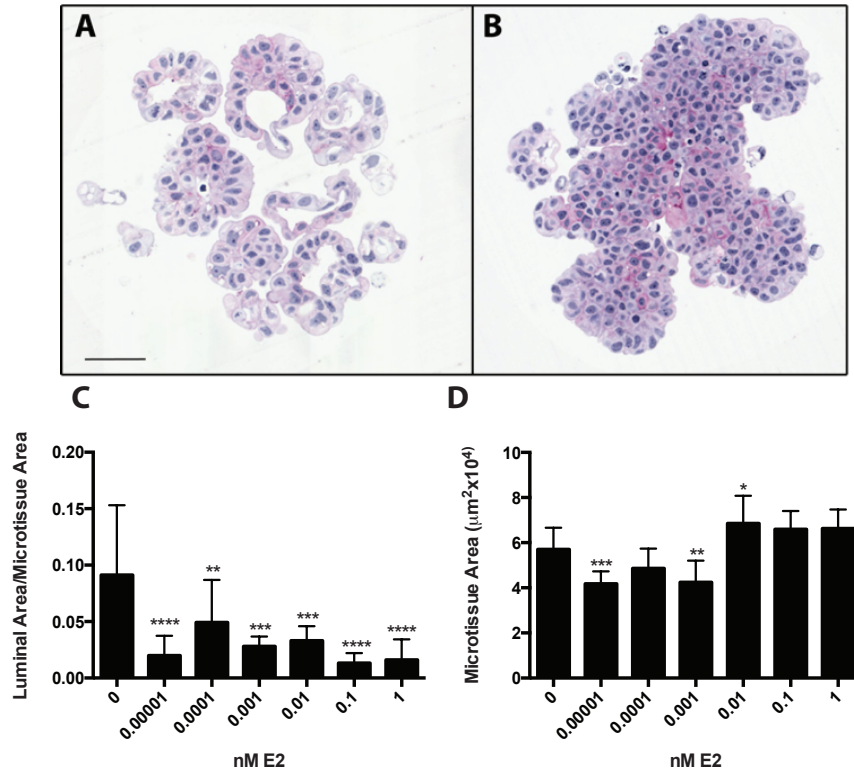


Figure 5.1 E2 treatment alters MCF-7 microtissue morphology. Histology of MCF-7 microtissues grown for 7 days in vehicle control (A) or 1nM estradiol (B) demonstrated distinct morphological differences resulting from treatment. MCF-7 microtissues exhibited decreased lumen formation, represented as luminal area/aggregate area in a concentration-dependent manner (C), and resulted in slight changes in microtissue area (D). Morphologic data is shown as mean $\pm$ SEM, and were analyzed using one-way ANOVA. Scale bar = 50 μm . * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

5.4.2 Specific ER α activation alters MCF-7 microtissue morphology and gene expression

Microtissues of MCF-7 cells treated for 7 days with the ER α selective agonist PPT reveal a marked decrease luminal area at concentrations of 1nM and 10nM (Figure 5.2A, B, C) with no significant increase in total microtissue cellular area (Figure 5.2C). Following 7 days of treatment with 5nM PPT, MCF-7 cells exhibited altered expression of 6 transcripts, with increased PDZK1 (q=0.0045), cyclin A1 (CCNA1, q=0.00098), trefoil factor 1 (TFF1, q=0.0166) and claudin 1 (CLDN1, q=0.040) expression. PPT-treated microtissues displayed decreased APOD (q=9.6E-05) and ESR1 (q=0.040) expression (Table 5.2). PPT-induced gene expression results are included in Supplemental Table 5.3.

Table 5.2: Significantly Altered Genes Following Exposure to 5nM PPT for 7 Days			
Gene name	Abbreviation	Fold change	q value
PDZ containing domain 1	PDZK1	2.68	0.004578
Cyclin A1	CCNA1	2.56	0.000985
Trefoil factor 1	TFF1	1.64	0.016571
Claudin 1	CLDN1	1.60	0.040437
Cytochrome P450 1A1	CYP1A1	-1.50	0.003628
Estrogen receptor alpha	ESR1	-1.70	0.040437
Apolipoprotein 1	APOD	-2.36	0.000096

Table 5.2: Significantly altered genes following exposure to 5nM PPT for 7 days. Treatment with the ER α specific agonist PPT for 7 days significantly altered expression of 7 transcripts. Absolute fold change values greater than or equal to 1.5 and q values less than 0.005 were included. Values are expressed relative to vehicle-treated controls.

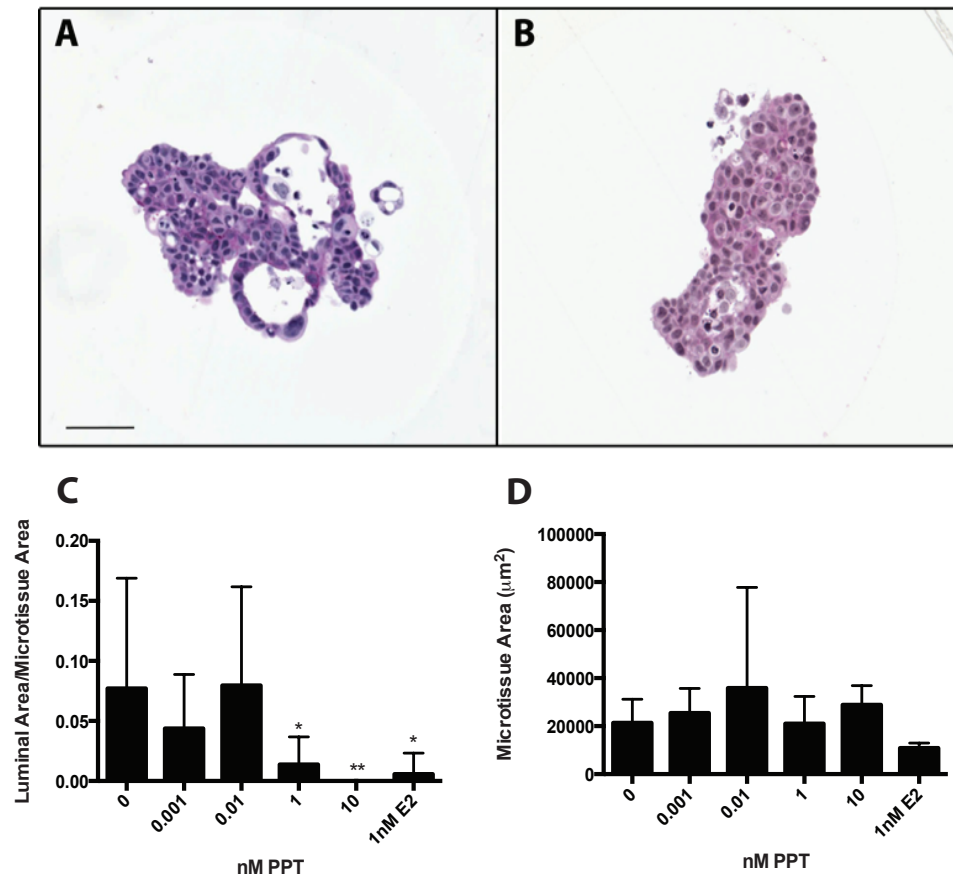


Figure 5.2 Selective estrogen receptor alpha activation by PPT disrupts MCF-7 microtissue morphology. Representative histological sections of 3D MCF-7 microtissues grown in vehicle control (A) or 5nM PPT (B) for 7 days. PPT treatment reduced the luminal formation within MCF-7 microtissues (C), and did not alter microtissue area (D). Data is displayed as mean $\pm$ SEM, with analysis using a one-way ANOVA. Scale bar = 50 μ m. * $p < 0.05$, ** $p < 0.01$

5.4.3 ER β activation does not significantly alter MCF-7 microtissue morphology

Treatment with the ER β agonist DPN resulted in an apparent decrease in the luminal area at concentrations 0.5 and 1nM following treatment for 7 days (Figure 5.3A and B) and an increase in cellular area at the high concentration of 10nM DPN (Figure 5.3C). Treatment with DPN for 7 days resulted in significantly decreased expression of APOD (q=5.2E-05; Supplemental Table 4).

Table 5.3: Significantly Altered Genes Following Exposure to 5nM DPN for 7 Days			
Gene name	Abbreviation	Fold change	q value
Apolipoprotein 1	APOD	-2.55	0.000052

Table 5.3. Significantly altered transcripts following exposure to 5nM DPN for 7 days. DPN, a specific Erb agonist, significantly decreased expression of APOD compared to vehicle control. Q-significant genes with an absolute fold change value of greater than or equal to 1.5 were included, and are expressed relative to vehicle-treated control.

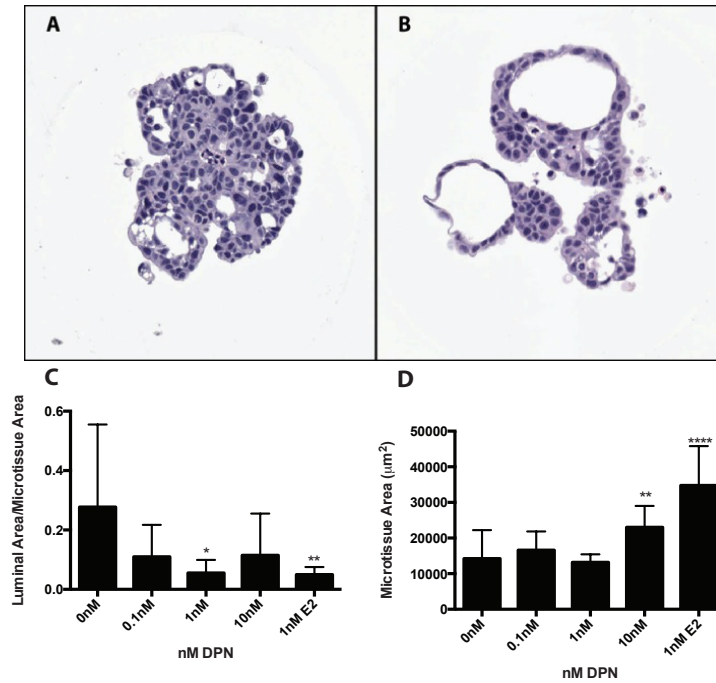


Figure 5.3 Estrogen receptor beta activation with DPN alters MCF-7 microtissue morphology at high concentrations. MCF-7 microtissues were grown for 7 days in the presence of vehicle control (A) or 5nM DPN (B). DPN treatment significantly reduced the luminal ratio at 1nM, while 10nM DPN treatment significantly increased cellular area. Data is represented as mean ± SEM, and was analyzed using one-way ANOVA. Scale bar = 50μm. * p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001

5.4.4 GPER activation by selective agonist G1 Alters MCF-7 microtissue morphology but not gene expression

Following 7 days of treatment with the GPER agonist G1, MCF-7 cells did not exhibit a significant change in luminal ratio (Figure 5.4), but did demonstrate an increase in cellular area at low concentrations compared to vehicle-treated controls (Figure 5.4). G1-treated microtissues did not significantly alter expression of transcripts (Supplemental Table 5.5).

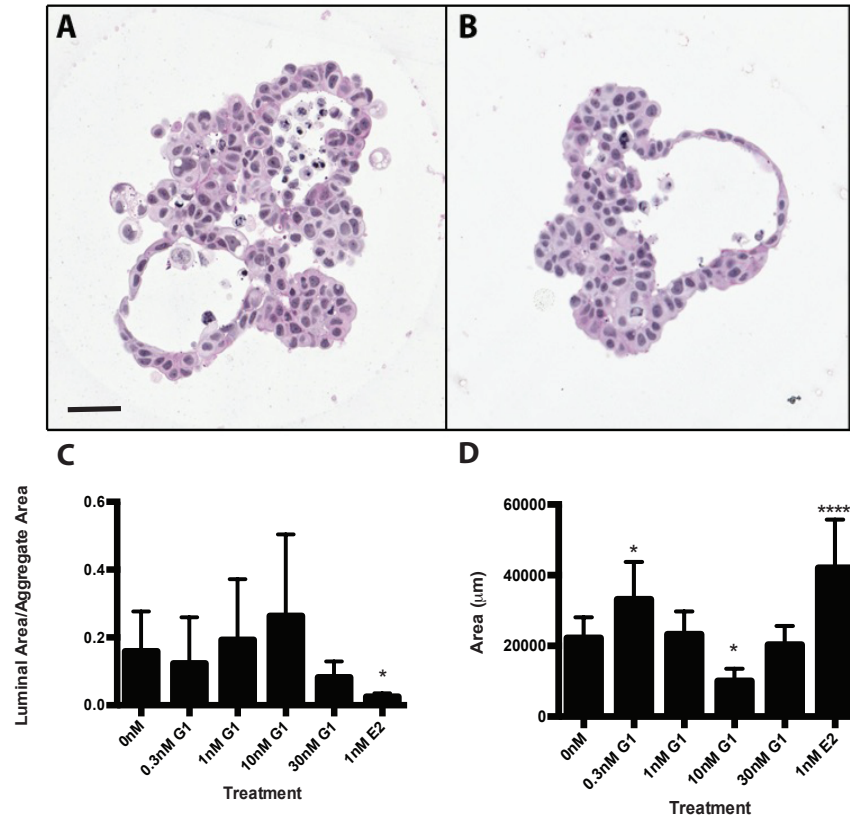


Figure 5.4 . Activation of g-protein coupled estrogen receptor does not significantly alter MCF-7 microtissue morphology. MCF-7 microtissues were grown in the presence of vehicle control (A) or 0.3nM G1 (B) for 7 days. G1 treatment did not significantly alter MCF-7 luminal morphology (C). At 0.3nM G1, microtissue area was significantly increased, while at 10nM microtissue area was significantly decreased (D). Morphologic data is shown as mean ± SEM, and were analyzed using one-way ANOVA. Scale bar = 50μm. * p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001

5.4.5 Gene expression signatures overlap but do display receptor-specificity

Several gene expression targets exhibited overlap between agonist treatments, as E2 and PPT induced similar changes in CYP1A1, PDZK1 and ESR1, while DPN, E2 and PPT changed expression of APOD (Figure 5.5). E2 significantly changed expression of 19 unique transcripts, while DPN exposure altered expression of only ENPP2. G1 treatment did not alter expression of any transcripts, while PPT treatment resulted in altered expression of 3 distinct transcripts (Figure 5.5).

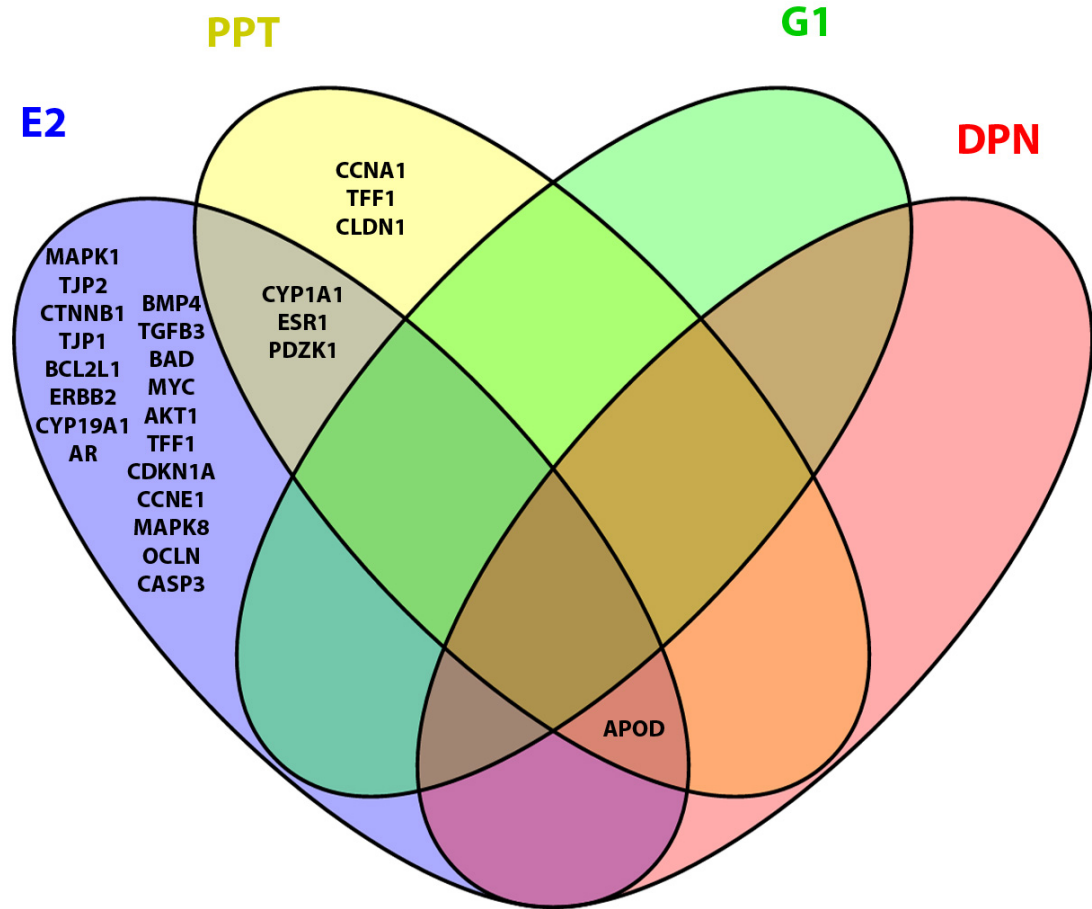


Figure 5.5 . Selective estrogen receptor activation results in unique gene expression profiles. E2 (blue) altered expression of 19 unique transcripts, PPT (yellow) altered expression of 3 transcripts and G1 (green) resulted in altered expression of 1 transcript. G1 treatment did not share any significantly differentially expressed genes with any other treatments, however, E2, DPN and PPT shared expression of 1 transcript, while E2 and PPT shared 3 transcripts.

4.5 Discussion

MCF-7 microtissues display altered morphology following culture for 7 days in the presence of estrogen receptor agonists, with distinct, quantifiable phenotypes associated with selective pathway activation (Figure 6). MCF-7 microtissues exhibited increased proliferation and decreased differentiation (as indicated by luminal ratio) following the broad activation of estrogen receptors with E2. Similarly, activation of ER α by PPT increased proliferation and decreased differentiation, while activation of ER β resulted in slight increases in proliferation, with not alteration in differentiation. Uniquely, specific activation of GPER by G1 resulted in no change in differentiation, and a decrease in proliferation at high concentrations. Together, these outcomes indicate that MCF-7 3D microtissues are sensitive to estrogenic compounds, and that activation of specific pathways yields unique morphologic changes.

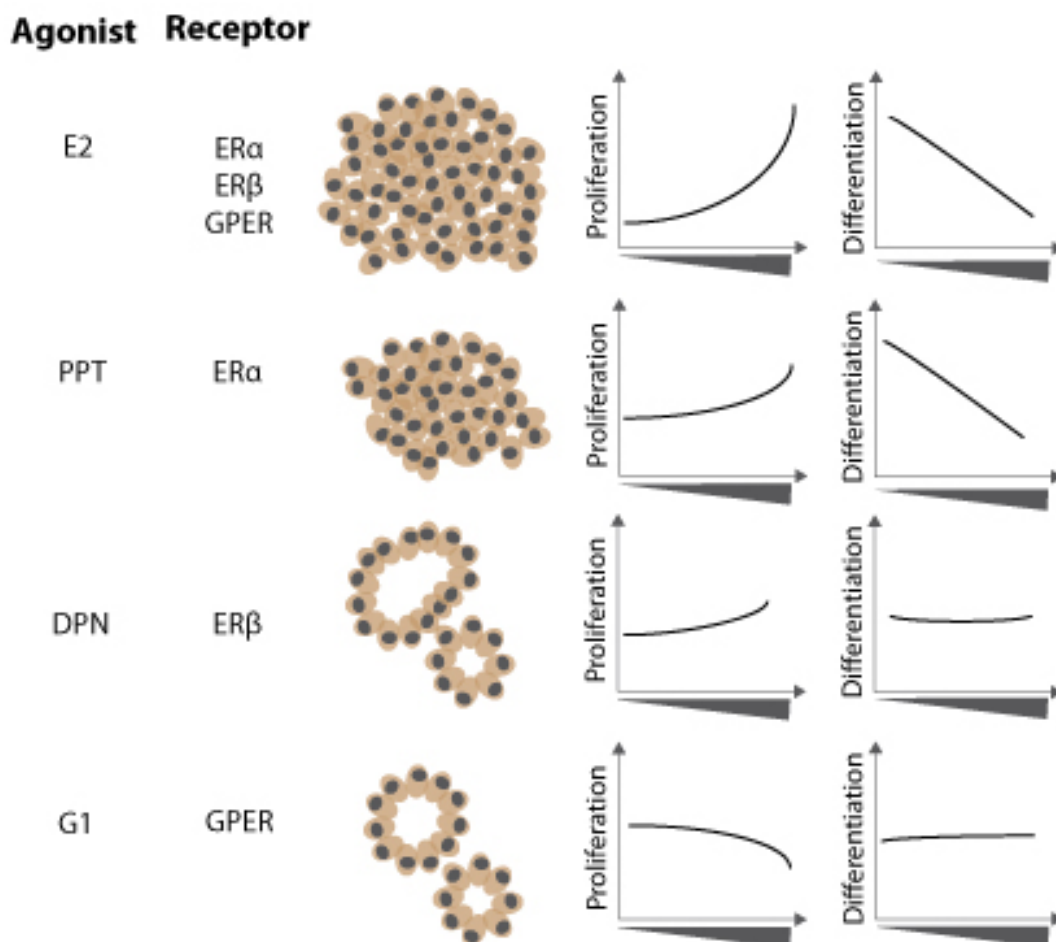


Figure 5.6 . Selective receptor activation resulted in unique MCF-7 microtissue morphology. E2 treatment, which activated ER α , ER β and GPER caused a concentration-dependent increase in proliferation and decrease in differentiation (luminal ratio). Selective activation of ER α using PPT resulted in a concentration-dependent increase in proliferation and decrease in microtissue differentiation. Activation of ER β using the selective agonist DPN resulted in a significant increase in proliferation at high concentrations and no change in differentiation. GPER activation with G1 resulted in a decrease in proliferation at high concentrations, with no changes in differentiation.

