Evaluation of Extracellular Matrix Composition and Rheology as Determinants of Growth, Invasion, and Response to Photodynamic Therapy in 3d Cell Culture Models of Pancreatic Ductal Adenocarcinoma

Gwendolyn M. Cramer

University of Massachusetts Boston

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EVALUATION OF EXTRACELLULAR MATRIX COMPOSITION AND RHEOLOGY AS DETERMINANTS OF GROWTH, INVASION, AND RESPONSE TO PHOTODYNAMIC THERAPY IN 3D CELL CULTURE MODELS OF PANCREATIC DUCTAL ADENOCARCINOMA

A Dissertation Presented
by
GWENDOLYN M. CRAMER

Submitted to the Office of Graduate Studies, University of Massachusetts Boston, in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2017

Molecular, Cellular and Organismal Biology Program
EVALUATION OF EXTRACELLULAR MATRIX COMPOSITION AND
RHEOLOGY AS DETERMINANTS OF GROWTH, INVASION, AND RESPONSE
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ABSTRACT

EVALUATION OF EXTRACELLULAR MATRIX COMPOSITION AND RHEOLOGY AS DETERMINANTS OF GROWTH, INVASION, AND RESPONSE TO PHOTODYNAMIC THERAPY IN 3D CELL CULTURE MODELS OF PANCREATIC DUCTAL ADENOCARCINOMA

December 2017

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Directed by Assistant Professor Jonathan Celli

Pancreatic ductal adenocarcinoma (PDAC) is a notoriously lethal disease characterized by prominent stromal involvement, which plays complex roles in regulating tumor growth and therapeutic response. The extracellular matrix (ECM)-rich stroma has been implicated as a barrier to drug penetration, although stromal depletion strategies have had mixed clinical success. It remains less clear how biophysical interactions with the ECM regulate invasive progression and susceptibilities to specific therapies. Here, an integrative approach combining 3D cell culture and quantitative imaging techniques is used to evaluate invasive behavior and motility as determinants of response to classical chemotherapy and photodynamic therapy (PDT), in which light activated agents induce site-directed cell death by generating reactive oxygen species. The 3D culture protocol
developed for these studies with transplanted multicellular PDAC spheroids in rheologically characterized ECM shows that in invasion-promoting ECM environments, PDT response is markedly enhanced in the most motile populations while the same cells exhibit chemoresistance. Conversely, drug-resistant sublines with characterized increase in invasive potential were generated to compare differential treatment response in identical ECM conditions, monitored by particle-tracking microrheology measurements of matrix remodeling. In both scenarios, ECM infiltrating cells exhibit increased PDT sensitivity, whether invasion is consequent to selection of chemoresistance, or whether chemoresistance is correlated with acquisition of invasive behavior. However, while ECM-infiltrating, chemoresistant cells exhibit mesenchymal phenotype, EMT induction in monolayers lacking ECM is not sufficient to enhance PDT sensitivity, yet does impart chemoresistance as expected. In further experiments seeking to elucidate intertwined roles of mechanical and biochemical interactions with ECM components, invasive progression and response to therapeutics were evaluated using ECM protein admixtures and collagen hydrogels with varying extent of crosslinking. In these studies, increased collagen stiffness or presence of laminin-rich ECM both inhibit invasion of PDAC cells, although cells that do infiltrate into ECM nevertheless exhibit chemoresistance and enhanced PDT sensitivity, independent of their ECM environment. In addition to containing platform development with broader applicability to inform microenvironment-dependent therapeutics, results of this work collectively reveal the efficacy of PDT for targeting the most aggressive, chemoresistant, and invasive PDAC cell populations associated with dismal outcomes for this disease.
ACKNOWLEDGEMENTS

I would like to thank my advisor, Jonathan Celli, and all of the undergraduates, graduate students, and postdoctoral researchers I have worked with the past few years. Undergraduates I’ve worked with and mentored at UMass-Boston include Rojin Jafari, Lucas Barbosa Gullo, Saipriya Sagiraju, Saikrishna Gourishetti, Khadija Boudarse, Joshua Hempstead, and Alexa Putnam, who have all been helpful assistants and collaborators, as well as excellent company, in the variety of projects contained by this dissertation.