As a result of culture in the presence of the endogenous estrogen receptor ligand E2, MCF-7 microtissues contained a smaller luminal space with

increased cellular area, indicating that exposure to E2 altered morphology, leading to a de-differentiated microtissue, compared to vehicle-treated controls. Previous work has demonstrated this to be true for E2 exposure in other breast cell lines cultured in scaffold-based 3D systems including Matrigel and collagen matrices. The spontaneously immortalized MCF-10F human breast cell line grown in a collagen matrix has demonstrated that exposure to E2 reduces ductogenesis [14], altering the phenotype of these cultures in a manner consistent with that caused by exposure to other carcinogens, including benzo-a-pyrene [15]. The MCF-10F cell line does not express high levels of estrogen receptors, and their expression must first be induced. To circumvent the need to induce estrogen receptors, more recent studies have focused on the use of normal, immortalized MCF-12A human breast epithelial cells grown in Matrigel [16]. MCF-12A cells grown in 3D form growth-arrested acini reminiscent of the *in vivo* structure of the human mammary gland. When exposed to estradiol this morphology is disrupted [16], similar to the results obtained in the current study.

When cultured in 2D in the presence of E2, MCF-7 cells have a more invasive phenotype, and exhibit a loss of polarity [17, 18]. In the present 3D study, MCF-7 microtissues treated with E2 developed an altered phenotype, with increased overall microtissue area and a decreased lumen, even at concentrations as low as 10fM. This finding suggests that in this model, upon exposure to estradiol, MCF-7 cells undergo a change in their morphogenesis

in 3D, resulting in a disorganized microtissue that is less differentiated than untreated samples. In 2D cultures, while invasion and migration can be assessed using traditional assays, the phenotype of the culture as a whole is difficult to ascertain. The induction of this phenotype at low concentrations of E2 in the 3D culture model indicates this is a sensitive model to assess potential estrogenic activity. Further supporting this observation is the decrease in gene expression of transcripts that play a role in cell adhesion, including TJP1, TJP2 and CTNNB1 after E2 exposure. Previous work has indicated that MCF-7 cells cultured in 2D monolayer culture decrease expression of TJP1 following exposure to estradiol, which may lead to a more mesenchymal and invasive phenotype [19].

In the present study, select activation of ER α using the specific agonist PPT yielded several results that overlapped with E2 treatment, both in morphological changes and altered gene expression. Both PPT and E2 decreased the luminal ratio, while only E2 altered total cellular area, indicating that the loss of the luminal phenotype can be attributed to signaling mediated by ER α . In addition to the morphological changes, treatment with both E2 and PPT increased expression of the estrogen responsive target PDZK1[20], and decreased expression of ESR1, indicating that in this system, ER α is functional and responsive to E2. Decreased expression of ESR1 correlates with increased breast cancer progression in mouse [21], and the decrease in ESR1 expression observed in the present

study may contribute to the observed loss of luminal phenotype in both E2- and PPT-treated microtissues. E2 exposure also altered genes that were significantly altered following both DPN and PPT treatment, indicating that there is overlap between ER α and ER β signaling pathways. Another explanation for the high overlap between PPT and DPN treatments may be the relatively long time point of 7 days examined in the present study and the potential for initiation of downstream responses. Both ER α and ER β have the capacity to bind genomic estrogen response elements, and at later time points, the specific transcriptional profiles resulting from activation through each isoform may be confounded. The role of ER β within this system needs to be further elucidated. However, the alteration of specific genes altered with the agonists demonstrates the activation of each specific pathway, regardless of the timing, further strengthening the use of this system to investigate receptor-specific effects. In this study, there is a degree of variability in the control samples due to the use of histological sectioning, where controlling for the difference in z-direction is difficult. We expect that the adaptation of this culture system to a high-throughput confocal platform will help reduce variability in measurements.

In contrast to the morphologic and transcriptional changes observed following exposure to E2, PPT and DPN, G1 exposure did not significantly alter the luminal ratio of MCF-7 microtissues, but did significantly alter cellular area in a bi-modal concentration response. Recent work has indicated a role for

GPER in E2-mediated proliferation, despite its role in rapid, non-genomic signaling [22], and the current study further supports that E2-induced proliferation is likely due to a balance in signaling dependent on both GPER and ER α . At high concentrations, GPER induction by G1 inhibited MCF-7 microtissue proliferation as previously reported [23], but at low concentrations G1 increased proliferation, in agreement with recent work [22].

Overall, the present study describes a system that can be used to assess potential estrogenicity by combining gene expression and morphologic analyses. Exposure to receptor agonists results in the generation of unique MCF-7 microtissue phenotypes, allowing for the potential to assess receptor-specific responses.

4.6 Conclusions

The present study is the first to examine and to quantify changes in MCF-7 3D scaffold-free microtissue morphology as an indicator of estrogenic exposure. Additionally, this system allows for the evaluation of receptor-specific effects, and for the combined examination of molecular changes and phenotypic alterations as a result of exposure. With its potential applications for phenotypic, high content screening, this sensitive system can provide information about the safety of chemicals and their estrogenic effects, which may allow for an improved understanding of adaptive versus adverse estrogenic endocrine disruptor responses.

4.7 Acknowledgements

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4.8 Competing Interests

Kim Boekelheide is an occasional expert consultant for chemical and pharmaceutical companies, and owns stock in CytoSolv, an early stage biotechnology company developing a wound healing therapeutic.

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CHAPTER SIX

Genistein and Bisphenol A Alter 3D MCF-7 Microtissue Morphology and Gene Expression

Genistein and Bisphenol A Alter 3D MCF-7 Microtissue Morphology and Gene Expression

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Contributed to Data or Statistical Analysis: M.M.V., S.W.

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6.1 Abstract

When cultured in scaffold-free conditions using agarose hydrogels, MCF-7 human breast cancer cells self-assemble and organize into differentiated microtissues. MCF-7 3D microtissues are sensitive to estrogenic stimuli, and that their morphology can be altered following specific estrogenic receptor activation. In the present study, exposure to the estrogenic endocrine disrupting chemicals (EDCs) bisphenol A (BPA) and genistein (GEN) for 7 days altered MCF-7 microtissue morphology and gene expression in a compound- and concentration-specific manner. BPA treatment of 1 μ M increased luminal ratio and altered expression of 26 transcripts, including cell adhesion markers CLDN1 and FN, and cell cycle markers CCND2 and CCNA2. Treatment with 1 μ M GEN increased proliferation of MCF-7 microtissues and altered expression of 36 transcripts. The sensitivity and unique expression and morphologic changes observed in MCF-7 3D microtissues offer further insight into mechanism and effects of exposure to EDCs.

6.2 Introduction

As a result of the high cost, length of time and species differences between humans and laboratory animals, there is a large effort underway to move away from animal-based *in vivo* toxicity testing. There has been increasing research focusing on the use of *in vitro* cultures based on human cells, in both two-dimensional (2D) monolayer and three-dimensional (3D) culture models. The US EPA and NIEHS have both designed and begun using *in vitro* high-throughput, tiered testing batteries to assess and evaluate safety of environmental compounds, through the respective ToxCast and Tox21 programs [1, 2]. However, many of the models employed by these programs rely on 2D culture models, an environmental condition distinctly different than *in vivo* biology.

Monolayer cultures are widely used, due in part to their low cost and applicability to high-throughput assays. Growth on plastic substrates alters cell morphology and gene expression, and many types of cells grown in 2D lose expression of cell-type specific markers. Upon culturing cells using 3D models, cells become more differentiated, regaining both normal structure and function [3-6]. The resemblance in 3D culture of many cell types to their *in vivo* counterparts makes these systems attractive platforms for assessing compound safety.

While imaging of 3D cultures can be considerably more difficult than imaging monolayer cultures, the ability to assess changes in cell and microtissue

structure is an invaluable attribute of 3D culture models. This method of assessment allows for evaluation of structural changes that can be linked to well-characterized *in vivo* pathologic endpoints. MCF-7 human breast carcinoma cells cultured in a 3D model have demonstrated that 3D microtissue morphologic changes are an important, sensitive endpoint when assessing estrogenic activity of compounds *in vitro* [7].

Estrogens exert their effects through several mechanisms, including genomic and non-genomic signaling. Various estrogen receptors (ERs) exist, and these include ER alpha (ER α), ER beta (ER β) and g-protein coupled estrogen receptor (GPER). Adverse reproductive effects in uterine and breast tissues have been linked to exposure to exogenous estrogens[8-11], and assessing the estrogenic potential of environmental chemicals is an important undertaking. The EPA utilizes several high-throughput assays as part of the estrogenic activity screen for environmental chemicals, however, these assessments are based on gene reporter, receptor activation and proliferation assays in which cells are grown in 2D monolayer. Previous studies have demonstrated that 3D culture of mammary epithelial cells alters gene expression when compared to 2D monolayer cultures, and that the morphology of MCF-7 3D microtissues can be altered by selective activation of specific estrogen receptors[7]. In this study, we demonstrate that the estrogenic endocrine disrupting chemicals (EDCs) genistein (GEN) and bisphenol A (BPA) alter MCF-7 microtissue morphology and gene expression in a consistent,

compound-specific manner. The morphologic changes correspond to specific estrogen receptor pathway activation, and support the use of 3D culture systems to assess the estrogenic potential of chemicals.

6.3 Materials and Methods

6.3.1 Chemicals

Cell culture media and supplements were purchased from Life Technologies, Inc. (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Flowery Branch, GA) and dextran-coated-charcoal (DCC) stripped serum was purchased from Gemini Bioscience (Sacramento, CA). Dimethylsulfoxide (DMSO), bovine insulin, estradiol (E2), bisphenol A (BPA) and genistein (GEN) were purchased from Sigma Aldrich (St. Louis, MO). Ultra-pure agarose was purchased from Fisher Scientific (Agawam, MA), and Technovit 7100 was purchased from Heraeus Kulzer GmbH (Wehrheim, Germany).

6.3.2 Cell Culture

Human breast carcinoma MCF-7 cells (ATCC, HTB-22) (Soule, 1973) were cultured in complete growth medium (phenol red-free DMEM-F12 supplemented with 10%FBS, MEM non-essential amino acids, gentamicin and 10 μ g/mL bovine insulin) as monolayer cultures as previously described [6, 12]. Media was changed every 2-3 days and cells were passaged when 65-80% confluent. Cells were limited to use within 10 passages of the original vial obtained from ATCC to control for genetic drift and genomic instability.

6.3.3 3D Culture Methods

To make nonadhesive agarose hydrogels, 3D Petri Dish molds (Microtissues, Inc., Providence, RI) were made as previously described [6, 12, 13]. MCF-7 cells grown as monolayer cultures were trypsinized, counted, and seeded in agarose hydrogels as previously described [6]. MCF-7 cells settled into recesses for 30 minutes, and 2mL of treatment media was added.

6.3.4 Compound Exposure

GEN, BPA and vehicle (DMSO) solutions were made in treatment media (phenol red-free DMEM-F12 supplemented with 5% DCC FBS, MEM nonessential amino acids, gentamicin and 6ng/mL bovine insulin). Following seeding of cells into agarose hydrogels, 2mL treatment media was added per well and returned to a 5% CO₂ incubator. Media was changed every 2-3 days, and samples collected for analysis after 7 days of growth.

6.3.5 Histological Examination

Agarose hydrogels containing 3D MCF-7 microtissues were fixed in 10% neutral-buffered formalin overnight at 4°C and embedded in glycol methacrylate (Technovit 7100) as previously described [12]. Three-micron sections were cut, affixed to slides and stained using periodic acid and Schiff's reagent, and counterstained with hematoxylin. Images were taken on an Aperio Scanscope CS (Aperio Technologies, Vista, CA) at 40x magnification.

6.3.6 Histological Quantification Using Image J

Image of individual microtissues were collected, blinded and quantified in ImageJ as previously described (E2 morphology paper). Luminal area, aggregate area, nuclear count and debris area was measured for each microtissue. The luminal ratio was calculated as the ratio of luminal area to total aggregate area. Cellular area was calculated as total aggregate area minus luminal area. Data from each endpoint was imported in GraphPad Prism for statistical analysis.

6.3.7 RNA Isolation and PCR Array Analysis

Microtissues were collected from hydrogels and RNA isolated as previously described. A custom PCR array (Qiagen) was used to examine expression of targets involved in estrogen signaling, breast morphogenesis, cell proliferation and differentiation, and epithelial-to-mesenchymal transition. PCR arrays also included 5 housekeeping genes and contamination controls. Samples were prepared per manufacturers instructions and were added to plated using an epMotion 5075 automated pipettor (Eppendorf, Hamburg, Germany). Plates were run on an ABI 7900HT PCR cycler using conditions specified by the manufacturer.

6.3.8 Statistical Analysis

Histological measurements from ImageJ were imported into GraphPad Prism and analyzed using a one-way analysis of variance (ANOVA).

The raw PCR cycle (Ct) values were imported into the R statistical environment (ref: A language and environment for statistical computing), and values were normalized (Ct) using the SLqPCR package. The LIMMA package in R was used to construct a linear model of adjusted dCt values, and the empirical Bayes statistic was used. Genes with an adjusted p-value significance for multiple comparisons (q value) of less than 0.05 were selected.

6.4 Results

MCF-7 cells grown in scaffold-free agarose hydrogels develop and form differentiated microtissues, which contain a luminal space. Previous work has demonstrated that microtissues treated with estrogen-receptor specific agonists display altered morphology and gene expression (insert reference). Treatment with BPA and GEN in the present study altered MCF-7 microtissue morphology and gene expression in compound-specific patterns.

6.4.1 Bisphenol A Morphology and Gene Expression

Treatment with BPA for 7 days altered MCF-7 microtissue morphology. Concentrations of 1 and 100nM did not significantly alter the luminal morphology, however, 1 μ M BPA significantly increased the luminal ratio (Figure 6.1A). Treatment with 1nM BPA significantly decreased nuclear count, while both 100nM and 1 μ M did not alter cell counts (Figure 6.1B). Cellular area of MCF-7 microtissues was significantly decreased by both 1nM and 100nM BPA, but treatment with 1 μ M BPA did not alter cellular area of MCF-7 microtissues (Figure 6.1C).

MCF-7 microtissues treated with 1nM BPA for 7 days had significantly increased expression of two transcripts, wingless MMTV-integration site 5A (WNT5A) and wingless MMTV-integration site 4 (WNT4) (Table 6.1). Exposure to 100nM BPA significantly altered two transcripts, and increased expression of progesterone receptor (PGR) and decreased expression of cyclin

D2 (CCND2). Treatment with 1 μ M BPA altered expression of 26 transcripts, and increased expression of PGR, cathepsin d (CTSD), PDZ-containing domain 1 (PDZK1), trefoil factor 1 (TFF1), growth response to estrogen in breast cancer 1 (GREB1), fibronectin 1 (FN1), Wnt-inducible signaling protein 2 (WISP2), cyclin A1 (CCNA1) and claudin 1 (CLDN1). BPA treatment also increased expression of WNT5A, v-myb avian myeloblastosis viral oncogene homolog 2 (MYBL2), breast cancer 2 (BRCA 2), breast cancer 1 (BRCA1), WNT4 and amphiregulin (AREG). Treatment for 7 days with 1 μ M BPA significantly decreased expression of breast cancer anti-estrogen resistance 1 (BCAR1), laminin 5 (LAMA5), GATA binding protein 3 (GATA3), bone morphogenic protein 7 (BMP7), CCND2, twist-family BHLH transcription factor 1 (TWIST1), snail family zinc finger 2 (SNAI2), leucine rich repeat containing 15 (LRCC15), apolipoprotein D (APOD) and cytochrome P450 1A1 (CYP1A1) (Table 6.1).

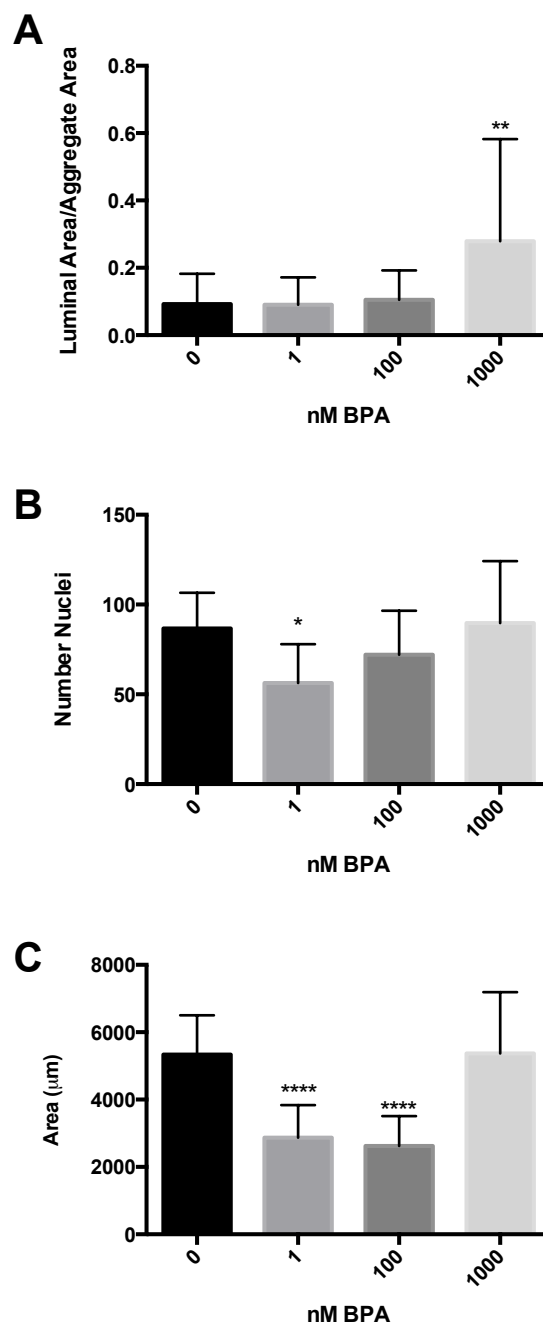


Figure 6.1 BPA exposure alters MCF-7 microtissue morphology at high concentrations. Exposure to 1 and 100nM BPA for 7 days does not alter the luminal morphology, but exposure to 1000nM BPA significantly increases the luminal ratio (A). BPA did not significantly alter nuclei number at high concentrations, but did decrease nuclei number at 1nM (B). Total cellular area was significantly decreased by exposure to 1nM and 100nM BPA, but exposure to 1000nM did not alter cellular area (C). * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$

Table 6.1

[BPA]	Gene name	Gene ID	Fold Change	q value
1nM	Wingless MMTV-integration site 5A	WNT5A	1.77	0.02640
	Wingless MMTV-integration site 4	WNT4	1.52	0.04389
100nM	Progesterone Receptor	PGR	1.59	0.00234
	Cyclin D2	CCND2	-1.66	0.01869
1μM	Progesterone Receptor	PGR	2.90	<0.0001
	Cathepsin D	CTSD	2.69	<0.0001
	PDZ-containing domain 1	PDZK1	2.46	<0.0001
	Trefoil factor 1	TFF1	2.30	<0.0001
	Growth response to estrogen, breast cancer 1	GREB1	2.27	0.00011
	Fibronectin 1	FN1	2.21	0.00018
	Wnt1 inducible signaling protein 2	WISP2	2.11	<0.0001
	Cyclin A1	CCNA1	1.91	<0.0001
	Claudin 1	CLDN1	1.82	0.00035
	Wingless MMTV-integration site 5A	WNT5A	1.73	0.03580
	v-myb avian myeloblastosis viral oncogene homolog-like 2	MYBL2	1.65	0.01512
	Breast cancer, 2	BRCA2	1.64	0.02378
	Breast cancer, 1	BRCA1	1.63	0.00269
	Wingless MMTV-integration site 4	WNT4	1.62	0.03439
	Amphiregulin	AREG	1.50	0.00486
	Breast cancer anti-estrogen resistance 1	BCAR1	-1.55	0.01038
	Laminin 5	LAMA5	-1.57	0.00875
	GATA binding protein 3	GATA3	-1.69	0.00438
	Bone morphogenic protein 7	BMP7	-1.84	0.00093
	Cyclin D2	CCND2	-1.95	0.00458
	Twist family BHLH transcription factor 1	TWIST1	-2.18	0.00013
	G-protein coupled estrogen receptor	GPER	-2.28	0.00054
	Snail family zinc finger 2	SNAI2	-2.46	0.00485
	Leucine rich repeat containing 15	LRRC15	-3.18	0.00350
	Apolipoprotein D	APOD	-3.20	<0.0001
	Cytochrome P450 1A1	CYP1A1	-3.47	<0.0001

6.4.2 Genistein Morphology and Gene Expression

MCF-7 3D microtissues morphology was significantly altered by GEN exposure. GEN treatment did not significantly alter MCF-7 microtissue luminal ratio (Figure 6.2A). Treatment with 1 and 100nM GEN did not significantly change nuclear count, though treatment with 1 μ M significantly increased nuclear count of MCF-7 3D microtissues (Figure 6.2 B). Cellular area of MCF-7 microtissues was significantly increased following exposure to both 100nM and 1 μ M GEN for 7 days (Figure 6.2 C).