Graduate students I have worked with since I started my PhD program include Hamid El-Hamidi and Ljubica Petrovic. I also need to thank Dustin Jones, Will Hanna, Michael Anderson, and Jeffrey La for all their help with MATLAB and image analysis, and Hui Liu for her varied engineering and photodynamic therapy expertise. All other friends and family members around the world have been wonderfully giving me breaks from science on occasion, such as Daniel Acuña for sitting and drinking tea and coffee with me.

I would also like to thank all of my dissertation committee members in addition to Jonathan Celli, Jill Macoska (and members of her research group, particularly Sathish Kasina, for help with westerns and unlocking doors), Alexey Veraksa, Linda Huang, and Imran Rizvi. Additionally, Tayyaba Hasan provided some conference funding support and importantly advised my advisors/committee members.
I am also, of course, grateful for funding sources covering these projects, most of which came from the NIH/NCI R00CA155045 grant to Jonathan Celli. The Sanofi-Genzyme Doctoral Research Fellowship was also helpful for allowing me to focus solely on research instead of teaching for three semesters of funding, and I want to thank Andrew Grosovsky and Velina Batchvarov for helping to make this funding available and organizing opportunities to interact with Sanofi-Genzyme financial supporters.
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CHAPTER 1
INTRODUCTION

Tumor growth behavior and response to therapies are influenced not just by inherent genetic traits of cancer cells themselves, but also by interactions with their environment. While significant research has been done in correlating chemotherapy efficacy, in particular, with tumor phenotype and microenvironment, fewer studies have been completed for determining tumor characteristics linked to photodynamic therapy (PDT) response. Furthering this research is particularly important for pancreatic ductal adenocarcinoma (PDAC), since its characteristic aggressiveness and resistance to chemotherapy interventions have limited substantial gains in survival or quality of life for those diagnosed. PDT has the potential to overcome the metastatic and chemoresistant pancreatic cancer phenotype because of its mechanistically distinct mode of cell death compared to standard chemotherapy regimens. The overall theme of this dissertation is that stromal components of the PDAC microenvironment affect cancer cell behavior which in turn impact susceptibilities to various therapeutic regimens. In this context, I determine the effectiveness of PDT at targeting chemoresistant and invasive populations of pancreatic tumor cells by establishing 3D culture conditions with tunable biophysical and biochemical properties to explore changes in pancreatic cancer phenotype and treatment response.
Pancreatic Cancer

Pancreatic ductal adenocarcinoma (PDAC) has a 5-year survival rate of only 6%, and about half of patients are diagnosed with advanced-stage metastatic cancer with a 5-year survival rate of merely 2% (Ghosn et al., 2014; Siegel et al., 2014). Additionally, median survival is only about 6 months (Goldstein et al., 2015; Gong et al., 2011). Major reasons for PDAC’s low survival rate are late stage diagnosis, early metastasis, and the common resistance to standard chemotherapy treatments.

Only approximately 15-20% of pancreatic tumors are surgically resectable at the time of diagnosis, and unfortunately many of these are already associated with micrometastases (Konstantinidis et al., 2013), so the 5-year survival for patients with resectable tumors is still less than 20% (Oettle et al., 2007). While surgery is the only potentially curative PDAC treatment, adjuvant therapies like radiotherapy or chemotherapy (discussed later in this chapter) are usually used to increase survival since surgical resection does not always produce negative margins.

A variety of risk factors are associated with an increased likelihood of developing pancreatic cancer, including those with environmental and behavior patterns such as cigarette smoking, chronic pancreatitis, diabetes, and obesity. Familial history of pancreatic cancer can also increase risk. Associated genetic mutations include \( p16 \) (involved in familial atypical multiple mole and melanoma syndrome) and \( BRCA1 \) and \( BRCA2 \) (linked to breast and ovarian cancers) (Makohon-Moore and Iacobuzio-Donahue, 2016; Oettle, 2014; Ryan et al., 2014).
While PDAC tumors are genetically heterogeneous, some specific somatic mutations are fairly common. For example, over 90% of PDAC tumors have KRAS mutations. Other mutations found at a high frequency in these tumors include those in p53, CDKN2A, and the TGFβ signaling pathway, specifically SMAD4 (Makohon-Moore and Iacobuzio-Donahue, 2016; Oettle, 2014; Ryan et al., 2014).

Stromal and Mechanoregulatory Crosstalk in the PDAC Microenvironment

As PDAC develops, it interacts with surrounding tissue and fashions a unique and heterogeneous microenvironment that plays complex roles in regulating PDAC behavior and therapeutic efficacy. This microenvironment is mostly composed of reciprocally interacting fibroblast-like cells, immune cells, and various extracellular matrix proteins (ECM) (Figure 1) (Bardeesy and DePinho, 2002; Bijlsma and van Laarhoven, 2015). The desmoplastic reaction involved in PDAC development is stronger than in many other cancers, and the resulting stroma can account for over 90% of tumor volume, which prevents tumor vascularization and thus inhibits drug perfusion (Erkan et al., 2012a; Neesse et al., 2011). Since the characteristically vast PDAC stroma stiffly envelops the cancer cells, it has been cast as a barrier to drug delivery that should be removed to improve chemotherapy penetration.

In a mouse model, an inhibitor of smoothened (SMO), required in the hedgehog signaling pathway for tumor-stroma communication, increased gemcitabine delivery and improved survival (Olive et al., 2009). Despite high hopes as an effective way to target PDAC, similar stromal depletion approaches in Infinity Pharmaceutical’s Phase 2 clinical
trial were actually observed to increase PDAC progression and this trial was discontinued (Garber, 2010; Madden, 2012). According to Rucki et al. (2017), heterogeneous stromal expression of sonic hedgehog (SHH) and hepatocyte growth factor (HGF) signaling that influences PDAC metastasis prevents therapeutic interventions targeting only one of these pathways. Clearly, the stroma’s complex regulatory roles in PDAC disease progression extend far beyond a functional barrier.

**Figure 1. Tumor-stroma interactions.** Major components of the tumor microenvironment include a scaffold of supportive extracellular matrix proteins (green), fibroblasts/pancreatic stellate cells (blue), and immune cells (purple) in addition to the cancer cells themselves. All these reciprocally interactive components influence tumor growth, invasive behavior, and treatment response.

Signaling pathways involved in the PDAC desmoplastic reaction and stromal generation lead to the secretion of various stroma structural components such as collagen, as well as enzymes like matrix metalloproteinases, and contribute to tumor growth and
metastasis (Hanahan and Weinberg, 2011; Mahadevan and Von Hoff, 2007). In normal tissue, the major players in the process of developing connective tissue stroma are fibroblasts, which produce most of these ECM proteins. This is especially prevalent after tissue injury when fibroblasts are recruited to these sites and converted into activated myofibroblasts to more dramatically repair and restructure the ECM (Hinz et al., 2007). In tumors, cancer associated fibroblasts (CAFs) are manipulated into a perpetually activated myofibroblast phenotype that supports cancer progression (Olumi et al., 1999). These fibroblasts can originate from a variety of sources, and in PDAC mostly stem from pancreas-resident fibroblasts and bone marrow-derived cells (Nielsen et al., 2016). Pancreatic cancer associated fibroblasts, pancreatic stellate cells (PSCs), become activated when interacting with cancer cells and switch to a myofibroblast phenotype expressing alpha smooth muscle actin (αSMA) (Apte et al., 2015). These activated PSCs are the main source of collagen synthesis in PDAC (Apte et al., 2004).

In addition to its role as a physical barrier to drug delivery, both the biochemical and biophysical characteristics of the stroma’s extracellular matrix can regulate PDAC behavior. According to Pickup et al. (2014), the ECM has regulatory roles in many of the hallmarks of cancer first listed by Hanahan and Weinberg (2000, 2011). Compared to normal tissue, the ECM around pancreatic tumors becomes stiffer and thicker, with more fibrillar collagen proteins. Therefore, it is important to discuss ECM context when studying factors influencing PDAC metastasis, growth, and therapeutic response.