Exposure of MCF-7 3D microtissues to GEN altered gene expression. Treatment with 1nM GEN significantly decreased expression of 4 transcripts including g-protein coupled estrogen receptor (GPER), bone morphogenic protein 4 (BMP4), BCAR1 and estrogen-related receptor alpha (ESRRA). Treatment with 100nM GEN altered expression of 14 transcripts and increased expression of ectonucleotide pyrophosphatase/phosphodiesterase 2 (ENPP2), BRCA2, PDZK1, PGR, cytochrome P450 19A1, AREG, CLDN1, CCNA1, MYC avian myelocytomatosis viral oncogene homolog (MYC), BRCA1, CTSD, FN1, and GREB1. Treatment with 100nM GEN decreased expression of GPER. Following treatment with 1 μ M GEN, 36 transcripts were differentially expressed, with increased expression of PDZK1, AREG, TFF1, BRCA1, PGR, CTSD and CCNA1. Expression of GREB1, CLDN1, FN1, MYC, ENPP2 and transforming growth factor beta 3 (TGFB3). Exposure to 1 μ M GEN significantly decreased expression of 24 transcripts

including Bcl2-associated death promoter (BAD), androgen receptor (AR), LAMA5, RAC-alpha serine/threonine-protein kinase (AKT1), cyclin E1 (CCNE1), WNT4, cyclin D dependent kinase inhibitor 1A (CDKN1A), tight junction protein 2 (TJP2), SNAIL1, mitogen-associated protein kinase 3 (MAPK3), BMP7 and twist family homolog 1 (TWIST1). BCAR1, estrogen receptor alpha (ESR1), ESRRA, BMP4, BCL2-like 1 (BCL2L1), GATA3 and LRRC15 were significantly decreased following 1 μ M GEN treatment. Expression of Erb-B2 receptor tyrosine kinase 2 (ERBB2), GPER, CYP1A1 and APOD were significantly decreased by 1 μ M GEN treatment (Table 6.2).

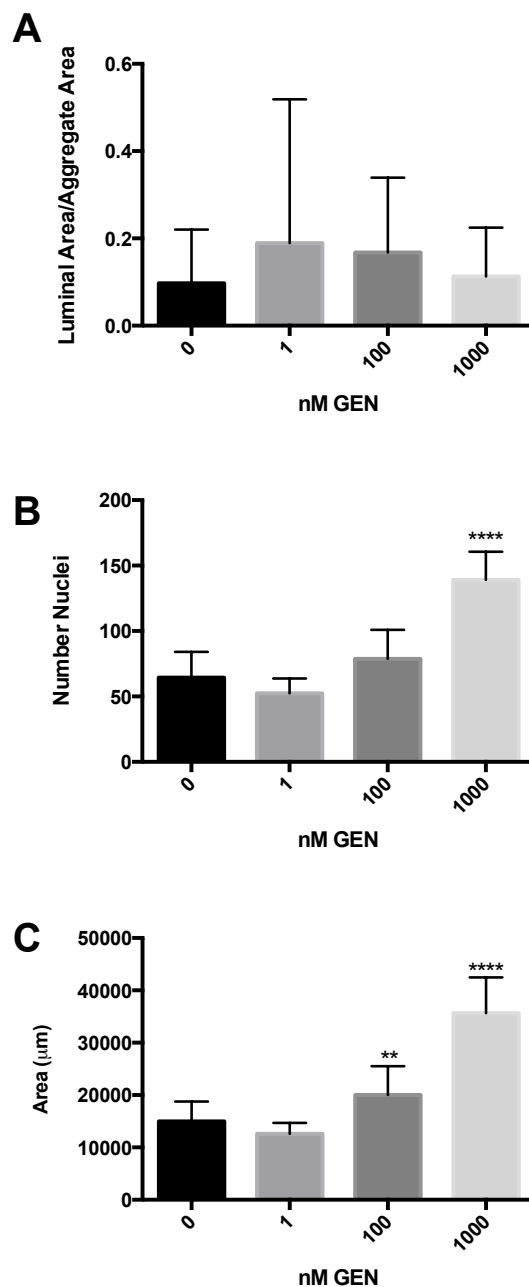


Figure 6.2 GEN exposure alters MCF-7 microtissue nuclear counts and cell area, but not luminal morphology. Luminal ratio did not change as a result of GEN exposure (A). MCF-7 microtissues exposed to 1 and 100nM GEN did not display any changes in nuclei number (B), but 100nM GEN significantly increased cellular area (C). Exposure to 1000nM GEN significantly increased nuclei number (B) and cellular area (C). ** $p < 0.01$, **** $p < 0.0001$

Table 6.2

[GEN]	Gene name	Gene ID	Fold Change	q value
1nM	G-protein coupled receptor	GPER	-1.51	0.01507
	Bone morphogenic protein 4	BMP4	-1.55	0.02173
	Breast cancer, anti estrogen 1	BCAR1	-1.60	0.00756
	Estrogen-related receptor alpha	ESRRA	-1.85	0.00109
100nM	Ectonucleotide pyrophosphatase/ phosphodiesterase 2	ENPP2	2.99	0.00087
	Breast cancer 2	BRCA2	2.75	0.00892
	PDZ-containing domain 1	PDZK1	2.26	0.00087
	Progesterone receptor	PGR	2.21	0.00125
	Cytochrome P450 19A1	CYP19A1	1.89	0.03887
	Amphiregulin	AREG	1.85	0.00033
	Claudin 1	CLDN1	1.74	0.01855
	Cyclin A1	CCNA1	1.62	0.03609
	MYC avian myelocytomatosis viral oncogene homolog	MYC	1.62	0.01238
	Breast cancer, anti estrogen 1	BRCA1	1.57	0.01286
	Cathepsin D	CTSD	1.57	0.00536
	Fibronectin 1	FN1	1.52	0.01909
	Growth response to estrogen in breast cancer 1	GREB1	1.51	0.04345
	G-protein coupled estrogen receptor	GPER	-1.59	0.00799
1μM	PDZ-containing domain 1	PDZK1	5.69	<0.0001
	Amphiregulin	AREG	4.86	<0.0001
	Trefoil factor 1	TFF1	3.61	0.04359
	Breast cancer 2	BRCA2	3.36	0.00263
	Progesterone receptor	PGR	3.33	<0.0001
	Cathepsin D	CTSD	3.10	<0.0001
	Cyclin A1	CCNA1	2.68	0.00023
	Growth response to estrogen in breast cancer 1	GREB1	2.54	0.00034
	Claudin 1	CLDN1	2.38	0.00139
	Fibronectin 1	FN1	2.07	0.00052
	MYC avian myelocytomatosis viral oncogene homolog	MYC	1.92	0.00160
	Ectonucleotide pyrophosphatase/phosphodiesterase 2	ENPP2	1.88	0.03171
	Transforming growth factor, beta 3	TGFB3	1.76	0.02255
	Bcl2-associated death promoter	BAD	-1.58	0.01444

	Androgen receptor	AR	-1.68	<0.0001
	Laminin 5	LAMA5	-1.71	0.00451
	RAC-alpha serine threonine-protein kinase	AKT1	-1.73	0.00299
	Cyclin E1	CCNE1	-1.74	0.00890
	Wingless MMTV integration site 4	WNT4	-1.80	0.01763
	cyclin D dependent kinase inhibitor 1A	CDKN1A	-1.80	0.00087
	Tight junction protein 2	TJP2	-1.83	0.00718
	Snail family transcription factor 2	SNAI1	-1.92	0.00263
	mitogen-associated protein kinase 3	MAPK3	-2.05	<0.0001
	Bone morphogenic protein 7	BMP7	-2.24	0.00028
	Twist family homolog 1	TWIST1	-2.48	0.00011
	Breast cancer, anti-estrogen 1	BCAR1	-2.53	<0.0001
	Estrogen receptor alpha	ESR1	-2.67	<0.0001
	estrogen-related receptor alpha	ESRRA	-2.74	<0.0001
	Bone morphogenic protein 4	BMP4	-2.88	<0.0001
	BCL2-like 1	BCL2L1	-2.97	<0.0001
	GATA binding protein 3	GATA3	-3.08	0.01455
	Leucine rich repeat containing 1	LRRC15	-3.67	0.01023
	Erb-B2 receptor tyrosine kinase 2	ERBB2	-3.74	<0.0001
	G-protein coupled receptor 1	GPER	-7.57	<0.0001
	Cytochrome P450 1A1	CYP1A1	-8.31	<0.0001
	Apolipoprotein D	APOD	-20.99	<0.0001

6.5 Discussion

Exposure to the xenoestrogens GEN and BPA significantly altered morphology and gene expression profiles of MCF-7 3D microtissues. Previous work has demonstrated that 3D MCF-7 microtissues generated using agarose hydrogels are sensitive to estrogenic stimuli, and display altered morphologies and gene expression profiles after selective estrogen receptor activation [7]. In the present study, BPA treatment decreased cellular area at 1 and 100nM, and did not significantly change cellular area of the MCF-7 microtissues at the highest concentration of 1 μ M. In contrast to previously published work demonstrating that both estradiol treatment and specific activation of estrogen receptor alpha (ER α) lead to a concentration-dependent loss of the luminal space and an increase in proliferation[7], treatment with BPA did not significantly disrupt microtissue morphology. Interestingly, in addition to not displaying large morphologic changes, MCF-7 microtissues treated with BPA displayed subtle changes in gene expression consistent with increased differentiation and the maintenance of morphologic feature of glandular phenotype. BPA induces changes in typical estrogen-responsive targets including PGR, CTSD, PDZK1, TFF1 and GREB1, though the fold-change values are lower than those reported following estradiol stimulation [7, 14, 15], indicating that BPA acted as weak estrogen, but only at high (1 μ M) concentrations. In contrast to traditional estrogenic stimuli that induce proliferation and de-differentiation of cells, BPA exposure decreased

expression of the epithelial to mesenchymal transition regulators TWIST1 and SNAI2. Both TWIST1 and SNAI2 repress e-cadherin transcription to initiate the epithelial to mesenchymal transition [16, 17]. The down regulation of TWIST and SNAI2 correlates with the increased expression of WNT5A and WNT4 observed in the present study, as both WNT5A and WNT4 promote mammary gland development [18, 19]. Further supporting BPA's role in promoting differentiation is the significant increase in CLDN1, a protein involved in tight-junctions important for cell-cell adhesion [20]. Overall, high concentrations of BPA promote cell differentiation, and this observation warrants further investigation. The relatively weak estrogenic response observed in the present study conflicts with previous data describing the effects of estradiol on MCF-7 microtissues[7], and indicates that BPA does not act as an ER α agonist, but may signal primarily through other receptors including GPER. Previous work has demonstrated that it binds strongly to estrogen-related receptor gamma, an orphan nuclear receptor[21]. The role estrogen-related receptor gamma plays in mediating BPA effects needs to be investigated. The lack of changes at low concentrations of BPA may be due to the lack of metabolism of BPA in this system, as BPA is metabolized into 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBP) and further studies need to be performed to elucidate the effects that MBP has on the morphology and gene expression of MCF-7 microtissues.

Treatment with the soy isoflavone genistein did not alter luminal ratio, but did increase proliferation and cellular area at high concentrations. Genistein has high affinity for ER β compared to ER α [22]. It has been demonstrated that genistein acts as an estrogen in normal mammary gland tissue *in vivo* and increases proliferation[23]. Genistein has high affinity for ER β [22], but at high concentrations (i.e. 1 μ M) can also activate ER α and lead to proliferation, like that observed in the present study. Our morphologic and gene expression results support this observation, as at concentrations of 1 μ M increases proliferation and altered expression of genes critical for cell cycle progression including CCNA1, CDKN1A, CCNE1 and AKT1. Studies have demonstrated that overexpression of ER β results in reduced proliferation and reduced tumor formation of MCF-7 cells *in vivo*. The data generated in this study further supports the previous observations that genistein activates ER β at physiologic levels [24], and that the observed reduction of proliferation was due to inhibition of S-phase transition [25] and G1 arrest[26]. In the present study, 1nM GEN treatment resulted in a decrease in proliferation of 3D MCF-7 microtissues, although it was not significant. This observation coupled with the gene expression results indicating cell cycle progression was altered further demonstrated that GEN signals through ER β at low levels and inhibits proliferation, and at higher concentrations can activate ER α and result in proliferation. Further studies are needed to determine the response of MCF-7 3D microtissues to GEN at concentrations lower than 1nM.

6.6 Conclusions

Overall, the present study demonstrates the utility of using 3D MCF-7 microtissues to better understand the effects of suspected estrogenic EDCs on breast cells. We demonstrate that exposure to different EDCs leads to altered microtissue morphology and gene expression that is consistent with specific receptor activation. This data supports the use of 3D microtissue models to assess potential estrogenic activity of compounds, as they allow for the assessment of morphologic changes and the underlying molecular alterations.

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6.8 Competing Interests

Kim Boekelheide is an occasional expert consultant for chemical and pharmaceutical companies, and owns stock in CytoSolv, an early stage biotechnology company developing a wound healing therapeutic.

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CHAPTER SEVEN

Discussion and Conclusions

7.1 Overview

There is a large need to develop higher-throughput, inexpensive and relevant assays for toxicity testing[1]. Historically, toxicity testing has relied on the use of animals, which are exposed to high doses of test compounds and evaluated for adverse responses. Animal testing is time-intensive, expensive, and small rodent biology does not completely recapitulate human biology. The use of *in vitro* assays is the basis for the Tox21 and ToxCast assays, though the cell culture assays are based on monolayer cultures. While monolayer culture models are inexpensive, technically simple and easily scalable to high-throughput screening approaches, cells grown in monolayer lose a number of their cell-type specific markers and functions. However, in contrast to monolayer cultures, where cells grow as flat sheets, three-dimensional (3D) cultures increase cell-cell contact, which alters the differentiation and gene expression of cells *in vitro* [2-7].

A specific area of concern is the potential of environmental chemicals to interfere with the endocrine system. These compounds are termed endocrine disrupting compounds (EDCs). Increased exposure to EDCs is associated with increased risk of certain cancers and reproductive tract malformations[8-10]. Therefore, it is important to screen for the potential of compounds to interfere with the signaling of the endocrine system.

A large focus of EDC research examines the ability of chemicals to alter estrogen signaling pathways. Environmental compounds inducing diethylstilbestrol (DES), bisphenol A (BPA) and genistein (GEN) bind estrogen receptors involved in both pregenomic and genomic estrogenic signaling pathways. Exogenous exposure to these and other estrogenic EDCs has been associated with altered reproductive tract development and function and an increased risk of reproductive cancers. To test for the ability of chemicals to interfere with endocrine signaling pathways, the US EPA developed the Endocrine Disruptor Screening Program (EDSP). The ToxCast program also includes a number of assays that assess for estrogen, androgen and thyroid hormone disruption. The *in vitro* assays cover receptor binding, activation and translocation, and there are cellular proliferation assays for the estrogen pathway. The cellular *in vitro* assays are based solely on monolayer culture models, which do not completely recapitulate *in vivo* biology.

This research effort explored the development and characterization of a 3D scaffold-free culture model of human MCF-7 human breast carcinoma cells, and the morphologic and molecular changes associated with exposure to estrogenic endocrine disruptors. The use of, and reliance on, relevant *in vitro* assays in toxicity testing is growing, due to the limitations of animal testing and the inherent advantages to using human cells for testing. This work first improved the tools needed to visualize 3D microtissues with histopathological

sections and using confocal imaging (**Chapter 2**). Next, we examined the differentiation and phenotype of MCF-7 cells cultured in this model, compared to monolayer cultures, using immunofluorescent and molecular markers of epithelial and luminal mammary differentiation (**Chapter 3**). Following the characterization of this system, the differences of 2D and 3D culture responses to E2 were explored using custom PCR arrays (**Chapter 4**). To determine the ability of the system to detect estrogenic compounds, morphologic and molecular changes were quantified following exposure to estrogen receptor-specific compounds (**Chapter 5**). Finally, the 3D MCF-7 microtissue system was used to assess morphologic and gene expression changes following exposure to several concentrations of two EDCs – the soy isoflavone genistein (GEN) and bisphenol A (BPA) (**Chapter 6**).

The results addressed the following questions:

- What modifications to existing histologic and imaging techniques are needed to visualize 3D microtissues grown in agarose hydrogels?
- Do MCF-7 human breast cancer cells form differentiated microtissues when cultured in scaffold-free hydrogels?

- Are 3D MCF-7 microtissue more differentiated than traditional monolayer cultures?
- Do 3D MCF-7 microtissues respond differently to E2 than monolayer cultures?
- Is MCF-7 microtissues morphology sensitive to disruption by receptor-specific agonists?
- Can alterations in MCF-7 microtissue morphology following exposure to estrogenic chemicals be correlated with gene expression changes?
- Can this model be used in future studies to evaluate the potential of environmental compounds to interfere with estrogen signaling?

These questions were answered using a variety of histological and molecular techniques, which provided new insights into the use of 3D cultures to assess potential estrogenic endocrine disruption, and addressed the following aims:

Specific Aim 1: Characterize the differentiation, phenotypic changes, and

molecular profiles of human breast cells grown in a 3D scaffold-free cell culture system.

Specific Aim 2: Determine differential 3D model responses to E2 and estrogen receptor-specific agonists.

Specific Aim 3: Investigate the response of the MCF-7 cells in the 3D scaffold-free system to estrogenic endocrine disrupting compounds including genistein (GEN) and bisphenol A (BPA).

The resulting data provided a results supporting the use of scaffold-free 3D culture models in toxicity testing assays, and has paved the way for designing future assays that combine the strengths of assessing histopathological changes with the convenience of *in vitro* cell culture systems.

7.2 Summary of Major Findings

7.2.1 Modifications to Histologic and Imaging Techniques Enables Improved Visualization of 3D Scaffold-free Microtissues

These studies focused on adapting existing histologic and imaging techniques for use with 3D scaffold-free agarose hydrogel cultures, to provide optimized protocols for use in this thesis. For these studies, LNCaP prostate adenocarcinoma and Beas-2B immortalized lung epithelial cells were grown in agarose hydrogels for up to 21 days. Using an amended glycol-methacrylate embedding protocol, the entire hydrogel was embedded and sectioned. The two-step *en-bloc* embedding protocol enabled visualization of up to 256 microtissues per histological section, and permitted examination of nuclear, cellular and overall microtissue morphology. The ability to assess histopathological changes in the microtissue *in vitro* system is a critical finding, as it is a unique aspect of 3D microtissues. This allows for bridging the gap between monolayer cultures and *in vivo* histopathological endpoints, further supporting the use of 3D cultures as relevant models for *in vitro* toxicity testing.

While histopathological assessment allows for detailed visualization of cellular morphology, the ability to image the entire 3D microtissue without sectioning allows for the analysis of microtissue structure. Confocal imaging is limited in thick tissue samples, due to scattering of light. To address this

concern, an organic solvent-free method for optical clearing, Clear^{T2}, improved imaging depth and greatly increased the number of discernable nuclei in cleared samples compared to uncleared controls. These results demonstrate the first application of the Clear^{T2} clearing method to non-neuronal microtissues. Clear^{T2} is a fast, detergent-free, immersion-based method, ideal characteristics for 3D *in vitro* applications and toxicity testing [11-16]. Increased imaging depth and better resolution allowed for better quantification of morphologic and phenotypic changes within 3D microtissues, enabling the assessment of cellular changes within a heterogeneous microtissue.

The use of non-adhesive micromold hydrogels gels to create 3D multicellular *in vitro* models is important platform with the capability to merge the complexities of *in vivo* tissues with the advantages and practicality of 2D *in vitro* models. The ability to clearly assess detailed morphology, evaluate longer-term endpoints with sub-lethal markers and discern adaptive responses from acute toxicity is an invaluable tool. The improved techniques presented in this study have addressed the visualization challenges of 3D microtissues, highlighting their utility for evaluating classical pathological changes, and laying the foundation for alternative platforms as efficient and effective screening tools, with the potential to contribute to the ongoing revolution in toxicity testing [17] (**Chapter 2**).