Mechanical properties of the ECM and microenvironment surrounding tumors enable complex signaling patterns that regulate tumor growth and malignancy (Kumar
and Weaver, 2009; Paszek et al., 2005). In breast cancer, for example, the presence of extensive collagen fibers aligned perpendicular to the tumor is associated with a poor prognosis (Conklin et al., 2011), since these fibers provide pathways for cancer cell invasion (Provenzano et al., 2008; Wang et al., 2007), and the increased stiffness and mechanical stress in aligned ECMs encourages tumor proliferation (Boghaert et al., 2012; Paszek et al., 2005; Schrader et al., 2011). The high interstitial fluid pressure and solid stress resulting from a stiff stroma and cancer growth also alter fluid mechanics in a way that limits diffusion and flow of chemotherapy drugs into the tumor (Nieskoski et al., 2017; Provenzano and Hingorani, 2013), thus influencing how the cells respond to treatments.

Tumors also modify rheological properties of the ECM during invasion and metastasis. Matrix metalloproteinases (MMPs), in normal tissues, are mostly produced by fibroblasts to control ECM remodeling in a well-regulated fashion (Li et al., 2007). These zinc-dependent peptidases have a wide variety of important biological roles in the extracellular space and can degrade all ECM components (Rodriguez et al., 2010). Cancer cells can also secrete MMPs, and their production is influenced by stiffness of the surrounding ECM. In vitro, for example, pancreatic cell lines may upregulate MMP expression in stiffer ECM substrates, although this seems to be cell line dependent (Haage and Schneider, 2014). The increase in MMP expression during cancer metastasis leads to matrix degradation in the area of invasion (Kenny et al., 2008).

ECM composition and stromal signaling, such as type 1 collagen, which composes much of the PDAC stroma (Mollenhauer et al., 1987), is linked to epithelial-
mesenchymal transition (EMT, shown in Figure 2) (Ellenrieder et al., 2001; Fuxe et al., 2010; Shintani et al., 2006) chemoresistance (Dangi-Garimella et al., 2011), and reduced patient survival (Whatcott et al., 2015). The PDAC microenvironment appears to be a potent stimulator of a metastatic phenotype. Indeed, this may happen even in the beginning of PDAC development, since one component of the tumor microenvironment is pancreatic cancer cells that undergo early EMT (Rhim et al., 2012).

Since pancreatic tumors are associated with such an extensive desmoplastic reaction, tumor-stroma interactions play a large role in cancer proliferation and metastasis. VEGF (vascular endothelial growth factor), HGF (hepatocyte growth factor), and IL-6 (interleukin-6) signaling, among others, allow stromal cells such as fibroblasts and cancer cells to communicate in a way that leads to invasive characteristics (Cirri and Chiarugi, 2011). Most common locations of PDAC metastases are the liver, lungs, and abdominal cavity. Cancer cells can metastasize individually, through an amoeboid process or acquisition of a mesenchymal phenotype, or collectively in chains or sheets, depending on the type of cell and microenvironment conditions (Friedl and Alexander, 2011). After metastasis, newly formed PDAC tumors in secondary locations develop desmoplasia similar to that of the primary tumor (Whatcott et al., 2015).
Figure 2. Epithelial to mesenchymal transition (EMT). As cells transition from an epithelial (left) to mesenchymal (right) phenotype, they lose tight cell-cell adhesion, apical-basal polarity, and E-cadherin expression. Mesenchymal cells express more vimentin and cancer stem cell (CSC) markers and, importantly, are also associated with chemoresistance.

A variety of other microenvironment and signaling conditions can promote EMT including hypoxia (Salnikov et al., 2012), hedgehog signaling (mentioned earlier in this chapter as a stromal depletion target), and TGFβ signaling (Figure 3). Activation of the hedgehog signaling pathway, for example, leads to Gli nuclear translocation and subsequent activation of transcription factors that repress E-cadherin and increase mesenchymal proteins, as well as inducing TGFβ secretion (Gonzalez and Medici, 2014).

TGFβ has a dual role in cancer. Early in the life history of a cancer cell, this pathway suppresses tumor growth and promotes apoptosis. But cancers commonly develop TGFβ signaling mutations (in TGFβ-R1 and TGFβ-R2 receptors, SMAD4, etc.)
and also stop responding to growth arrest signaling, causing TGFβ activation to promote EMT (see Figure 3, below, for a diagram of this signaling pathway) (Heldin et al., 2009; Massague, 2008). In the canonical signaling pathway, TGFβ activates its receptors TGFβ-R1 and TGFβ-R2 at the cell membrane, transducing the signal to the nucleus via a variety of SMAD protein complexes. This activates transcription factors like Snail that downregulate E-cadherin expression, thus minimizing epithelial characteristics. TGFβ activation also promotes fibroblast to myofibroblast phenoconversion (O'Connor and Gomez, 2014) that encourages collagen accumulation, as discussed earlier in this section.
Figure 3. The TGFβ signaling pathway. Canonical TGFβ signaling (SMAD4 dependent) is shown in blue. Additional signaling pathways that can respond to TGFβ receptor activation to influence EMT characteristics are Ras and PI3K pathways, also shown above.

3D Tumor Models

Classically, most cancer research has been done with monolayer culturing of cancer cell lines derived from patient tumor samples. The first isolated pancreatic cancer cell line (called PaCa) was reported in 1963, a few years after the generation of HeLa cells, and originated from an abdominal metastatic node of pancreatic adenocarcinoma.
These cell lines have been vital for understanding molecular processes that can occur in cells, including the process of malignant transformation, but their utility is diminished when looking to predict cancer treatment response. Other in vitro systems, namely 3D cultures, better model cell-cell and cell-ECM interactions and biomechanical influences, and represent an important stepping stone that may help with high attrition rates in drug development (Antoni et al., 2015; Fang and Eglen, 2017). The concept of using 3D in vitro models to better represent the relationships between tumors and their environments was popularized by Mina Bissell, who has consistently defended the importance of communication between the extracellular matrix and the cells within it for growth and development of normal and malignant tissues (Barcellos-Hoff et al., 1989; Bissell et al., 1999; Nelson and Bissell, 2006).

By restoring stromal interactions in vitro, physiologically relevant cell signaling patterns and influences of the biomechanical microenvironment should also be restored. This can be partially accomplished through growing cancer cells in 3D structures on or embedded in an extracellular matrix, and co-culturing cancer cells with fibroblasts on ECM beds to mimic in vivo signaling even more realistically (Debnath and Brugge, 2005). A variety of ECM products are used for 3D model development, including collagen and growth factor reduced Matrigel. Matrigel is a basement membrane extracted from Engelbreth–Holm–Swarm mouse sarcomas, and contains ECM proteins such as collagen IV and laminin.

Compared to monolayer, various 3D models of cancer including breast (Kobayashi et al., 1993; Nicholson et al., 1997; Ohmori et al., 1998), colon (Nicholson et
al., 1997), oral (Fischbach et al., 2007), ovarian (Rizvi et al., 2010), and pancreatic (Dufau et al., 2012) have increased resistance to chemotherapeutic agents. Growing cancer cells in 3D conditions with ECM influences their phenotype and therapeutic response due to a variety of factors. For example, multicellular tumor spheroids in 3D cultures are more resistant to drugs because of decreased penetration, increased hypoxia, increased anti-apoptotic signaling, and more cell-cell and cell-ECM contacts (Celli et al., 2011; Evans et al., 2011; Longati et al., 2013; Minchinton and Tannock, 2006; Nicholson et al., 1997; Weaver et al., 2002; Yang et al., 2009b). In more complicated 3D culture models with stromal cell involvement, in addition to the influence of direct contact between stromal cells and cancer cells, fibroblasts also secrete growth factors including TGFβ and HGF (Ding et al., 2013; Lebret et al., 2007) that can induce EMT in cancer cell lines.