7.2.2 MCF-7 3D Microtissues Are More Differentiated Than Monolayer Cultures

These studies characterized the growth of human MCF-7 breast carcinoma cells in the scaffold-free conditions provided by using agarose hydrogels, addressing *Specific Aim 1*. MCF-7 cells were selected due to their extensive use and their reported expression of estrogen receptors α , β and GPER. MCF-7 cells are reported to expression orphan estrogen receptors as well, but these were not examined in this study. Environmental chemicals bind the estrogen receptors with varying affinities, making it critical to select a cell line with expression of all receptors. ER α , ER β and GPER work in concert to modulate estrogen signaling, and the pathways activated by each receptor can converge downstream.

Previous work has examined the growth of various normal and malignant breast cell lines to grow in 3D culture models. Various cell lines grow in scaffolds including collagen and Matrigel, however, imaging these cultures can be difficult. Additionally, cells need to be detached from the matrix before use in downstream molecular techniques, which can alter gene and protein expression. Finally, cell density within collagen and Matrigel scaffolds is generally much lower than the dense cellularity of epithelial tissues. To address these concerns, a scaffold-free culture model was chosen.

These studies began by establishing that MCF-7 cells could survive in scaffold-free agarose hydrogels. MCF-7 cells were trypsinized and seeded into agarose hydrogels, and allowed to grow for periods up to 35 days, with media changes throughout. In contrast to many cell types cultured in scaffold-free conditions, MCF-7 cells formed microtissues containing luminal spaces. Luminal spaces were observed up to 35 days in culture (data not shown). Histological analysis revealed that the luminal spaces contained PAS-positive secretions that increased over time, and these results were confirmed using TEM. This luminal morphology is similar to the *in vivo* morphology of the adult mammary gland, and the presence of secretory products in the luminal space suggests that MCF-7 cells regain functionality and differentiate when cultured under scaffold-free conditions.

To further characterize the differentiation of MCF-7 cells cultured in this model, expression and localization of mammary epithelial and luminal differentiation markers was examined using molecular and immunofluorescent techniques, and compared to monolayer cultures. The use of a custom PCR array containing targets involved in estrogen signaling, cell adhesion, cell differentiation and cell death was used to compare gene expression of 2D and 3D cultures, and revealed increased differentiation of 3D microtissue cultures. Differences in gene expression between 2D and 3D cultures correlated with observed proteins detected using immunofluorescence. Together, these results demonstrate that MCF-7 cells

cultured as 3D microtissues are more differentiated than 2D monolayer cultures (**Chapter 3**).

7.2.3 MCF-7 3D Microtissues Respond Differently to E2 When Compared to Monolayer Cultures

This study focused on determining how differentiated MCF-7 3D microtissues and 2D monolayer cultures responded to E2, to address *Specific Aim 1*. MCF-7 microtissues were more differentiated than monolayer cultures[18], and therefore were likely to respond in a different manner to E2. Monolayer and 3D cultures of the same cells have distinct differences in their gene expression profiles[19]. In this study, MCF-7 cells were grown as 2D or 3D microtissues and exposed to vehicle control or 1nM E2 for 4, 8 and 24 hours, and gene expression was analyzed using custom PCR arrays. Overall, the gene expression changes overlapped between the 2D and 3D treatments at 4 hours, particularly in terms of well-characterized estrogen-responsive genes. However, the 2D versus 3D culture responses began to diverge at 8 hours, and the cultures had distinctly different gene expression at 24 hours. These results demonstrate that 2D and 3D cultures respond to E2 differently, likely due to their differences in underlying gene expression profiles as a result of the culture method, which increases cell-cell interactions and cell differentiation. Interestingly, the 3D cultures showed large changes in gene expression of cell adhesion and communication

markers. This suggests that 3D microtissues are a better model for understanding the downstream responses to compounds, as it is possible to detect dynamic changes in these pathways. These findings demonstrate the importance of selecting a relevant cell system for determining the downstream responses to toxicants (**Chapter 4**).

7.2.4 MCF-7 3D Microtissue Morphology is Altered by Estrogenic Compounds

These studies began by determining how MCF-7 microtissue formation and morphogenesis is altered by exposure to estrogen, designed to address ***Specific Aim 2***. MCF-7 cells were seeded into agarose hydrogels and treated with a range of concentrations of E2 for 7 days. Following 7 days of treatment, it was evident that even at concentrations as low as 10 pM, E2 altered 3D microtissue morphology in a concentration dependent manner, with significant changes in luminal morphology. E2 treatment significantly decreased the luminal ratio, indicating a loss of differentiation compared to vehicle-treated controls. This was further supported by the observation that E2 increased nuclear count and 3D microtissue size. As E2 is well documented to promote mammary cell proliferation, this result was not in and of itself striking; however, the ability to quantify morphologic changes in this *in vitro* system is an important finding. In addition to assessing the morphologic changes, changes in gene expression as a result of exposure to

1nM E2 were examined. Classical estrogen responsive genes including PDZK1, ESR1 and APOD were altered in a manner consistent with previous studies, and alterations of expression of genes involved in cell adhesion and communication were decreased compared to vehicle-treated controls. Additionally, genes involved in cell cycle progression were significantly changed, further supporting the observed increase in nuclear counts in the morphologic assessment.

To determine which estrogen receptor pathway mediated the observed luminal changes, MCF-7 microtissues were grown in the presence of a range of concentrations of PPT, the ER α -specific agonist. Like E2, PPT treatment resulted in decreased lumen formation, increased nuclear count and non-significant changes in microtissue area. Gene expression analysis also demonstrated significant changes in ER α responsive genes, cell cycle regulators and genes involved in cell adhesion following exposure to 5nM PPT. The panel of genes altered by PPT was much more condensed, which is consistent with the receptor-specific properties of the compound. As a result of these observations, it was determined that ER α signaling was driving the loss of luminal morphology observed in the PPT and E2-treated microtissues.

Next, the response of the microtissues to the ER β -specific agonist DPN was examined across a concentration range. DPN treatment caused reductions in luminal formation at higher concentrations; however, this was likely due to cross activation of the ER α receptor, due in part to the relatively

long time of exposure (7 days), low observed levels of mRNA and protein of the ER β gene, and documented cross activation of ER α by DPN at high concentrations. Due to the convergence of these possible contributors, ER β agonism significantly altered only one target transcript, APOD, which was also altered in E2 and PPT treatments.

Finally, the response of the system to the GPER agonist G1 was examined across concentrations, and G1 was found to promote an interesting morphologic effect. GPER maintained the luminal formation, regardless of microtissue area. GPER did not induce significant changes in any gene included on the PCR array, though it is possible that changes are induced in genes not included on the array. Despite this observation, this finding is significant as it demonstrates the sensitivity of both the morphologic and mRNA expression changes in the 3D MCF-7 microtissues.

Overall, this study showed that histopathological changes within 3D MCF-7 microtissues can be quantified and were sensitive indicators of estrogen exposure. Additionally, the morphologic and gene expression changes were dependent on selective receptor activation. These findings support the further use of this system, and others like it, to assess morphologic changes in a simple *in vitro* system (**Chapter 5**).

7.2.5 Xenoestrogens Disrupt MCF-7 Morphology and mRNA Expression

Characterization and use of the MCF-7 3D microtissues to assess pathway-specific estrogen receptor effects allowed for its use in assessing the effects of estrogenic endocrine disrupting chemicals, to address *Specific Aim 3*. Monolayer cultures were used to determine a NOAEL, LOAEL and high concentrations of both GEN and BPA to inform concentration selection based on proliferation and expression of estrogen-responsive genes CCND1 and PGR. From these studies, it was determined for each compound that morphologic and gene expression analysis would be performed on exposure of 1, 100 and 1000nM concentrations, following 7 days of exposure.

BPA treatment across concentration elicited unique responses in terms of both morphologic changes and molecular changes. In assessing luminal morphologic changes, BPA did not significantly alter MCF-7 lumen formation at concentrations of 1 or 100nM. BPA treatment of 1nM significantly reduced the nuclear number of MCF-7 microtissues, while both 1nM and 100nM treatment was sufficient to significantly reduce microtissue cellular area. Assessment of select genes using the custom PCR arrays revealed interesting changes, where 1nM altered expression of genes involved in tissue differentiation, though the magnitude of fold change was small. PGR and GREB1, ER α -specific targets, were increased though fold change magnitude was again modest when compared with PPT and E2 stimulation. At 1000nM BPA, several striking morphologic changes were observed, with a highly

increased luminal area, paired with no observable changes in proliferation or microtissue cellular area. Another observation was that high concentration of BPA increased thinning of the luminal wall, though this data has not yet been quantified. A large subset of genes was altered by treatment, including those involved in promotion of differentiation (WNT4, TWIST1 and BMP7), though this was confounded by altered expression of differentiation markers FN1 and LAMA5 that are consistently implicated in promoting invasion and a mesenchymal transition. These observations, coupled with the increased expression of some ER α target genes, was likely due to crosstalk between estrogen signaling pathways at high concentrations.

Treatment with GEN did not alter the luminal morphology, though more cellular debris was present in sections of samples treated with the higher concentrations of GEN for 7 days. Both nuclear number and microtissue area were significantly increased at the highest concentration of 1000nM, but no changes were detected at the lower concentrations. Gene expression analysis revealed significant changes in expression at all treatments, indicating that the microtissues may be more sensitive to GEN than the monolayers observed in the preliminary study, though the targets altered were not assessed in the early study. Exposure to 1nM GEN altered targets involved in estrogen signaling and morphogenesis, notably GPER and ESRRA, both demonstrated to be targets of GEN [20]. Expression of 14 targets was altered following 100nM GEN treatment for 7 days, and the

altered targets included classical ER α responsive genes, such as PGR and GREB1, as well as increased expression of the ER β target ENPP2. The combination of these responses indicated that at this concentration, there was a tipping point from an ER β -mediated response to an ER α response. This is consistent with the observations regarding both morphologic and molecular changes at the highest concentrations, as the results indicate an even stronger ER α -dependent response, demonstrated by increased altered expression of ER α targets. This finding of cross-activation of ER α by GEN at high concentrations has been described in other *in vitro* and *in vivo* studies[21]. It is worth noting that a 1000nM exposure to GEN is exceptionally high and likely not relevant for human exposure *in vivo*. Overall, we have preliminarily demonstrated the utility of this system for better characterizing and understanding alterations caused by exposure to various estrogenic endocrine disrupting compounds (**Chapter 6**).

To summarize, the data presented in this thesis demonstrates that histological techniques for *in vivo* assessment of histopathological endpoints can be optimized and applied to 3D *in vitro* microtissue cultures. Data from ***Specific Aim 1*** supports the increased differentiation of MCF-7 microtissues when compared to monolayer cultures, and that MCF-7 microtissues respond in a more sensitive manner than MCF-7 monolayer cultures following treatment with estradiol. This system is sensitive to both morphological and molecular disruption by E2 and estrogen-receptor specific agonists, and

activation of each receptor leads to specific downstream changes, addressing the goals of ***Specific Aim 2***. The morphologic changes are quantifiable and reproducible, indicating that MCF-7 microtissues are useful in assessing histopathological changes *in vitro*. In ***Specific Aim 3***, this approach was applied to two model xenoestrogens, GEN and BPA, and the molecular and morphologic changes were correlated and provide insights into the mechanism of disruption as a result of concentration. In summary, this model combines the utility of *in vitro* systems with the assessment of *in vivo* histopathological endpoints in a simple system.

7.3 Limitations

While this model has many advantages, there are several limitations to using cancer-derived cell lines. These limitations are centered on problems with genetic drift, which can alter the cellular genetics, molecular and phenotypic responses between lots, passages and lab cell stocks. Additionally, cell line contamination by other cell lines as well as mycoplasma has been described previously[22-26].

Although they are one of the most widely used cell lines, MCF-7 cells have their downside. MCF-7 cells are highly genetically unstable, which has resulted in stocks of cells have different chromosomal numbers. This genetic instability has contributed to intra- and interlaboratory variability, and influences the gene expression, phenotype and responses of the cells to estrogens. Due to this high amount of variability, most test nominations avoid use of the MCF-7 cell line.

Despite advances described in this thesis and by others, there are limitations to the imaging of 3D microtissues. First, the histological technique utilized throughout this study renders it difficult to accurately assess microtissue size at the same z-location across multiple microtissues in a mold, which results in the noise and variability in assessing the morphologic data sets. This limitation is of particular concern with regards to the analysis of compounds that may agonize several receptors and pathways, as this can lead to competing effects. Additionally, despite advances in

development and application of various optical clearing techniques, confocal imaging is still limited to an approximate depth of 150-200 μ m into the sample. Multiphoton microscopy improves this depth, though there are currently not high-throughput multiphoton platforms available. Additionally, the imaging protocols utilized here are time-intensive, limiting the number of samples, timepoints and concentrations that can be tested for a given chemical.

Another limitation of the present study is the reliance on pharmacologic agonists to specifically target receptors, which confounds the data, particularly at high concentrations. As described previously, at high concentrations, non-specific activation of non-target receptors was observed, and altered morphologic and gene expression outcomes.

Finally, a perceived limitation of the study described in this thesis is the reliance on PCR arrays for gene expression analysis. While convenient and informative, the examination of approximately 80 mRNA transcripts missed substantial amounts of biology. It is likely that the use of microarrays would have resulted in both shared and compound-specific profiles. Additionally, the use of microarrays would provide information with regard to multiple pathway activation.

In spite of these issues, the benefits of using MCF-7 cells overwhelm the limitations. The information gathered from these studies does not

require any form of species extrapolation, and allows for evaluation of xenoestrogens in a simple, human cell-based system.

7.4 Future Directions

The studies presented in this thesis have paved the way for a variety of future experiments using this and similar cell models. This model has provided insight into both morphologic and molecular endpoints affected by estrogen exposure, and these findings can be greatly expanded going forward.

7.4.1 Adapt MCF-7 microtissues to use in a high-content confocal platform

The main focus of this work was to characterize MCF-7 microtissues and their response to estrogens. The experiments presented here have demonstrated that MCF-7 microtissue morphology and gene expression are sensitive to estrogenic compounds, and that the responses are compound and receptor-specific. While the data presented has demonstrated that MCF-7 microtissue morphology is sensitive to disruption, the data is highly variable due in large part to the reliance on traditional histological techniques and the lack of control over the location of spheroids in the z-direction. This resulted in large variances in microtissue area, which can alter the luminal endpoint readouts as well. These techniques are also time and labor-intensive, so the use of a confocal imaging platform where the entire 3D microtissue can be imaged and reconstructed for analysis would allow for the generation of more

complete data sets. However, this approach is not without its challenges, as the analysis of such large data sets require the development of intricate image analysis protocols. Despite this challenge, the adaptation of the microtissue model to a high-content confocal setting would allow for more comprehensive analysis of morphologic changes in a more efficient and reproducible manner.

These studies have already begun, and focus on the optimization of seeding density to ensure that microtissue size is within the imaging limit of the high-content confocal. Additionally, we are focused on the development of image analysis protocols that would allow for the volumetric analysis of 3D renderings of the microtissues. Following the development of these protocols, we will focus on studies examining the response of the microtissues to specific agonists across concentration and time.

7.4.2 Use CRISPR technology coupled with pharmacologic agonists to target specific receptor pathways

The focus of this thesis was to examine responses of MCF-7 microtissues following estrogenic stimulation. In the work presented in **Chapter 5**, pharmacological agonists of each estrogen receptor were used to specifically target genomic and nongenomic signaling pathways. While the data from each of these experiments is convincing, at high concentrations,

there is likely cross-activation of pathways, which has the potential to confound results. In future studies, it would be useful to combine the use of pharmacologic agonists and antagonists with the use of genetic manipulations, such as that provided by CRISPR technology. Using CRISPR allows for the selective deletion of specific genes, without the incomplete and unstable knockdowns generated using siRNA and shRNA techniques. CRISPR technology would allow for the deletion of each specific receptor. This method would allow for the characterization of microtissue morphology without the presence of a specific receptor. Following this initial characterization, these cells would then be exposed to selective agonists and morphologic endpoints examined. This combined approach would allow for better characterization of the ER α , ER β and GPER responses. As these signaling pathways converge on shared downstream targets, the analysis in an intact system can be complex.

Another benefit of using CRISPR in this system would allow for the elucidation of receptor isoform effects, particularly with regard to both the ER α 66, 46 and 36 isoforms. By deleting the ESR1 gene and then expressing each isoform, the effect of each isoform could be determined independently. Previous research has demonstrated that these isoforms bind xenoestrogens with varying affinities[27], and that the isoforms play different roles in estrogen signaling. These experiments would provide more information about

the biology of estrogen signaling, and also provide insight into the mechanisms of estrogenic endocrine disruption.

7.4.3 Development of co-culture models using MCF-7 cells

When cultured as monocultures in this model, MCF-7 microtissues are differentiated and sensitive to estrogenic stimuli. While the MCF-7 microtissues form a more differentiated culture system than monolayer cultures, monolayer cultures do not contain all of the cell types present in the *in vivo* mammary gland. The stromal compartment of the mammary gland, which is primarily composed of fibroblasts, is critical for maintenance of the mammary epithelium, plays a large role in modulating hormonal signaling, and may play a large role in promotion of breast carcinogenesis[28,29].

In these proposed experiments, MCF-7 cells would be cultured as microtissues using the same scaffold-free agarose hydrogels used in the present study. However, instead of only seeding trypsinized MCF-7 cells into the hydrogels, a mixed cell population made of MCF-7 cells and primary normal fibroblasts would be used. When fibroblasts and epithelial cells are mixed and seeded into agarose hydrogels, they will self-sort and self-assemble into organized microtissues[30]. Based on the results obtained in this thesis, MCF-7 and normal mammary fibroblasts should interact.

Preliminary studies would need to focus on optimization of cell culture medium and the fibroblast-to-epithelial cell ratios.

It is unclear what effect the addition of fibroblasts would have on microtissue morphology, though manipulation of the seeding protocol may control tissue organization. Based on literature using other cell types, fibroblasts may migrate to the center of the tissue, and result in polarization of the epithelium towards the outer surface of the microtissues, with no lumen formation. It is also possible that microtissues containing luminal spaces may still form, which would allow for the assessment of endpoints like those used in the present study. Either structure would allow for the assessment of estrogenic responses in the context of combined stromal and epithelial signaling in co-cultures.

7.4.4 Adaptation of other cell types for use in this model

To transition from MCF-7 cells, initial studies have been undertaken to examine the ability of T47D human ductal carcinoma cells to form microtissues using agarose hydrogels. T47D cells express ER α , ER β and GPER, and appear to be more genetically stable than MCF-7 cells. Preliminary results indicate that T47D microtissues form large aggregates that contain areas of ductal differentiation. These ducts contain PAS-positive secretions, as well as membrane-bound secretory vesicles when examined

using TEM. A pilot study designed to examine the morphologic response of these microtissues to E2, PPT and DPN demonstrated that T47D microtissues are sensitive to estrogenic disruption in a similar manner to MCF-7 cells, where E2 and PPT treatment reduced ductal formation and increased nuclear counts. The corresponding molecular analysis has not yet been performed for the T-47D system.

To move away from pitfalls associated with cancer-derived cell lines, it is also possible to use this approach to generate microtissues using normal human mammary epithelial cells (HMECs), which are primary cells isolated from reduction mammoplasty tissue. A pilot study was performed to determine if HMECs were able to survive in this system, and it was determined that HMECs form microtissues which appear to begin to undergo apoptosis around day 10, in a process that appears to be luminal clearing (data not shown). Additionally, the use of HMECs and normal human fibroblasts as a co-culture model would provide an interesting and relevant system. This system needs to be further characterized for estrogen receptor and differentiation status.

7.4.5 Assessment of anti-estrogenic compounds

This thesis examined the effects of selected estrogenic compounds on MCF-7 microtissue morphology. The effects of anti-estrogens need to be further elucidated, as environmental compounds and drugs that inhibit or block estrogen signaling can have detrimental effects. Studies using the ER α -

selective antagonist fulvestrant (ICI) demonstrated that ICI treatment for 7 days significantly decreases nuclei counts and microtissue area compared to vehicle treated controls. However, ICI treatment did not prevent the ER α -mediated luminal effects when combined with PPT and E2 treatments. This observation warrants further investigation, as ICI binds ER α 66 and ER α 46 with different affinities[27], and these experiments may help to elucidate the biological roles of the isoforms in this system.

7.5 Closing Statements

There is significant value in developing low cost, simple, and fast assays for toxicity testing that examine human biology. Animal models are expensive, time-intensive and do not completely recapitulate human biology, making dose and exposure extrapolation difficult. Current *in vitro* models used for high throughput screening are improved in that they are based on molecular changes following exposure to test compounds; however, most are based on receptor binding or translocation, which does not necessarily translate to an adverse effect at the tissue or organism level. The assays that study cellular endpoints, such as proliferation, are based on cells grown in 2D monolayer models, which have been demonstrated to proliferate far more than cells grown in 3D cultures, confounding results.