3D culturing of tumor cells also allows for more realistic modeling of invasion than monolayer cultures. These invasion processes include disruption of spheroid architecture and cell junctions, EMT, migration, and ECM/basement membrane breakdown (Debnath and Brugge, 2005; Vidi et al., 2013). Of course, the density, stiffness, and composition of the ECM model used will affect how the cancer cells behave, so this context must be kept in mind when designing 3D models in an effort to recapitulate in vivo scenarios (Herrmann et al., 2014).

This system allows for a more direct interpretation of results of treatment response than is possible in in vivo models, which are more challenging experimentally, financially, and ethically. Mouse models of tumors, for example, most frequently utilize
subcutaneous tumor growth (Blanchard, 2016). While these experiments can definitely provide useful information in certain scenarios, subcutaneous tumors exist in a completely different microenvironment than a naturally developing mouse (or especially human) tumor, and this will affect how it grows and responds to tested therapies. So, depending on the experimental question, an in vitro mechanobiological platform for characterizing PDAC behavior may offer the appropriate amount of complexity.

Popularizing 3D tumor models for pancreatic cancer research may allow for studies distilling complex tumor-host interactions to evaluate effects of therapeutic options on the mechanical and biochemical environment surrounding tumors. Two of the main methods for studying 3D tumors in ECM, cancer cells grown on top of or embedded in extracellular matrix, are shown below (Figure 4), and are discussed in more detail in the Methods and relevant Results chapters.

**Figure 4.** 3D tumor models used in this dissertation. In the overlay model (left) cancer nodules grow on top of an extracellular matrix gel with media also containing a low concentration of the ECM proteins. In the embedded model (right) a larger tumor spheroid is placed into the extracellular matrix before gelation.
Chemotherapies for PDAC include gemcitabine treatment and the multi-drug cocktail FOLFIRINOX, which contains folinic acid, 5-fluorouracil, irinotecan, and oxaliplatin. For early stage PDAC, in the unlikely scenarios that surgical resection is possible, standard clinical management is surgery followed by an adjuvant chemotherapy drug such as gemcitabine, a pyrimidine nucleoside analog that is actively transported across the cell membrane (Gesto et al., 2012; Plunkett et al., 1995; Oettle 2014; Bujanda et al., 2012).

For metastatic pancreatic cancer, gemcitabine is the standard palliative treatment option (Burris et al., 1997; Gesto et al., 2012; Plunkett et al., 1995). Unfortunately, less than 25% of patients experience any clinical benefit from gemcitabine (Burris et al., 1997). While FOLFIRINOX is somewhat more effective, it is also leads to a higher percentage of adverse side effects, so it can only be used in patients who are still reasonably healthy (Conroy et al., 2011; Ghosn et al., 2014). Oxaliplatin, a platinum-based DNA alkylating agent, also plays a role in metastatic pancreatic cancer treatment as the major component of the FOLFIRINOX cocktail, but its effectiveness comes at a cost of more side effects for patients (Bordin et al., 2013; Ghosn et al., 2014).

Several other combination treatments are available, and a clinical trial meta-analysis of these shows improved survival odds over gemcitabine alone for metastatic pancreatic cancer, also with increased side effects compared to gemcitabine (Gresham et al., 2014). Currently, either FOLFIRINOX or combined gemcitabine and nab-paclitaxel are standard regimens in patients that can tolerate the increased toxicity compared to
gemcitabine monotherapy (Ryan et al., 2014). Still, the increase in median overall survival from 6.8 months with gemcitabine to 11.1 months with FOLFIRINOX (Conroy et al., 2011), while significant, is disappointingly small. Due to pancreatic cancer chemoresistance, median survival for those diagnosed remains less than one year.

**Drug Resistance and the Epithelial to Mesenchymal Transition**

The pancreatic cancer microenvironment, characterized by poor vascularization and an extensive desmoplastic reaction, can promote the cancer stem cell (CSC) phenotype, a tumor subpopulation with self-renewing and tumor-initiating potential, that is associated with chemoresistance and EMT (Cabrera et al., 2015; Lamouille et al., 2014). In addition to the physical barrier to chemotherapy delivery linked to the pancreatic cancer microenvironment, PDAC cells (and developing cancers more generally) become resistant to chemotherapy through a variety of mechanisms.