This thesis has showed that MCF-7 cells cultured in 3D are more differentiated than those cultured in 2D, and that microtissue morphology is sensitive to disruption by estrogenic compounds. Molecular and morphologic analysis of 2D and 3D cultures demonstrated that 3D cultures are more differentiated than 2D cultures, more sensitive to E2 stimulation, and exhibit unique gene expression alterations in downstream pathways that are not observed in 2D cultures. Additionally, exposure of MCF-7 microtissues to receptor-specific agonists and environmental xenoestrogens revealed that morphology and gene expression is altered in a pathway-and compound

specific manner, allowing for the assessment of histopathological endpoints *in vitro*.

This body of research has demonstrated that MCF-7 microtissues generated using agarose hydrogels can be used to assess morphologic and molecular effects of exposure to estrogen-active compounds. The data presented supports the use of the MCF-7 platform and similar cellular assays for the investigation of suspected estrogenic and anti-estrogenic compounds, as well as the exploration of this approach for applications in the safety assessment of other cell types.

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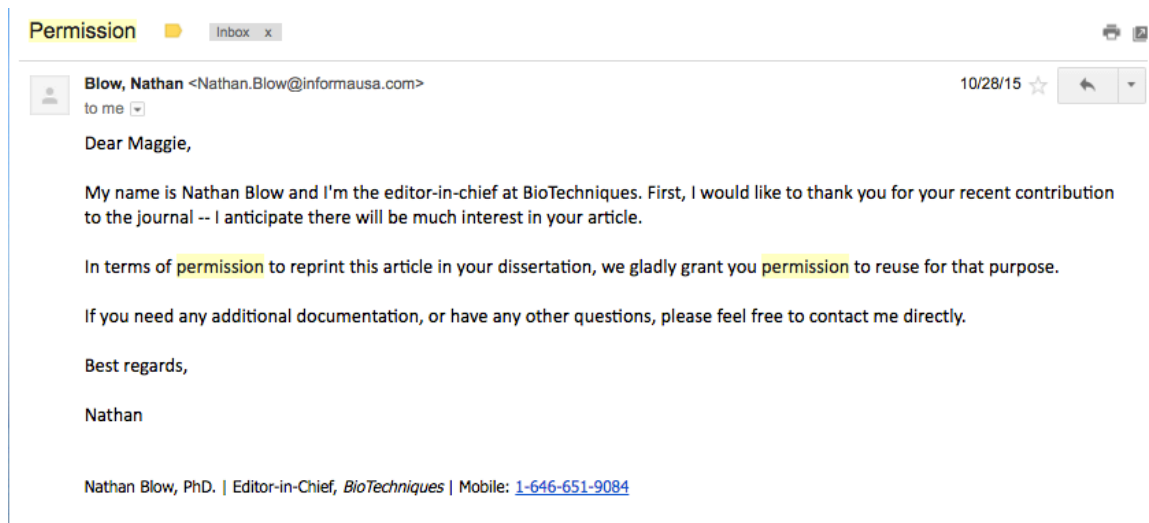
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Appendix A

Supplemental Materials

Appendix A.1

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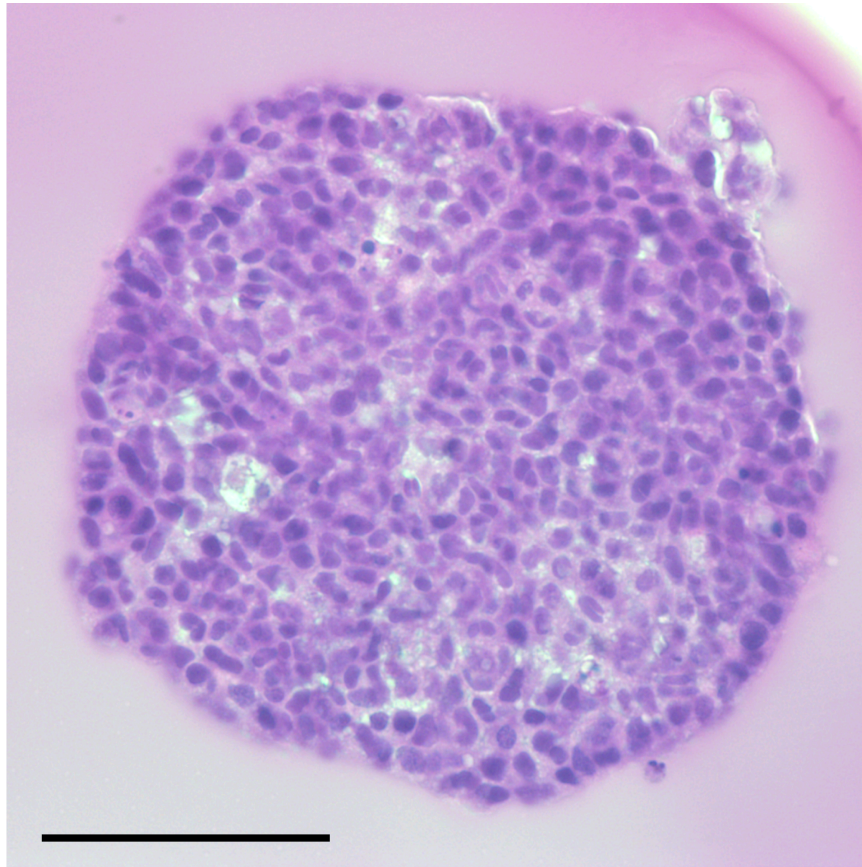
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Appendix A.2 Materials

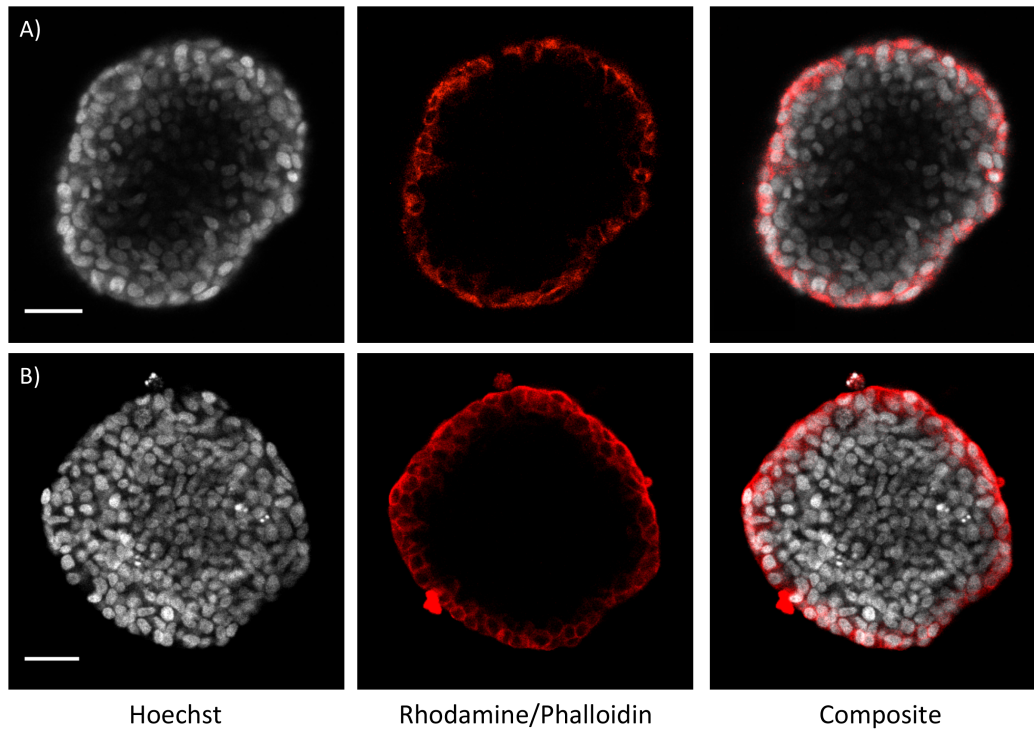
Chapter 2 Supplemental

A2.1 Supplemental Figure 2.1



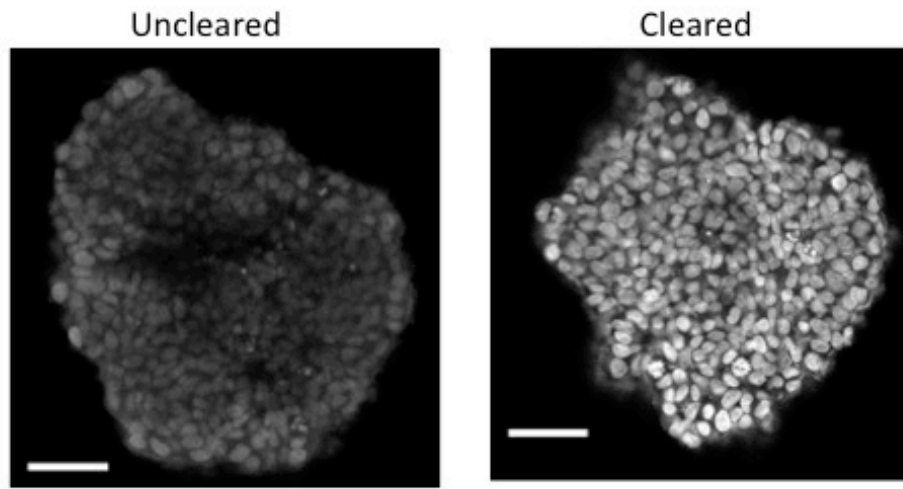
Brightfield imaging of 7 day PLHC-1 microtissues embedded in gelatin. PLHC-1 microtissues were grown for 7 days using the Microtissues, Inc. system, fixed in formalin, removed from hydrogels and pelleted. Microtissues were then suspended in PBS for a gentle rinse, depicting the potential for microtissues grown using other scaffold -free systems to be collected and used for histology. Following the rinse, samples were resuspended in warm 10% porcine gelatin. Spheroids sank to the bottom of the conical tube and were allowed to cool. Once hardened, the gelatin was removed, place in formalin, and processed for paraffin embedding. The paraffin blocks were sectioned and stained with hematoxylin and eosin. As seen in the image, the gelatin used for embedding stained strongly with eosin, increased the difficulty of staining and imaging the microtissues. Furthermore, as these microtissues were small in size, it was challenging to locate and identify samples within a section of block. Scale bar = 50 μ m.

A2.2 Supplemental Figure 2.2



Attenuated confocal fluorescence during imaging of immunostained microtissues under un-optimized staining conditions. LNCaP microtissues, at a 3 day time point, were permeabilized for 15 minutes with 0.25% Triton X-100 prior to immunostaining for E-cadherin (red), and counterstaining with Hoechst 33342 (grey). Spheroids were then imaged in PBS without clearing (A) or cleared with formamide and PEG prior to imaging (B), using confocal microscopy. Cross sections (z axis) were acquired through the middle of each spheroid. Scale bar = 50 μ m.

A2.3 Supplemental Figure 2.3

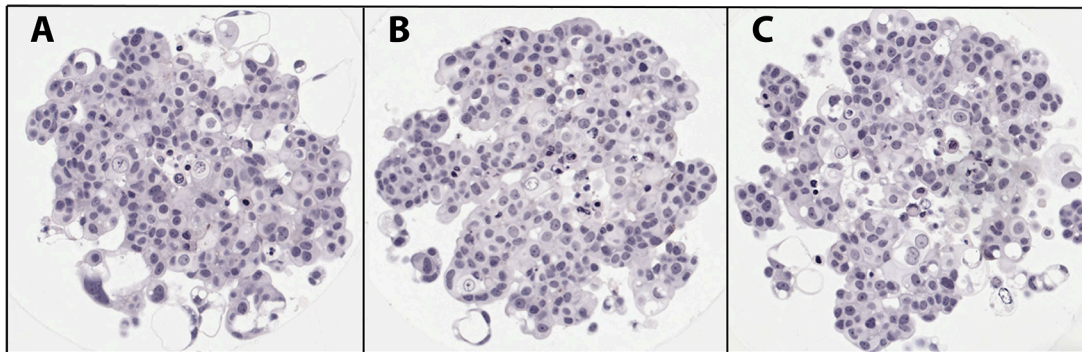


Confocal fluorescence imaging of microtissues collected by centrifugation and stained within hydrogels mimic the formation of microtissues from other scaffold-free systems and the applicability of the adapted Clear^{T2} clearing techniques. LNCaP microtissues, at a 3-day time point, were fixed within agarose hydrogels in 10% formalin for 30 minutes and then removed from hydrogels. Samples were removed from hydrogels and pelleted by centrifugation for collection, then resuspended in PBS to illustrate the transfer of samples from other potential scaffold free systems into hydrogels, as seen here, though less efficient and potentially compromising microtissue integrity. Similar to microtissues that might be grown in other scaffold free systems and collected in suspension, these samples were added to new agarose hydrogels for staining and clearing. Spheroids were stained as described previously using Hoechst 33342 (grey) and placed in PBS without clearing or cleared with formamide and PEG prior to imaging with confocal microscopy. Cross sections (z axis) were acquired through the middle of each spheroid. Scale bar = 50μm.

Appendix A.3 Materials

Chapter 3 Supplemental

A3.1 Supplemental Figure 3.1



MCF-7 cells derived in separate locations using identical culture methods exhibit different morphologies in 3D. MCF-7 cells derived in another lab were cultured for 5 (A), 7 (B) and 10 (C) days in 3D. MCF-7 cells were purchased from the same lot number from ATCC and derived in two locations utilizing identical protocols. When cultured in scaffold-free agarose hydrogels, MCF-7 cells derived by the Yager lab form large microtissues that do not contain defined luminal spaces up to 10 days in culture.

A3.2 Supplemental Table 3.1

Protein	Abbreviation	Primary Antibody	Primary Dilution	Secondary Antibody	Secondary Dilution
Milk fat globule-EGF factor 8	MFGE8	Abcam #ab17787	1:2000	AlexaFluor #A11001	1:500
Mucin 1	MUC1	Abcam #ab15481	1:1000	AlexaFluor #A11008	1:500
Trans-acting T-cell-specific transcription factor GATA-3	GATA3	Cell Signaling #5852	1:500	AlexaFluor #A11008	1:500
Keratin 8	KRT8	Abcam #ab9023	1:100	AlexaFluor #A11001	1:500
Keratin 5	KRT5	Abcam #ab24647	1:100	AlexaFluor #A11008	1:500
E-cadherin	CDH1	BD #610181	1:500	AlexaFluor #A11001	1:500
GM130	GM130	Cell Signaling #12480	1:500	AlexaFluor #A11008	1:500
Human Disc Large	hDlg	Santa Cruz Sc-9961	1:200	AlexaFluor #A11001	1:500
Vimentin	VIM	Sigma Aldrich V6630	1:200	AlexaFluor #A11008	1:500

Antibodies used for immunofluorescence. Antibody catalog numbers and dilutions used for immunofluorescence on 3D MCF-7 cryosections.

A3.3 Supplemental Table 3.2

Protein	Abbreviation	Primary Antibody	Primary Dilution	Secondary Antibody	Secondary Dilution
Snail family zinc finger 1	SNAI1	Cell Signaling #3879	1:500	Cell Signaling #7074	1:1000
Wnt-induced signaling protein 2	WISP2	Abcam #ab38317	1:500	Cell Signaling #7076	1:1000
Milk fat globule-EGF factor 8	MFGE8	Abcam #ab17787	1:2000	Cell Signaling #7076	1:1000
Mucin 1	MUC1	Abcam #ab15481	1:1000	Cell Signaling #7074	1:1000
Vimentin	VIM	Sigma #V6630	1:1000	Cell Signaling #7076	1:1000
Amphiregulin	AREG	Abcam #ab180722	1:1000	Cell Signaling #7074	1:1000
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	Cell Signaling #2118	1:5000	Cell Signaling #7074	1:5000

Antibodies used for Western blotting. Antibody catalog numbers and dilutions used for Western blotting.

A3.4 Supplemental Table 3.3

<u>Gene</u> <u>Abbre</u> <u>v.</u>	<u>Full gene ID</u>	<u>Main function</u>
AHR	Aryl hydrocarbon receptor	Activated by xenobiotics
AKT1	V-akt murine thymoma viral oncogene homolog 1	Suppression of apoptosis
APOD	Apolipoprotein D	ESR1 target, lipid metabolism
AR	Androgen receptor	Activated by androgen binding
AREG	Amphiregulin	Growth factor regulated by estrogen
BCAR1	Breast cancer anti-estrogen resistance 1	Pro-survival
BAD	BCL2-associated agonist of cell death	Positive regulation of apoptosis
BAX	BCL2-associated X protein	Activated apoptosis
BCL2	B-cell CLL/lymphoma 2	Apoptotic inhibitor
BCL2L1	BCL2-like 1	Apoptotic inhibitor
BCL2L2	BCL2-like 2	Apoptotic inhibitor
BDNF	Brain-derived neurotrophic factor	Anti-apoptotic
BMP4	Bone morphogenetic protein 4	Increased metastasis
BMP7	Bone morphogenetic protein 7	increased migration and invasion
BRCA1	Breast cancer 1, early onset	Tumor suppressor
BRCA2	Breast cancer 2, early onset	Tumor suppressor
CASP1	Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	Pro-apoptotic
CASP3	Caspase 3, apoptosis-related cysteine peptidase	Pro-apoptotic
CLDN1	Claudin 1	Tight junction component
CCNA1	Cyclin A1	G1/S and G2/M transition
CCND1	Cyclin D1	G1/S transition
CCND2	Cyclin D2	G1/S transition
CCNE1	Cyclin E1	G1/S progression

CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	cell adhesion, EMT
CDH2	Cadherin 2, type 1, N-cadherin (neuronal)	cell adhesion, EMT
CDK2	Cyclin-dependent kinase 2	cell cycle progression
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	G1/S progression
CST5	Cystatin D	ESR2 target loss associated with ER and PR loss in breast cancer
CST6	Cystatin E/M	component of adherens junctions
CTNNB1	Catenin (cadherin-associated protein), beta 1, 88kDa	ESR1 target
CTSD	Cathepsin D	estrogen synthesis
CYP19A1	Cytochrome P450, family 19, subfamily A, polypeptide 1	PAH metabolism
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1	ESR2 target regulates MAPK and PI3K pathways
ENPP2	Ectonucleotide pyrophosphatase/phosphodiesterase 2	Binds estradiol proliferative response
ERBB2	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	Bind estradiol, inhibitory response
ESR1	Estrogen receptor 1	Related to estrogen signaling
ESR2	Estrogen receptor 2 (ER beta)	Related to estrogen signaling
ESRRA	Estrogen-related receptor alpha	Related to estrogen signaling
ESRRB	Estrogen-related receptor beta	Related to estrogen signaling
ESRRG	Estrogen-related receptor gamma	Cell adhesion and migration
FN1	Fibronectin 1	Transcription factor
FOS	FBJ murine osteosarcoma viral oncogene homolog	transcription factor, marker of breast epithelium
GATA3	GATA binding protein 3	cell adhesion and communication
GJA1	Gap junction protein, alpha 1, 43kDa	

GPGR	G protein-coupled estrogen receptor 1	Rapid, nongenomic estrogen signaling
GREB1	Growth regulation by estrogen in breast cancer 1	Estrogen responsive gene
IGF1	Insulin-like growth factor 1 (somatomedin C)	Mediates growth and development
IGF1R	Insulin-like growth factor 1 receptor	Anti-apoptotic
IGFBP4	Insulin-like growth factor binding protein 4	Mediates growth and development
ITGA1	Integrin, alpha 1	Myoepithelial cell marker
ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	Breast differentiation
KRT14	Keratin 14	Breast differentiation
KRT18	Keratin 18	Epithelial marker
KRT19	Keratin 19	Epithelial marker
KRT5	Keratin 5	Epithelial marker
KRT8	Keratin 8	Epithelial marker
LALBA	Lactalbumin, alpha-	Milk component
LAMA5	Laminin, alpha 5	Basal marker
LRRC15	Leucine rich repeat containing 15	ESR2 target
LTF	Lactotransferrin	Milk component
MAPK1	Mitogen-activated protein kinase 1	Signal kinase
MAPK3	Mitogen-activated protein kinase 3	Signal kinase
MAPK8	Mitogen-activated protein kinase 8	Signal kinase
MFGE8	Milk fat globule-EGF factor 8 protein	Milk component
MMP2	Matrix metalloproteinase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	Tissue remodeling
MMP9	Matrix metalloproteinase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	Tissue remodeling
MUC1	Mucin 1, cell surface associated	Mucus membrane component
MYBL2	V-myb myeloblastosis viral oncogene homolog (avian)-like 2	ESR2 target
MYC	V-myc myelocytomatosis viral oncogene homolog (avian)	Cell cycle progression
NOTCH1	Notch 1	cell adhesion and communication
NOTCH2	Notch 2	Cell adhesion and communication
OCLN	Occludin	Tight junction component
PGR	Progesterone receptor	Steroid receptor
PDZK1	PDZ domain containing 1	Scaffolding, estrogen responsive

PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	Forms prostaglandin
SNAI1	Snail homolog 1 (Drosophila)	Transcription factor associated with EMT
SNAI2	Snail homolog 2 (Drosophila)	Transcription factor associated with EMT
TFF1	Trefoil factor 1	ESR1 target
TGFA	Transforming growth factor, alpha	Proliferation
TGFB3	Transforming growth factor, beta 3	Proliferation
TJP1	Tight junction protein 1 (zona occludens 1)	Tight junction component
TJP2	Tight junction protein 2 (zona occludens 2)	Tight junction component
TWIST 1	Twist homolog 1 (Drosophila)	Transcription factor associated with EMT
VEGFA	Vascular endothelial growth factor A	Angiogenesis
VIM	Vimentin	Cytoskeletal, involved in EMT
WISP2	WNT1 inducible signaling pathway protein 2	Inhibits breast cancer progression
WNT4	Wingless-type MMTV integration site family, member 4	Mammary gland development
WNT5A	Wingless-type MMTV integration site family, member 5A	Mammary gland development
XBP1	X-box binding protein 1	Stress response

List of genes selected for the custom SABiosciences PCR Array. Gene names, abbreviations and main functions of genes included on the custom PCR arrays.