Reduced nucleoside transporter expression (hENT1, hCNT1, etc.) is associated with gemcitabine resistance by preventing drug uptake (de Sousa Cavalcante and Monteiro, 2014). Increased expression of certain ATP-binding cassette (ABC) transporters can increase the rate of chemotherapy drug efflux and decrease cell damage (Abdullah and Chow, 2013). Additionally, PDAC cells resistant to gemcitabine are characterized by a more mesenchymal phenotype (Shah et al., 2007). Induction of EMT alone, in the breast cancer cell line MCF7 using TGFβ, led to a significant increase in the number of ABC transporters compared to untreated cells (Saxena et al., 2011). Therefore, PDAC cells with a mesenchymal phenotype, generated either through activation of
signaling pathways or chemoresistance defense mechanisms, are likely to have fewer nucleoside transporters and/or increased ABC transporters to modify influx/efflux rates for a range of drugs.

Slower proliferation and more active DNA repair can also lead to chemoresistance. For example, changes in the base excision repair pathway can lead to resistance to alkylating chemotherapy drugs (Sarkaria et al., 2008). Additionally, PARP-1, a nuclear enzyme involved in DNA single-strand break repair, also regulates transcription of the Snail protein and thus increases in PARP-1 effect EMT (Broustas and Lieberman, 2014). In pancreatic cancers with \textit{BRCA} mutations, PARP inhibitors combined with platinum-based chemotherapy drugs are currently being explored in clinical trials, and it seems most beneficial to utilize the PARP inhibitors before resistance to the chemotherapy drugs can develop (Golan and Javle, 2017).

Alterations in death pathway signaling or defective apoptosis machinery can occur after chemotherapy exposure, thus reducing tumor response to these drugs. (See Figure 6 for a simplified image of apoptosis induction). Apoptosis induced by gemcitabine involves Bcl-2 family signaling mediated by mitochondria (Schniewind et al., 2004a), so anti-apoptotic Bcl-2 upregulation can decrease chemosensitivity. Bcl-xL and MCL-1, members of the anti-apoptotic Bcl-2 family, are both upregulated in PDAC (Boucher et al., 2000; Miyamoto et al., 1999).

Regulation and generation of reactive oxygen species (ROS) in cancer cells is also linked to chemoresistance. Cancer cells tend to generate more intracellular ROS, through increased metabolism, oncogene activity, mitochondrial dysfunction, etc., so their redox
balance is altered compared to normal cells (Giannoni et al., 2012; Kobayashi and Suda, 2012). Higher ROS sequestration abilities are associated with greater chemoresistance, such as upregulation of the antioxidant-regulating Nrf2 transcription factor or mutations in its gene \textit{NFE2L2} (Arumugam et al., 2009; Ishikawa et al., 2013). Mechanisms of resistance to ROS damage include higher levels of ROS sequestration molecules like glutathione (GSH) and antioxidant upregulation. Resistance to the chemotherapy drug 5-FU, for example, is partially mediated by an increase in the ROS modulating protein Romo1 (Hwang et al., 2007). Alkylating agents and platinum-based chemotherapy drugs (oxaliplatin, carboplatin, etc.) generate higher ROS levels than nucleoside analogs (such as gemcitabine) (Conklin, 2004) (Sosa et al., 2013), so developing oxaliplatin resistance may involve more ROS sequestration abilities than gemcitabine resistance.