A3.5 Supplemental Table 3.4

Gene ID	Full Name	Fold Change	q value
BMP7	Bone morphogenetic protein 7	-6.94	0.001896
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1	6.36	0.001896
CST6	Cystatin E/M	-4.35	0.006492
KRT19	Keratin 19	3.56	0.013046
WISP2	WNT1 inducible signaling pathway protein 2	3.50	0.013046
AREG	Amphiregulin	-3.20	0.018219
APOD	Apolipoprotein D	-3.98	0.022577
SNAI1	Snail homolog 1 (Drosophila)	47.18	0.023391
ESR2	Estrogen receptor 2 (ER beta)	-4.23	0.025454
FOS	FBJ murine osteosarcoma viral oncogene homolog	-2.57	0.032530
MFGE8	Milk fat globule-EGF factor 8 protein	2.56	0.032530
VIM	Vimentin	-2.56	0.032530
CYP19A1	Cytochrome P450, family 19, subfamily A, polypeptide 1	-4.27	0.032690
CCND2	Cyclin D2	-2.31	0.036126
GPGR	G protein-coupled estrogen receptor 1	2.48	0.036126
KRT14	Keratin 14	-29.19	0.036126
KRT8	Keratin 8	2.29	0.036126
LRRC15	Leucine rich repeat containing 15	2.91	0.036396
MUC1	Mucin 1, cell surface associated	2.21	0.039501
IGFBP4	Insulin-like growth factor binding protein 4	2.10	0.050408
PDZK1	PDZ domain containing 1	2.09	0.054065
SNAI2	Snail homolog 2 (Drosophila)	-2.01	0.060817
AHR	Aryl hydrocarbon receptor	-1.91	0.063189
BCL2L1	BCL2-like 1	-1.94	0.063189
MYC	V-myc myelocytomatosis viral oncogene homolog (avian)	-1.94	0.063189
CASP3	Caspase 3, apoptosis-related cysteine peptidase	-2.15	0.064501
BMP4	Bone morphogenetic protein 4	-1.87	0.065298
GREB1	Growth regulation by estrogen in breast cancer 1	2.04	0.078960
KRT18	Keratin 18	1.76	0.078960
FN1	Fibronectin 1	1.81	0.084200
TFF1	Trefoil factor 1	1.75	0.084200
BCL2	B-cell CLL/lymphoma 2	-1.74	0.097744

MYBL2	V-myb myeloblastosis viral oncogene homolog (avian)-like 2	1.67	0.097744
NOTCH1	Notch 1	1.70	0.097744
NOTCH2	Notch 2	-1.78	0.097744
AR	Androgen receptor	-1.63	0.102528
BAX	BCL2-associated X protein	-1.57	0.103499
ERBB2	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2	3.26	0.103499
GJA1	Gap junction protein, alpha 1, 43kDa	-5.61	0.103499
MAPK3	Mitogen-activated protein kinase 3	1.58	0.103499
TWIST1	Twist homolog 1 (Drosophila)	2.29	0.103499
CTSD	Cathepsin D	1.55	0.108156
WNT5A	Wingless-type MMTV integration site family, member 5A	-1.54	0.109953
CCNA1	Cyclin A1	1.56	0.110222
CCNE1	Cyclin E1	-1.46	0.123017
ENPP2	Ectonucleotide pyrophosphatase/phosphodiesterase 2	-2.32	0.125364
IGF1R	Insulin-like growth factor 1 receptor	-1.47	0.125364
WNT4	Wingless-type MMTV integration site family, member 4	1.45	0.125364
VEGFA	Vascular endothelial growth factor A	1.39	0.147894
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	0.75	0.152293
ESRRA	Estrogen-related receptor alpha	1.36	0.152293
ITGB1	Integrin, beta 1	-1.35	0.152293
MAPK8	Mitogen-activated protein kinase 8	-1.33	0.152293
TGFA	Transforming growth factor, alpha	1.34	0.152293
XBP1	X-box binding protein 1	1.36	0.152293
ESR1	Estrogen receptor 1	-1.30	0.154909
AKT1	V-akt murine thymoma viral oncogene homolog 1	-1.31	0.161156
MMP9	Matrix metalloproteinase 9	1.33	0.168636
PGR	Progesterone receptor	1.73	0.169560
CDK2	Cyclin-dependent kinase 2	1.26	0.171028
BCL2L2	BCL2-like 2	-1.26	0.188193
TJP2	Tight junction protein 2 (zona occludens 2)	-1.22	0.191742
MAPK1	Mitogen-activated protein kinase 1	-1.19	0.196285
BAD	BCL2-associated agonist of cell death	1.16	0.200382
BRCA1	Breast cancer 1, early onset	-1.18	0.200382
CLDN1	Claudin 1	-1.19	0.200382
BRCA2	Breast cancer 2, early onset	-1.14	0.215875
TGFB3	Transforming growth factor, beta 3	-1.14	0.215875

CTNNB1	Catenin (cadherin-associated protein), beta 1, 88kDa	-1.12	0.220533
GATA3	GATA binding protein 3	-1.10	0.221360
BCAR1	Breast cancer anti-estrogen resistance 1	1.08	0.233553
CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	-1.08	0.233553
CCND1	Cyclin D1	-1.02	0.258692
ITGA1	Integrin, alpha 1	1.03	0.258692
LAMA5	Laminin, alpha 5	1.01	0.258692

Complete PCR-results for PCR arrays. Fold change values of all RT-PCR targets, sorted by ascending q-value.

Appendix A.4 Materials

Chapter 4 Supplemental

A4.1 Supplemental Table 4.1

Gene Name	Gene Symbol	Fold Change	q value
Transforming growth factor, alpha	TGFA	3.61	0.0001
Growth regulation by estrogen in breast cancer 1	GREB1	3.23	0.0001
FBJ murine osteosarcoma viral oncogene homolog	FOS	3.35	0.0001
Cyclin D1	CCND1	2.75	0.0002
Transforming growth factor, beta	TGFB3	2.50	0.0004
Xbox binding protein 1	XPB1	2.10	0.0011
Twist homolog 1 (Drosophila)	TWIST1	-2.10	0.0023
Snail homolog 1 (Drosophila)	SNAI1	2.60	0.0052
Vascular endothelial growth factor A	VEGFA	2.01	0.0089
Wingless-type MMTV integration site family, member 4	WNT4	1.68	0.0123
Amphiregulin	AREG	1.84	0.0207
Occludin	OCLN	-1.45	0.0283
BCL2-like 1	BCL2L1	-1.58	0.0306
V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	ERBB2	-1.78	0.0454
B-cell CLL/lymphoma 2	BCL2	1.50	0.0760
Tight junction protein 1 (zona occludens 1)	TJP1	2.36	0.0772
Trefoil factor 1	TFF1	2.64	0.0854
Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CDKN1A	-1.26	0.0882
Estrogen receptor 1	ESR1	-1.40	0.0934
Bone morphogenetic protein 4	BMP4	-1.34	0.1117

Matrix metalloproteinase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	MMP9	-1.32	0.1196
Cathepsin D	CTSD	0.67	0.1400
Cyclin E1	CCNE1	1.27	0.1779
Mitogen-activated protein kinase 3	MAPK3	-1.26	0.1840
GATA binding protein 3	GATA3	-1.57	0.1902
Wingless-type MMTV integration site family, member 5A	WNT5A	-2.33	0.1927
Breast cancer anti-estrogen resistance 1	BCAR1	-0.61	0.2118
Beta-catenin	CTNNB1	-0.72	0.2146
Claudin 1	CLDN1	-0.91	0.2364
Estrogen-related receptor alpha	ESRRA	-1.56	0.2437
Vimentin	VIM	-1.17	0.2723
Keratin 19	KRT19	-0.71	0.3702
E-cadherin	CDH1	0.50	0.3703
Mucin 1, cell surface associated	MUC1	-1.20	0.3804
Tight junction protein 2 (zona occludens 2)	TJP2	-1.25	0.3855
Insulin-like growth factor 1 receptor	IGF1R	0.49	0.3899
BCL2-associated X protein	BAX	-1.12	0.3909
Breast cancer 2, early onset	BRCA2	-0.48	0.4122
Breast cancer	BRCA1	-0.49	0.4435
Estrogen-related receptor gamma	ESRRG	-1.39	0.4494
Notch 1	NOTCH1	0.57	0.4908
Apolipoprotein D	APOD	0.43	0.4977
Keratin 18	KRT18	0.49	0.5650
BCL2-like 2	BCL2L2	1.16	0.5706
Cyclin A1	CCNA1	-0.55	0.5809

Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	ITGB1	-0.42	0.5919
Snail homolog 2 (Drosophila)	SNAI2	-0.46	0.6305
Cystatin E/M	CST6	0.50	0.6514
Aryl hydrocarbon receptor	AHR	0.23	0.6679
V-akt murine thymoma viral oncogene homolog 1	AKT1	0.24	0.6896
Milk fat globule-EGF factor 8 protein	MFGE8	0.26	0.7161
Cytochrome P450 19A1	CYP19A1	0.69	0.7195
V-myb myeloblastosis viral oncogene homolog (avian)-like 2	MYBL2	0.38	0.7576
Fibronectin 1	FN1	-0.45	0.7710
BCL2-associated agonist of cell death	BAD	0.38	0.7744
Cyclin D2	CCND2	0.44	0.8443
Mitogen-activated protein kinase 1	MAPK1	0.29	0.8445
Mitogen-activated protein kinase 8	MAPK8	0.37	0.8647
Notch 2	NOTCH2	-0.37	0.8729
Bone morphogenetic protein 7	BMP7	-0.37	0.8820
WNT1 inducible signaling pathway protein 2	WISP2	1.11	0.8869
Caspase 3	CASP3	0.36	0.9049
Androgen receptor	AR	0.35	0.9216
Laminin, alpha 5	LAMA5	-0.31	0.9454
Keratin 8	KRT8	0.27	0.9777
Cytochrome P450, family 1, subfamily A, polypeptide 1	CYP1A1	-3.27	<0.0001
G protein-coupled estrogen receptor 1	GPER	-3.06	<0.0001
Insulin-like growth factor binding protein 4	IGFBP4	5.78	<0.0001
V-myc myelocytomatosis viral oncogene homolog (avian)	MYC	3.72	<0.0001

PDZ domain containing 1	PDZK1	3.17	<0.0001
Progesterone receptor	PGR	4.59	<0.0001

Supplemental Table 4.1

Complete expression results for MCF-7 monolayers treated with 1nM E2 for 4 hours. Fold changes and q-values for all mRNA targets in monolayer cultures following 4 hours of exposure to 1nM E2. Positive fold change values indicate increased expression, and results are compared to vehicle-treated controls

A4.2 Supplemental Table 4.2

Gene Name	Gene Symbol	Fold Change	q values
Cyclin D1	CCND1	3.31	0.0000
Transforming growth factor, alpha	TGFA	2.95	0.0002
G protein-coupled estrogen receptor 1	GPER	-2.14	0.0009
Amphiregulin	AREG	2.57	0.0012
Xbox binding protein 1	XBP1	2.07	0.0017
FBJ murine osteosarcoma viral oncogene homolog	FOS	2.02	0.0049
Snail homolog 1 (Drosophila)	SNAI1	2.40	0.0061
Notch 2	NOTCH2	1.97	0.0063
WNT1 inducible signaling pathway protein 2	WISP2	8.37	0.0126
Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CDKN1A	-1.37	0.0258
Insulin-like growth factor 1 receptor	IGF1R	1.45	0.0350
Cathepsin D	CTSD	1.50	0.0380
B-cell CLL/lymphoma 2	BCL2	1.57	0.0431
Wingless-type MMTV integration site family, member 5A	WNT5A	-4.26	0.0457
Twist homolog 1 (Drosophila)	TWIST1	-1.48	0.0525
Snail homolog 2 (Drosophila)	SNAI2	1.59	0.0584
Transforming growth factor, beta	TGFB3	1.46	0.0622
E-cadherin	CDH1	1.37	0.0642
V- homolog (avian)	ERBB2	-1.56	0.0802
GATA binding protein 3	GATA3	1.81	0.0903
Vascular endothelial growth factor A	VEGFA	1.53	0.0982
Occludin	OCLN	-1.28	0.1116
Tight junction protein 2 (zona occludens 2)	TJP2	-1.59	0.1122

Fibronectin 1	FN1	-1.35	0.1131
Keratin 18	KRT18	1.42	0.1866
Cyclin E1	CCNE1	0.58	0.2492
Estrogen-related receptor gamma	ESRRG	-2.67	0.2833
Breast cancer 2, early onset	BRCA2	0.53	0.2896
Trefoil factor 1	TFF1	1.67	0.3400
Keratin 19	KRT19	1.44	0.3484
Aryl hydrocarbon receptor	AHR	1.18	0.3507
Tight junction protein 1 (zona occludens 1)	TJP1	1.55	0.3567
Bone morphogenetic protein 4	BMP4	-1.17	0.3670
Estrogen receptor 1	ESR1	-1.18	0.3800
BCL2-like 1	BCL2L1	-1.17	0.3889
Mitogen-activated protein kinase 3	MAPK3	-0.48	0.4119
Cytochrome P450 19A1	CYP19A1	0.87	0.4460
BCL2-associated agonist of cell death	BAD	-1.12	0.4561
Notch 1	NOTCH1	0.58	0.4638
Cyclin D2	CCND2	-1.34	0.4924
Breast cancer anti-estrogen resistance 1	BCAR1	-1.13	0.5128
Wingless-type MMTV integration site family, member 4	WNT4	1.12	0.5376
Keratin 8	KRT8	-1.20	0.5635
Beta-catenin	CTNNB1	-0.48	0.5649
Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	ITGB1	-0.42	0.5952
V-myb myeloblastosis viral oncogene homolog (avian)-like 2	MYBL2	-0.25	0.5982

Mucin 1, cell surface associated	MUC1	0.46	0.6079
Mitogen-activated protein kinase 8	MAPK8	1.09	0.6468
Cyclin A1	CCNA1	0.16	0.6671
BCL2-associated X protein	BAX	0.39	0.6904
Estrogen-related receptor alpha	ESRRA	1.17	0.7014
Androgen receptor	AR	0.43	0.7019
Milk fat globule-EGF factor 8 protein	MFGE8	-1.06	0.7022
Breast cancer	BRCA1	0.41	0.7088
Matrix metalloproteinase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	MMP9	1.06	0.7292
Mitogen-activated protein kinase 1	MAPK1	-0.28	0.7753
Laminin, alpha 5	LAMA5	-0.40	0.7981
Cystatin E/M	CST6	0.41	0.8202
Bone morphogenetic protein 7	BMP7	-0.38	0.8438
Caspase 3	CASP3	-0.36	0.9024
Claudin 1	CLDN1	-0.37	0.9096
V-akt murine thymoma viral oncogene homolog 1	AKT1	0.35	0.9308
BCL2-like 2	BCL2L2	0.34	0.9659
Apolipoprotein D	APOD	-0.34	0.9730
Vimentin	VIM	-0.34	0.9804
Cytochrome P450, family 1, subfamily A, polypeptide 1	CYP1A1	-3.78	<0.0001
Growth regulation by estrogen in breast cancer 1	GREB1	5.18	<0.0001

Insulin-like growth factor binding protein 4	IGFBP4	5.33	<0.0001
V-myc myelocytomatosis viral oncogene homolog (avian)	MYC	3.39	<0.0001
PDZ domain containing 1	PDZK1	5.00	<0.0001
Progesterone receptor	PGR	10.49	<0.0001

Complete expression results for MCF-7 microtissues treated with 1nM E2 for 4 hours. Fold changes and q-values for all mRNA targets in 3D cultures following 4 hours of exposure to 1nM E2. Positive fold change values indicate increased expression, and results are compared to vehicle-treated controls.

A4.3 Supplemental Table 4.3

Gene Name	Gene Symbol	Fold Change	q value
Cytochrome P450, family 1, subfamily A, polypeptide 1	CYP1A1	-2.10	<0.0001
Breast cancer	BRCA1	2.37	0.00010
WNT1 inducible signaling pathway protein 2	WISP2	1.92	0.00013
Wingless-type MMTV integration site family, member 5A	WNT5A	-5.29	0.00013
Cathepsin D	CTSD	2.05	0.00019
Insulin-like growth factor binding protein 4	IGFBP4	3.33	0.00043
V-myc myelocytomatosis viral oncogene homolog (avian)	MYC	1.72	0.00158
Amphiregulin	AREG	3.99	0.00257
B-cell CLL/lymphoma 2	BCL2	1.93	0.00340
G protein-coupled estrogen receptor 1	GPER	-1.49	0.00465
Breast cancer 2, early onset	BRCA2	2.13	0.00473
Transforming growth factor, alpha	TGFA	1.55	0.00516
Notch 2	NOTCH2	1.40	0.00594
Ectonucleotide pyrophosphatase/phosphodiesterase 2	ENPP2	15.49	0.00627
Cyclin D2	CCND2	-2.32	0.00898
V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	ERBB2	-1.75	0.00946
Leucine rich repeat containing 15	LRRC15	2.71	0.01055

Transforming growth factor, beta	TGFB3	1.53	0.01083
Twist homolog 1 (Drosophila)	TWIST1	-1.44	0.01886
V-myb myeloblastosis viral oncogene homolog (avian)-like 2	MYBL2	1.36	0.02275
Mucin 1, cell surface associated	MUC1	-1.37	0.04060
Aryl hydrocarbon receptor	AHR	1.67	0.04977
Androgen receptor	AR	1.80	0.05026
N-cadherin	CDH2	1.29	0.06682
Insulin-like growth factor 1 receptor	IGF1R	1.31	0.07895
FBJ murine osteosarcoma viral oncogene homolog	FOS	1.25	0.08307
Xbox binding protein 1	XBP1	1.17	0.12070
V-akt murine thymoma viral oncogene homolog 1	AKT1	1.56	0.12110
GATA binding protein 3	GATA3	12.11	0.14174
Brain-derived neurotrophic factor	BDNF	-1.44	0.15518
Tight junction protein 2 (zona occludens 2)	TJP2	-1.18	0.16162
BCL2-associated X protein	BAX	1.28	0.16252
Cyclin A1	CCNA1	1.39	0.16336
Mitogen-activated protein kinase 3	MAPK3	-1.24	0.16651
Snail homolog 1 (Drosophila)	SNAI1	1.22	0.18198
Tight junction protein 1 (zona occludens 1)	TJP1	1.23	0.19012
Fibronectin 1	FN1	1.23	0.20256
E-cadherin	CDH1	1.22	0.21176
Claudin 1	CLDN1	1.19	0.23288
Cystatin D	CST5	-2.68	0.27232
Vascular endothelial growth factor A	VEGFA	0.47	0.31489
Laminin, alpha 5	LAMA5	-1.11	0.34956

Apolipoprotein D	APOD	1.26	0.35411
Cyclin E1	CCNE1	1.13	0.35576
Vimentin	VIM	1.22	0.35656
Mitogen-activated protein kinase 1	MAPK1	1.13	0.39078
Keratin 19	KRT19	1.16	0.41891
Beta-catenin	CTNNB1	-0.54	0.46442
BCL2-associated agonist of cell death	BAD	1.09	0.55794
Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	ITGB1	0.42	0.57918
Bone morphogenetic protein 7	BMP7	-0.45	0.58340
Breast cancer anti-estrogen resistance 1	BCAR1	0.08	0.60613
Cytochrome P450 19A1	CYP19A1	0.45	0.60887
Estrogen receptor 1	ESR1	-1.05	0.63391
Cyclin D1	CCND1	2.47	0.65349
Matrix metalloproteinase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	MMP9	0.29	0.67133
Notch 1	NOTCH1	-0.42	0.71314
Caspase 3	CASP3	0.37	0.73338
Cystatin E/M	CST6	0.51	0.75572
Occludin	OCLN	-1.13	0.76896
Lactotransferrin	LTF	0.45	0.77824
Keratin 8	KRT8	0.30	0.87755
Estrogen-related receptor alpha	ESRRA	0.36	0.89281
Mitogen-activated protein kinase 8	MAPK8	-0.32	0.91059
BCL2-like 1	BCL2L1	-0.36	0.92484
Bone morphogenetic protein 4	BMP4	-0.35	0.93430
Milk fat globule-EGF factor 8 protein	MFGE8	-0.34	0.94904

Wingless-type MMTV integration site family, member 4	WNT4	0.33	0.95256
Snail homolog 2 (Drosophila)	SNAI2	-0.31	0.95600
BCL2-like 2	BCL2L2	0.30	0.96498
Keratin 18	KRT18	-0.33	0.96833
Gap junction protein, alpha 1, 43kDa	GJA1	0.25	0.97527
Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CDKN1A	-0.33	0.98972
Growth regulation by estrogen in breast cancer 1	GREB1	3.83	<0.0001
PDZ domain containing 1	PDZK1	3.31	<0.0001
Progesterone receptor	PGR	5.63	<0.0001
Trefoil factor 1	TFF1	1.80	<0.0001

Complete expression results for MCF-7 monolayers treated with 1nM E2 for 8 hours. Fold changes and q-values for all mRNA targets in monolayer cultures following 8 hours of exposure to 1nM E2. Results are compared to vehicle-treated, time-matched controls. Positive fold change values indicate increased expression.

A4.4 Supplemental Table 4.4

Gene Name	Gene Symbol	Fold Change	q value
Brain-derived neurotrophic factor	BDNF	-4.34	0.0001
Matrix metalloproteinase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	MMP9	-1.75	0.0001
FBJ murine osteosarcoma viral oncogene homolog	FOS	1.88	0.0002
Xbox binding protein 1	XBP1	1.62	0.0003
Cathepsin D	CTSD	1.88	0.0004
Snail homolog 1 (Drosophila)	SNAI1	1.99	0.0006
G protein-coupled estrogen receptor 1	GPER	-1.71	0.0006
V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	ERBB2	-2.05	0.0020
Twist homolog 1 (Drosophila)	TWIST1	-1.70	0.0021
Trefoil factor 1	TFF1	1.42	0.0023
Cyclin D2	CCND2	-2.86	0.0026
Breast cancer	BRCA1	1.72	0.0031
Estrogen receptor 1	ESR1	-1.50	0.0031
Claudin 1	CLDN1	1.63	0.0052
Amphiregulin	AREG	3.41	0.0055
Insulin-like growth factor 1 receptor	IGF1R	1.59	0.0073
WNT1 inducible signaling pathway protein 2	WISP2	1.44	0.0082
Tight junction protein 2 (zona occludens 2)	TJP2	-1.42	0.0083
Fibronectin 1	FN1	1.61	0.0120
Vascular endothelial growth factor A	VEGFA	1.42	0.0138
Keratin 18	KRT18	1.36	0.0165
B-cell CLL/lymphoma 2	BCL2	1.64	0.0173
Breast cancer 2, early onset	BRCA2	1.70	0.0233
V-myb myeloblastosis viral oncogene homolog (avian)-like 2	MYBL2	1.33	0.0308
Cyclin A1	CCNA1	1.74	0.0353

N-cadherin	CDH2	1.33	0.0414
Occludin	OCLN	-3.92	0.0452
Mucin 1, cell surface associated	MUC1	-1.32	0.0634
Laminin, alpha 5	LAMA5	-1.24	0.0891
Wingless-type MMTV integration site family, member 4	WNT4	-1.26	0.0963
Keratin 8	KRT8	1.38	0.1137
Caspase 3	CASP3	-1.22	0.1162
Keratin 19	KRT19	1.35	0.1311
Milk fat globule-EGF factor 8 protein	MFGE8	1.15	0.2429
Aryl hydrocarbon receptor	AHR	0.69	0.2536
Cyclin E1	CCNE1	-1.16	0.2596
Cyclin D1	CCND1	2.20	0.2661
E-cadherin	CDH1	1.19	0.2684
Leucine rich repeat containing 15	LRRC15	0.93	0.2787
Bone morphogenetic protein 4	BMP4	-0.75	0.2840
Snail homolog 2 (Drosophila)	SNAI2	-0.65	0.3187
BCL2-like 1	BCL2L1	-1.23	0.3466
Notch 1	NOTCH1	-1.24	0.3535
Bone morphogenetic protein 7	BMP7	-0.53	0.4027
Ectonucleotide pyrophosphatase/phosphodiesterase 2	ENPP2	1.98	0.4097
Androgen receptor	AR	1.27	0.4117
Mitogen-activated protein kinase 3	MAPK3	-0.44	0.5222
Estrogen-related receptor alpha	ESRRA	-1.12	0.5252
BCL2-associated X protein	BAX	1.09	0.6176
V-akt murine thymoma viral oncogene homolog 1	AKT1	0.51	0.6435
Notch 2	NOTCH2	0.38	0.6524
Tight junction protein 1 (zona occludens 1)	TJP1	1.07	0.6759

Mitogen-activated protein kinase 8	MAPK8	0.38	0.6995
Mitogen-activated protein kinase 1	MAPK1	-0.27	0.7078
BCL2-associated agonist of cell death	BAD	-0.39	0.7393
Cystatin D	CST5	-0.55	0.8012
BCL2-like 2	BCL2L2	0.42	0.8146
Wingless-type MMTV integration site family, member 5A	WNT5A	-0.27	0.8327
Gap junction protein, alpha 1, 43kDa	GJA1	-0.24	0.8448
Beta-catenin	CTNNB1	-0.38	0.8543
Lactotransferrin	LTF	-0.39	0.8614
GATA binding protein 3	GATA3	-1.29	0.8764
Cystatin E/M	CST6	-0.43	0.8840
Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CDKN1A	-0.33	0.9424
Breast cancer anti-estrogen resistance 1	BCAR1	0.35	0.9541
Vimentin	VIM	-0.34	0.9654
Apolipoprotein D	APOD	0.32	0.9681
Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	ITGB1	0.33	0.9709
Cytochrome P450 19A1	CYP19A1	-0.32	0.9725
Cytochrome P450, family 1, subfamily A, polypeptide 1	CYP1A1	-2.22	<0.0001
Growth regulation by estrogen in breast cancer 1	GREB1	7.05	<0.0001
Insulin-like growth factor binding protein 4	IGFBP4	6.10	<0.0001
V-myc myelocytomatosis viral oncogene homolog (avian)	MYC	2.99	<0.0001
PDZ domain containing 1	PDZK1	5.43	<0.0001
Progesterone receptor	PGR	8.52	<0.0001
Transforming growth factor, alpha	TGFA	2.38	<0.0001
Transforming growth factor, beta	TGFB3	3.09	<0.0001

Gene expression results for all PCR array targets for MCF-7 microtissues treated for 8 hours with 1nM E2. Fold changes and q-values for all mRNA targets in microtissue cultures following 8 hours of exposure to 1nM E2. Results are compared to vehicle-treated, time-matched controls. Positive fold change values indicate increased expression.

A4.5 Supplemental Table 4.5

Gene Name	Gene Symbol	Fold Change	q value
Insulin-like growth factor binding protein 4	IGFBP4	3.68	0.0002
Progesterone receptor	PGR	6.98	0.0017
PDZ domain containing 1	PDZK1	7.27	0.0030
Amphiregulin	AREG	3.84	0.0184
V-myb myeloblastosis viral oncogene homolog (avian)-like 2	MYBL2	2.47	0.0468
Transforming growth factor, beta	TGFB3	1.88	0.0794
FBJ murine osteosarcoma viral oncogene homolog	FOS	-2.09	0.0910
Apolipoprotein D	APOD	-1.45	0.1623
V-myc myelocytomatosis viral oncogene homolog (avian)	MYC	1.74	0.1782
Breast cancer	BRCA1	2.45	0.2225
Cyclin-dependent kinase 2		1.84	0.2472
G protein-coupled estrogen receptor 1	GPER	-2.08	0.2593
Brain-derived neurotrophic factor	BDNF	-13.68	0.2884
Growth regulation by estrogen in breast cancer 1	GREB1	5.07	0.3445
V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	ERBB2	-1.84	0.3850
Insulin-like growth factor 1 receptor	IGF1R	1.35	0.3933
Breast cancer 2, early onset	BRCA2	2.72	0.3966
Transforming growth factor, alpha	TGFA	1.83	0.4184
Cytochrome P450, family 1, subfamily A, polypeptide 1	CYP1A1	-1.98	0.4601
E-cadherin	CDH1	1.22	0.4960
Estrogen-related receptor beta	ESRRB	-8.47	0.5004
Snail homolog 2 (Drosophila)	SNAI2	-2.35	0.5041
Mucin 1, cell surface associated	MUC1	-1.34	0.5066
N-cadherin	CDH2	4.84	0.5202
Estrogen-related receptor gamma	ESRRG	3.11	0.5414
Mitogen-activated protein kinase 1	MAPK1	1.31	0.5510
Cyclin A1	CCNA1	1.86	0.5594

Twist homolog 1 (Drosophila)	TWIST1	-1.58	0.5832
integrin, alpha 1	ITGA1	2.98	0.5929
Cathepsin D	CTSD	2.53	0.6018
Cyclin D1	CCND1	2.25	0.6064
Cyclin D2	CCND2	-2.02	0.6097
B-cell CLL/lymphoma 2	BCL2	1.53	0.6179
Trefoil factor 1	TFF1	-13.85	0.6202
Keratin 19	KRT19	1.14	0.6288
Keratin 5	KRT5	-2.38	0.6508
Breast cancer anti-estrogen resistance 1	BCAR1	-0.69	0.6529
Gap junction protein 1	GJA1	1.63	0.6623
Aryl hydrocarbon receptor	AHR	1.25	0.6644
Cystatin D	CST5	2.31	0.6834
Vimentin	VIM	1.41	0.6841
Milk fat globule-EGF factor 8 protein	MFGE8	1.30	0.6942
Prostaglandin synthase 2	PTGS2	-1.87	0.6954
Keratin 14	KRT14	1.34	0.6997
V-akt murine thymoma viral oncogene homolog 1	AKT1	0.48	0.7089
Insulin-like growth factor 1	IGF1	2.09	0.7206
Matrix metalloproteinase 2	MMP2	-1.67	0.7370
Fibronectin 1	FN1	1.37	0.7392
Androgen receptor	AR	1.59	0.7469
Mitogen-activated protein kinase 8	MAPK8	1.20	0.7596
Lactalbumin	LALBA	1.84	0.7633
BCL2-like 1	BCL2L1	-1.18	0.7740
Cystatin E/M	CST6	-1.79	0.7751
Keratin 18	KRT18	-0.44	0.7833
BCL2-associated agonist of cell death	BAD	0.52	0.7901
Ectonucleotide pyrophosphatase/phosphodiesterase 2	ENNP2	1.37	0.7913

Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	ITGB1	0.44	0.7940
WNT1 inducible signaling pathway protein 2	WISP2	1.81	0.8052
Notch 2	NOTCH2	1.37	0.8123
Wingless-type MMTV integration site family, member 5A	WNT5A	-0.74	0.8135
Occludin	OCLN	-0.45	0.8192
BCL2-associated X protein	BAX	1.11	0.8213
Caspase 1	CASP1	-1.29	0.8251
Vascular endothelial growth factor A	VEGFA	0.44	0.8256
Xbox binding protein 1	XBP1	0.44	0.8287
Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CDKN1A	-1.51	0.8360
Cytochrome P450 19A1	CYP19A1	0.62	0.8522
Estrogen receptor 1	ESR1	0.44	0.8529
Tight junction protein 1 (zona occludens 1)	TJP1	1.30	0.8535
Keratin 8	KRT8	-0.41	0.8581
Mitogen-activated protein kinase 3	MAPK3	-0.41	0.8661
Notch 1	NOTCH1	-0.51	0.8735
GATA binding protein 3	GATA3	-0.42	0.8745
BCL2-like 2	BCL2L2	0.69	0.8763
Matrix metalloproteinase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	MMP9	-0.48	0.8964
Wingless-type MMTV integration site family, member 4	WNT4	0.44	0.9018
Claudin 1	CLDN1	1.11	0.9034
Beta-catenin	CTNNB1	-0.41	0.9060
leucine-rich repeat containing 15	LRRC15	0.52	0.9168
Laminin, alpha 5	LAMA5	-0.40	0.9200

Snail homolog 1 (Drosophila)	SNAI1	0.47	0.9203
Bone morphogenetic protein 7	BMP7	-0.38	0.9321
Bone morphogenetic protein 4	BMP4	-0.37	0.9325
Estrogen receptor beta	ESR2	0.50	0.9357
Cyclin E1	CCNE1	0.40	0.9369
Estrogen-related receptor alpha	ESRRA	-0.36	0.9630
Tight junction protein 2 (zona occludens 2)	TJP2	-0.36	0.9652
Caspase 3	CASP3	0.35	0.9825
lactotransferrin	LTF	0.29	0.9846

Gene expression results for all PCR array targets for 2D MCF-7 cultures treated for 24 hours with 1nM E2. Fold changes and q-values for all mRNA targets in monolayer cultures following 24 hours of exposure to 1nM E2. Results are compared to vehicle-treated, time-matched controls. Positive fold change values indicate increased expression, and results are compared to vehicle-treated controls.

A4.6 Supplemental Table 4.6

Gene Name	Gene Symbol	Fold Change	q value
Insulin-like growth factor binding protein 4	IGFBP4	3.35	0.0002
PDZ domain containing 1	PDZK1	4.49	0.0004
Amphiregulin	AREG	4.01	0.0006
Progesterone receptor	PGR	3.08	0.0010
Transforming growth factor, beta	TGFB3	2.35	0.0015
V-myc myelocytomatosis viral oncogene homolog (avian)	MYC	2.05	0.0026
Claudin 1	CLDN1	2.54	0.0042
V-myb myeloblastosis viral oncogene homolog (avian)-like 2	MYBL2	1.87	0.0049
Transforming growth factor, alpha	TGFA	1.84	0.0070
Snail homolog 2 (Drosophila)	SNAI2	2.85	0.0072
leucine-rich repeat containing 15	LRRC15	3.66	0.0073
Gap junction protein 1	GJA1	2.18	0.0079
Cyclin A1	CCNA1	2.02	0.0081
Breast cancer	BRCA1	1.65	0.0084
Fibronectin 1	FN1	1.66	0.0103
FBJ murine osteosarcoma viral oncogene homolog	FOS	1.55	0.0105
Cyclin-dependent kinase 2		1.42	0.0112
Prostaglandin synthase 2	PTGS2	2.97	0.0117
Insulin-like growth factor 1 receptor	IGF1R	1.48	0.0129
B-cell CLL/lymphoma 2	BCL2	1.39	0.0136
Milk fat globule-EGF factor 8 protein	MFGE8	1.31	0.0141
integrin, alpha 1	ITGA1	4.07	0.0147
Xbox binding protein 1	XBP1	1.48	0.0149
Aryl hydrocarbon receptor	AHR	1.34	0.0151
Mitogen-activated protein kinase 8	MAPK8	1.31	0.0155
Vascular endothelial growth factor A	VEGFA	1.40	0.0156
BCL2-like 2	BCL2L2	34.16	0.0192

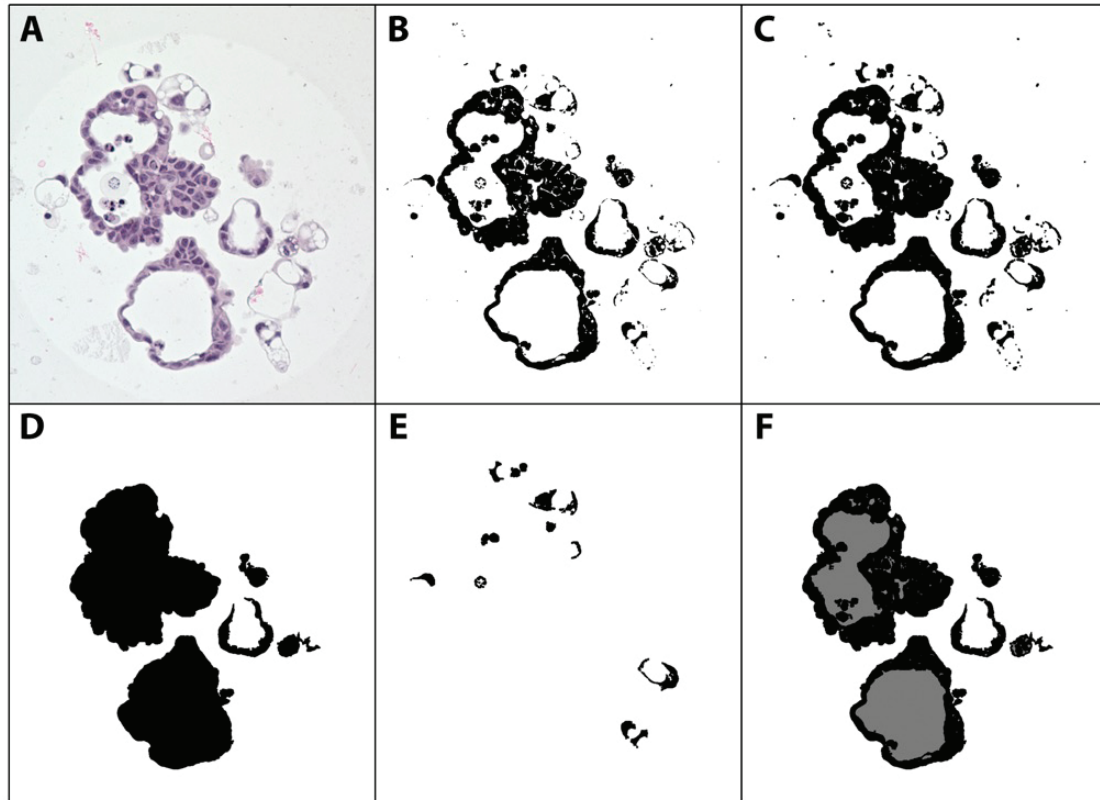
Cathepsin D	CTSD	- 57458.61	0.0194
Cystatin D	CST5	6.95	0.0197
Caspase 1	CASP1	1.18	0.0245
Vimentin	VIM	1.01	0.0251
Caspase 3	CASP3	1.00	0.0257
Insulin-like growth factor 1	IGF1	1.15	0.0275
Cyclin E1	CCNE1	1.00	0.0276
Keratin 5	KRT5	1.15	0.0277
Cytochrome P450 19A1	CYP19A1	-1.11	0.0286
Notch 1	NOTCH1	-1.10	0.0303
Wingless-type MMTV integration site family, member 5A	WNT5A	-1.10	0.0308
Brain-derived neurotrophic factor	BDNF	1.28	0.0311
N-cadherin	CDH2	1.82	0.0311
Lactalbumin	LALBA	1.15	0.0323
Mitogen-activated protein kinase 1	MAPK1	1.09	0.0324
BCL2-associated agonist of cell death	BAD	1.05	0.0324
Cytochrome P450, family 1, subfamily A, polypeptide 1	CYP1A1	-1.17	0.0329
Cystatin E/M	CST6	-1.47	0.0329
Snail homolog 1 (Drosophila)	SNAI1	1.48	0.0371
Laminin, alpha 5	LAMA5	-1.08	0.0377
Ectonucleotide pyrophosphatase/phosphodiesterase 2	ENNP2	-1.02	0.0396
Tight junction protein 2 (zona occludens 2)	TJP2	-1.13	0.0409
Matrix metalloproteinase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	MMP9	-1.34	0.0430
BCL2-like 1	BCL2L1	-1.14	0.0442
Estrogen-related receptor beta	ESRRB	1.26	0.0443
Twist homolog 1 (Drosophila)	TWIST1	-1.36	0.0480

Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	ITGB1	1.15	0.0493
Cyclin D2	CCND2	-2.08	0.0530
Matrix metalloproteinase 2	MMP2	-2.21	0.0544
Breast cancer anti-estrogen resistance 1	BCAR1	-1.09	0.0546
Bone morphogenetic protein 7	BMP7	-1.19	0.0569
BCL2-associated X protein	BAX	-1.06	0.0594
lactotransferrin	LTF	-2.62	0.0599
Estrogen-related receptor gamma	ESRRG	-1.92	0.0624
Mitogen-activated protein kinase 3	MAPK3	-1.00	0.0644
Estrogen receptor 1	ESR1	-1.22	0.0660
Keratin 14	KRT14	-1.01	0.0666
G protein-coupled estrogen receptor 1	GPER	-1.38	0.0675
Wingless-type MMTV integration site family, member 4	WNT4	-1.34	0.0683
V-akt murine thymoma viral oncogene homolog 1	AKT1	1.00	0.0695
Estrogen-related receptor alpha	ESRRA	-1.44	0.0701
Breast cancer 2, early onset	BRCA2	2.23	0.0703
Beta-catenin	CTNNB1	-1.06	0.0706
Mucin 1, cell surface associated	MUC1	-1.20	0.0886
V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	ERBB2	-1.65	0.1024
Occludin	OCLN	-1.05	0.1059
Bone morphogenetic protein 4	BMP4	-1.07	0.1102
Estrogen receptor beta	ESR2	-5.41	0.1206
Keratin 8	KRT8	1.13	0.1583
GATA binding protein 3	GATA3	-1.28	0.2688
E-cadherin	CDH1	1.23	0.3045
Androgen receptor	AR	1.03	0.3281
Tight junction protein 1 (zona occludens 1)	TJP1	1.01	0.4351

Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CDKN1A	-1.15	0.4731
Notch 2	NOTCH2	1.04	0.4735
Trefoil factor 1	TFF1	1.41	0.4826
Growth regulation by estrogen in breast cancer 1	GREB1	3.78	0.5262
Cyclin D1	CCND1	1.55	0.6878
Keratin 19	KRT19	-1.02	0.7607
Keratin 18	KRT18	-1.04	0.7755
WNT1 inducible signaling pathway protein 2	WISP2	1.79	0.8997
Apolipoprotein D	APOD	-1.35	0.9640

Complete expression results for MCF-7 microtissues treated with 1nM E2 for 24 hours. Fold changes and q-values for all mRNA targets in 3D cultures following 24 hours of exposure to 1nM E2. Positive fold change values indicate increased expression, and results are compared to vehicle-treated controls.

A5.1 Supplemental Figure 5.1



Images of individual microtissues were collected and blinded before quantification in ImageJ (A). Images were prepared first by determining a pixel/micron ratio using embedded scale markers present in 16-bit images (B). Background (noncellular debris) was removed in ImageJ (C). Brightness and contrast were adjusted to allow for generation of binary images through the default automatic thresholding procedure, and this was followed by morphological dilation ($d = 3$) (D). Using an automated count, the area of cellular objects was quantified. Particles of less than $500 \mu\text{m}^2$ were excluded as cellular debris (E), while the area of cellular aggregates with and without luminal spaces were quantified. Luminal area and total aggregate area were measured directly in ImageJ (F).

A5.2 Supplemental Table 5.1

Full Gene Name	Gene ID	Fold Change	q value
Apolipoprotein D	APOD	-4.66	<0.0001
Bone morphogenetic protein 4	BMP4	-1.88	1
Cytochrome P450, family 1, subfamily A, polypeptide 1	CYP1A1	-2.47	0.0001
V-erb-b2 erythroblastic leukemia viral oncogene homolog 2	ERBB2	-1.95	1
PDZ domain containing 1	PDZK1	1.76	0.0003
Caspase 3, apoptosis-related cysteine peptidase	CASP3	-0.99	1
Catenin (cadherin-associated protein), beta 1, 88kDa	CTNNB1	-1.14	0.0007
Tight junction protein 1 (zona occludens 1)	TJP1	-1.19	9
BCL2-like 1	BCL2L1	-1.36	0.0012
Cyclin E1	CCNE1	-0.75	8
Androgen receptor	AR	-1.33	0.0016
Cytochrome P450, family 19, subfamily A, polypeptide 1	CYP19A1	-2.08	3
Estrogen receptor 1	ESR1	-2.25	0.0034
Mitogen-activated protein kinase 1	MAPK1	-1.03	1
Occludin	OCLN	-0.78	0.0036
Transforming growth factor, beta 3	TGFB3	1.56	7
V-myc myelocytomatosis viral oncogene homolog (avian)	MYC	0.72	0.0036
Tight junction protein 2 (zona occludens 2)	TJP2	-1.11	7
V-akt murine thymoma viral oncogene homolog 1	AKT1	-0.95	0.0040
BCL2-associated agonist of cell death	BAD	-0.77	2
Mitogen-activated protein kinase 8	MAPK8	-0.78	0.0042
Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CDKN1A	-0.58	3
Trefoil factor 1	TFF1	0.65	0.0043
Growth regulation by estrogen in breast cancer 1	GREB1	1.40	4

			4
			0.0074
Keratin 18	KRT18	-0.74	4
			0.0087
Breast cancer anti-estrogen resistance 1	BCAR1	-1.10	2
			0.0087
B-cell CLL/lymphoma 2	BCL2	-0.90	2
			0.0087
Claudin 1	CLDN1	0.69	2
			0.0087
G protein-coupled estrogen receptor 1	GPOR	-1.44	2
			0.0087
Insulin-like growth factor 1 receptor	IGF1R	-0.94	2
			0.0088
Mitogen-activated protein kinase 3	MAPK3	-0.91	8
			0.0103
Cyclin D2	CCND2	-1.03	8
			0.0103
Keratin 8	KRT8	-1.03	8
			0.0197
Bone morphogenetic protein 7	BMP7	-0.51	0
			0.0237
GATA binding protein 3	GATA3	-0.86	4
			0.0255
Twist homolog 1 (Drosophila)	TWIST1	-0.71	8
			0.0273
Cyclin A1	CCNA1	0.51	8
			0.0330
FBJ murine osteosarcoma viral oncogene homolog	FOS	-0.63	4
			0.0331
Fibronectin 1	FN1	-0.89	8
			0.0331
Laminin, alpha 5	LAMA5	-0.62	8
			0.0331
X-box binding protein 1	XBP1	0.46	8
			0.0354
Integrin, beta 1	ITGB1	-0.38	9
			0.0473
Vascular endothelial growth factor A	VEGFA	0.45	2
			0.0502
Estrogen-related receptor alpha	ESRRA	-0.72	2
			0.0528
Mucin 1, cell surface associated	MUC1	-1.68	4
Wingless-type MMTV integration site family, member 5A	WNT5A	-0.82	2
			0.0831
Transforming growth factor, alpha	TGFA	0.67	9
			0.0968
Amphiregulin	AREG	0.83	1
	NOTCH		0.1191
Notch 2	2	-0.40	3

Breast cancer 1, early onset	BRCA1	-0.43	0.1995 3
BCL2-associated X protein	BAX	-0.30	0.2028 0
Notch 1	NOTCH 1	0.24	0.3585 9
Milk fat globule-EGF factor 8 protein	MFGE8	0.32	0.4054 7
Wingless-type MMTV integration site family, member 4	WNT4	-0.39	0.4097 7
Progesterone receptor	PGR	0.26	0.4397 5
Gap junction protein, alpha 1, 43kDa	GJA1	-0.30	0.4775 6
Vimentin	VIM	-0.40	0.5365 2
WNT1 inducible signaling pathway protein 2	WISP2	1.14	0.5456 1
Cyclin D1	CCND1	-0.19	0.6037 8
Snail homolog 2 (Drosophila)	SNAI2	0.40	0.6037 8
Ectonucleotide pyrophosphatase/phosphodiesterase 2	ENPP2	-0.12	0.6832 2
Keratin 19	KRT19	0.07	0.7253 5
Cadherin 1, type 1, E-cadherin (epithelial)	CDH1	-0.84	0.7815 3
V-myb myeloblastosis viral oncogene homolog (avian)-like 2	MYBL2	-0.07	0.7815 3
Insulin-like growth factor binding protein 4	IGFBP4	0.07	0.8069 4
Integrin, alpha 1	ITGA1	-0.02	0.9719 5

A5.3 Supplemental Table 5.2

Full Gene Name	Gene ID	Fold Change	q value
Apolipoprotein D	APOD	-2.36	0.00010
Cyclin A1	CCNA1	1.36	0.00099
Cytochrome P450, family 1, subfamily A, polypeptide 1	CYP1A1	-1.50	0.00363
PDZ domain containing 1	PDZK1	1.42	0.00458
Trefoil factor 1	TFF1	0.71	0.01657
Bone morphogenetic protein 7	BMP7	-0.67	0.02234
Claudin 1	CLDN1	0.68	0.04044
Estrogen receptor 1	ESR1	-1.70	0.04044
Bone morphogenetic protein 4	BMP4	-0.60	0.07808
Growth regulation by estrogen in breast cancer 1	GREB1	1.04	0.09095
Cytochrome P450, family 19, subfamily A, polypeptide 1	CYP19A1	-1.19	0.09142
Fibronectin 1	FN1	0.93	0.09142
Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CDKN1A	-0.37	0.09252
Wingless-type MMTV integration site family, member 5A	WNT5A	0.96	0.10109
Keratin 19	KRT19	0.44	0.10515
Milk fat globule-EGF factor 8 protein	MFGE8	0.82	0.11385
Tight junction protein 1 (zona occludens 1)	TJP1	-0.46	0.12710
Cyclin E1	CCNE1	-0.34	0.14058
V-erb-b2 erythroblastic leukemia viral oncogene homolog 2	ERBB2	-0.54	0.14509
Ectonucleotide pyrophosphatase/phosphodiesterase 2	ENPP2	0.56	0.15887
X-box binding protein 1	XBP1	0.36	0.16888
Cadherin 1, type 1, E-cadherin (epithelial)	CDH1	-5.32	0.20666
G protein-coupled estrogen receptor 1	GPER	-0.74	0.20666

V-myb myeloblastosis viral oncogene homolog (avian)-like 2	MYBL2	0.41	0.2066 6
Occludin	OCLN	-0.30	0.2066 6
Progesterone receptor	PGR	0.54	0.2066 6
BCL2-like 1	BCL2L1	-0.43	0.2112 1
V-myc myelocytomatosis viral oncogene homolog (avian)	MYC	0.26	0.2571 1
Tight junction protein 2 (zona occludens 2)	TJP2	-0.40	0.2661 4
Caspase 3, apoptosis-related cysteine peptidase	CASP3	-0.24	0.3156 2
Twist homolog 1 (Drosophila)	TWIST1	-0.37	0.3156 2
Vascular endothelial growth factor A	VEGFA	0.25	0.3834 8
Notch 2	NOTCH 2	-0.28	0.4276 0
FBJ murine osteosarcoma viral oncogene homolog	FOS	0.28	0.4425 3
Insulin-like growth factor binding protein 4	IGFBP4	-0.35	0.4447 9
Notch 1	NOTCH 1	0.27	0.4447 9
Catenin (cadherin-associated protein), beta 1, 88kDa	CTNNB 1	-0.21	0.4552 5
Mitogen-activated protein kinase 8	MAPK8	-0.20	0.4560 8
WNT1 inducible signaling pathway protein 2	WISP2	-1.64	0.5319 6
Androgen receptor	AR	-0.26	0.5435 6
Amphiregulin	AREG	0.41	0.5435 6
Cyclin D1	CCND1	0.29	0.5813 3
Gap junction protein, alpha 1, 43kDa	GJA1	0.31	0.6023 3
Transforming growth factor, alpha	TGFA	0.24	0.6786 7
Vimentin	VIM	0.37	0.7254 9
Transforming growth factor, beta 3	TGFB3	-0.21	0.7267 3
Insulin-like growth factor 1 receptor	IGF1R	0.15	0.7514 8
GATA binding protein 3	GATA3	-0.16	0.7729 6

BCL2-associated agonist of cell death	BAD	-0.09	0.8071 6
Breast cancer 1, early onset	BRCA1	0.13	0.8422 6
V-akt murine thymoma viral oncogene homolog 1	AKT1	-0.09	0.8460 5
Keratin 8	KRT8	0.12	0.8511 2
Laminin, alpha 5	LAMA5	-0.09	0.8511 2
Cyclin D2	CCND2	-0.08	0.9306 4
Snail homolog 2 (Drosophila)	SNAI2	0.19	0.9306 4
BCL2-associated X protein	BAX	-0.05	0.9522 4
B-cell CLL/lymphoma 2	BCL2	-0.04	0.9522 4
Integrin, alpha 1	ITGA1	0.08	0.9522 4
Keratin 18	KRT18	0.03	0.9522 4
Mitogen-activated protein kinase 1	MAPK1	0.04	0.9522 4
Mucin 1, cell surface associated	MUC1	0.12	0.9522 4
Breast cancer anti-estrogen resistance 1	BCAR1	0.03	0.9562 4
Estrogen-related receptor alpha	ESRRA	0.03	0.9562 4
Integrin, beta 1	ITGB1	-0.01	0.9588 5
Mitogen-activated protein kinase 3	MAPK3	-0.01	0.9939 1
Wingless-type MMTV integration site family, member 4	WNT4	0.00	0.9939 1

A5.4 Supplemental Table 5.3

Full Gene Name	Gene ID	Fold Change	q value
Apolipoprotein D	APOD	-2.55	0.0000 5
Cyclin A1	CCNA1	1.08	0.0051 7
Bone morphogenetic protein 4	BMP4	-1.07	0.0055 2
Cytochrome P450, family 1, subfamily A, polypeptide 1	CYP1A1	-1.35	0.0055 2
Ectonucleotide pyrophosphatase/phosphodiesterase 2	ENPP2	1.24	0.0103 6
PDZ domain containing 1	PDZK1	1.19	0.0103 6
Bone morphogenetic protein 7	BMP7	-0.66	0.0199 0
Claudin 1	CLDN1	0.75	0.0199 0
Trefoil factor 1	TFF1	0.61	0.0228 8
X-box binding protein 1	XBP1	0.49	0.0987 9
Amphiregulin	AREG	1.16	0.1057 7
Growth regulation by estrogen in breast cancer 1	GREB1	0.99	0.1057 7
Keratin 19	KRT19	0.44	0.1214 2
V-erb-b2 erythroblastic leukemia viral oncogene homolog 2	ERBB2	-0.58	0.1461 2
Fibronectin 1	FN1	0.78	0.1461 2
Vascular endothelial growth factor A	VEGFA	0.44	0.1461 2
BCL2-like 1	BCL2L1	-0.53	0.1642 8
Progesterone receptor	PGR	0.62	0.1764 0
Breast cancer 1, early onset	BRCA1	-0.61	0.1872 7
Cyclin E1	CCNE1	-0.30	0.1872 7
Estrogen receptor 1	ESR1	-0.95	0.1912 2
Milk fat globule-EGF factor 8 protein	MFGE8	0.67	0.1912 2
Snail homolog 2 (Drosophila)	SNAI2	1.37	0.1912 2

G protein-coupled estrogen receptor 1	GPER	-0.72	0.2163 6
Tight junction protein 1 (zona occludens 1)	TJP1	-0.36	0.2163 6
Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CDKN1A	-0.20	0.3968 0
V-myb myeloblastosis viral oncogene homolog (avian)-like 2	MYBL2	0.32	0.3968 0
Integrin, beta 1	ITGB1	0.20	0.4347 6
V-akt murine thymoma viral oncogene homolog 1	AKT1	-0.24	0.4765 5
Androgen receptor	AR	-0.31	0.4765 5
Cyclin D2	CCND2	-0.35	0.4765 5
Catenin (cadherin-associated protein), beta 1, 88kDa	CTNNB1	-0.21	0.4765 5
Cytochrome P450, family 19, subfamily A, polypeptide 1	CYP19A1	-0.51	0.4765 5
Insulin-like growth factor binding protein 4	IGFBP4	-0.34	0.4765 5
Mitogen-activated protein kinase 8	MAPK8	-0.21	0.4765 5
Vimentin	VIM	-0.64	0.4765 5
Wingless-type MMTV integration site family, member 4	WNT4	-0.49	0.4765 5
Integrin, alpha 1	ITGA1	0.43	0.5923 3
Notch 1	NOTCH1	0.21	0.5923 3
Cyclin D1	CCND1	0.29	0.6247 5
Gap junction protein, alpha 1, 43kDa	GJA1	0.29	0.6777 0
V-myc myelocytomatosis viral oncogene homolog (avian)	MYC	0.12	0.6777 0
Caspase 3, apoptosis-related cysteine peptidase	CASP3	-0.12	0.6904 8
Transforming growth factor, alpha	TGFA	0.23	0.7099 3
Twist homolog 1 (Drosophila)	TWIST1	-0.16	0.7189 7
WNT1 inducible signaling pathway protein 2	WISP2	1.11	0.7189 7
GATA binding protein 3	GATA3	-0.18	0.7343 9
Occludin	OCLN	-0.09	0.7416 9

Wingless-type MMTV integration site family, member 5A	WNT5A	0.21	0.7416 9
BCL2-associated agonist of cell death	BAD	-0.08	0.8122 5
Keratin 8	KRT8	0.14	0.8122 5
Estrogen-related receptor alpha	ESRRA	-0.14	0.8153 5
Tight junction protein 2 (zona occludens 2)	TJP2	-0.10	0.8206 0
B-cell CLL/lymphoma 2	BCL2	-0.10	0.8215 8
FBJ murine osteosarcoma viral oncogene homolog	FOS	0.09	0.8215 8
Keratin 18	KRT18	0.06	0.8704 5
Laminin, alpha 5	LAMA5	-0.06	0.8704 5
Mucin 1, cell surface associated	MUC1	0.23	0.8704 5
Transforming growth factor, beta 3	TGFB3	0.09	0.8704 5
Insulin-like growth factor 1 receptor	IGF1R	0.05	0.9035 1
Mitogen-activated protein kinase 3	MAPK3	-0.05	0.9035 1
BCL2-associated X protein	BAX	0.02	0.9694 2
Breast cancer anti-estrogen resistance 1	BCAR1	-0.02	0.9734 9
Mitogen-activated protein kinase 1	MAPK1	0.01	0.9734 9
Cadherin 1, type 1, E-cadherin (epithelial)	CDH1	-0.12	0.9753 3
Notch 2	NOTCH 2	0.01	0.9753 3

A5.5 Supplemental Table 5.4

Full Gene Name	Gene ID	Fold Change	q value
Growth regulation by estrogen in breast cancer 1	GREB1	-1.11	0.01256
V-myb myeloblastosis viral oncogene homolog (avian)-like 2	MYBL2	-1.21	0.07508
Bone morphogenetic protein 7	BMP7	0.44	0.08440
Cytochrome P450, family 1, subfamily A, polypeptide 1	CYP1A1	-0.35	0.08440
Estrogen receptor 1	ESR1	-0.37	0.08440
PDZ domain containing 1	PDZK1	-1.44	0.08440
B-cell CLL/lymphoma 2	BCL2	-1.31	0.08445
Progesterone receptor	PGR	-0.17	0.08445
Estrogen-related receptor alpha	ESRRA	-1.15	0.08856
G protein-coupled estrogen receptor 1	GPER	-0.34	0.11291
Androgen receptor	AR	-1.11	0.21643
Amphiregulin	AREG	-0.41	0.32838
Trefoil factor 1	TFF1	-0.48	0.42976
Insulin-like growth factor 1 receptor	IGF1R	-0.37	0.46348
BCL2-like 1	BCL2L1	-0.39	0.54334
Mucin 1, cell surface associated	MUC1	1.06	0.54334
V-erb-b2 erythroblastic leukemia viral oncogene homolog 2	ERBB2	-0.34	0.57398
Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CDKN1A	-1.09	0.59127
Cyclin D1	CCND1	-0.37	0.65667
Breast cancer anti-estrogen resistance 1	BCAR1	-0.34	0.68580
Cyclin D2	CCND2	-0.34	0.68580
Cyclin E1	CCNE1	1.38	0.68580
Mitogen-activated protein kinase 8	MAPK8	-1.15	0.68580

Breast cancer 1, early onset	BRCA1	-0.62	0.7083 9
Fibronectin 1	FN1	1.45	0.7503 9
Caspase 3, apoptosis-related cysteine peptidase	CASP3	0.33	0.7688 6
Wingless-type MMTV integration site family, member 4	WNT4	-0.60	0.7688 6
Transforming growth factor, beta 3	TGFB3	-0.23	0.8039 6
BCL2-associated agonist of cell death	BAD	-1.09	0.8039 6
Claudin 1	CLDN1	0.33	0.8039 6
Insulin-like growth factor binding protein 4	IGFBP4	-0.39	0.8106 2
Tight junction protein 2 (zona occludens 2)	TJP2	-0.35	0.8450 1
V-myc myelocytomatosis viral oncogene homolog (avian)	MYC	-1.25	0.8964 7
Cyclin A1	CCNA1	-0.37	0.9026 0
X-box binding protein 1	XBP1	-0.32	0.9350 2
Transforming growth factor, alpha	TGFA	0.02	0.9671 9
Arylhydrocarbon receptor	AHR	-0.41	0.9671 9
V-akt murine thymoma viral oncogene homolog 1	AKT1	1.02	0.9671 9
Apolipoprotein D	APOD	0.44	0.9671 9
BCL2-associated X protein	BAX	0.20	0.9671 9
Bone morphogenetic protein 4	BMP4	-1.22	0.9671 9
Cadherin 1, type 1, E-cadherin (epithelial)	CDH1	0.53	0.9671 9
Cyclin D kinase 2	CDK2	0.35	0.9671 9
Cathepsin D	CTSD	-1.13	0.9671 9
Cytochrome P450, family 19, subfamily A, polypeptide 1	CYP19A 1	-0.48	0.9671 9
FBJ murine osteosarcoma viral oncogene homolog	FOS	-2.00	0.9671 9
GATA binding protein 3	GATA3	-0.37	0.9671 9
Gap junction protein, alpha 1, 43kDa	GJA1	-1.61	0.9671 9

Integrin, beta 1	ITGB1	-0.35	0.9671 9
Keratin 18	KRT18	-1.11	0.9671 9
Keratin 19	KRT19	-1.09	0.9671 9
Keratin 8	KRT8	-1.15	0.9671 9
Laminin, alpha 5	LAMA5	-0.38	0.9671 9
Mitogen-activated protein kinase 1	MAPK1	-0.38	0.9671 9
Mitogen-activated protein kinase 3	MAPK3	0.37	0.9671 9
Milk fat globule EGF 8	MFGE8	-1.16	0.9671 9
Notch 1	NOTCH 1	0.40	0.9671 9
Occludin	OCLN	0.43	0.9671 9
Twist homolog 1 (Drosophila)	TWIST1	0.35	0.9671 9
Vascular endothelial growth factor A	VEGFA	0.42	0.9671 9
Vimentin	VIM	-1.68	0.9671 9
Catenin (cadherin-associated protein), beta 1, 88kDa	CTNNB 1	-0.50	0.9771 1