Furthermore, CSCs tend to be even more resistant to chemotherapy-induced apoptosis because of higher glycolytic rates, slower proliferation, augmented transporter expression, and increased ROS defenses (Abdullah and Chow, 2013; Kobayashi and Suda, 2012). EMT regulators Twist, Snail, ZEB, and others involved in TGF\(\beta\) signaling, can promote cells to acquire CSC traits (Cabrera et al., 2015). In pancreatic cancer, CSC markers include CD133, CD44, CD24, ESA, ALDH, and c-met (Fitzgerald and McCubrey, 2014). Furthermore, CD44 is regulated by a ZEB1-miR-200 feedback loop, and helps to regulate intracellular reactive oxygen species adaption (Wagner et al., 2014; Wellner et al., 2009). CD44 is a target of the micro-RNA miR-200c, and is associated with EMT, cancer aggressiveness, and high intracellular ROS (Lin et al., 2013).
Photodynamic Therapy

Since the highly invasive potential of pancreatic cancer cells is associated with resistance to chemotherapy (Shah et al., 2007), improvements in treatment options for patients will be essential for enhancing their life expectancy and quality. Building on previous literature demonstrating that photodynamic therapy (PDT) can bypass established mechanisms of chemoresistance (Celli et al., 2011; Duska et al., 1999; Spring et al., 2015), this thesis explores the potential for PDT to target therapeutically resistant PDAC populations associated with stromal crosstalk in PDAC. This work is timely with recent clinical studies, in which PDT using interstitial light delivery has been shown to be technically, feasible, safe and effective for treatment of locally advanced but unresectable pancreatic cancer (Huggett et al., 2014).

During PDT, photosensitized cells are exposed to light (generally 600-800 nm), initiating photochemistry that results in targeted tumor destruction primarily by generation of cytotoxic singlet oxygen (Dougherty et al., 1998) (Type 2 Reactions shown in Figure 5). A variety of photosensitizers (PS) are available for this, at different stages of regulatory approval, including the first-generation PS hematoporphyrin derivative, HpD (Photofrin), and the strategy of using a heme biosynthesis precursor, aminolevulinic acid (ALA), which is metabolized into the endogenous photosensitizer protoporphyrin IX (Abrahamse and Hamblin, 2016; Castano et al., 2004).
Figure 5. Photodynamic Therapy: Photochemistry. The light-activated photosensitizer ($^1$PS*) undergoes intersystem crossing to a long-lived triplet excited state ($^3$PS*), which can interact with cellular components in Type 1 (electron transfer to biological substrates) and Type 2 (energy transfer to oxygen) reactions to generate reactive oxygen species (ROS).

The second-generation photosensitizer verteporfin (BPD: benzoporphyrin derivative monoacid ring A) is already licensed in the U.S. and other countries for treatment of age-related macular degeneration (Wu and Murphy, 1999). Huggett et al. (2014) used BPD in their recent clinical trial because it is rapidly cleared by excretion in bile, has peak tissue concentration within 1-2 hours with only 24-hour skin sensitivity (Houle and Strong, 2002), has high singlet oxygen yield (Celli et al., 2011; Tekrony et al., 2011), and its absorbance at 690 nm (within the optical window) allows for deep tissue penetration (Figure 6). The photosensitizer Photofrin, with its 630 nm absorbance, has an effective tissue penetration depth of 3-5 mm (Wang et al., 2005). As the wavelength for photosensitizer activation increases, so does the distance the light can travel, depending on the specific tissue type. Since BPD has a higher wavelength peak
absorption, the light in this case can reach about 50% farther than light used to activate Photofrin (Stables and Ash, 1995).

**Figure 6. Photodynamic therapy: BPD characteristics.** Left: the chemical structure of the photosensitizer BPD. Right: absorbance curve for BPD at a concentration of 15 µM in DMSO. The red arrow shows the wavelength used for PDT (690 nm).

PDT leads to cell death by apoptosis, autophagy, and necrosis (Yoo and Ha, 2012), depending on the photosensitizer type and fluence dose. BPD uptake and ROS generation is localized in mitochondria, and preferential damage to anti-apoptotic mitochondrial membrane proteins (Bcl-2 family) leads to cytochrome c release and apoptotic pathway induction (Figure 7) (Almeida et al., 2004; Spring et al., 2015). The preferential accumulation of BPD in mitochondria is particularly advantageous because the direct release of cytochrome c after mitochondrial PDT damage bypasses tumor anti-apoptotic signaling mechanisms (Kessel and Luo, 1999).
While multi-chemotherapy drug resistance is common, determinants of PDT efficacy are unrelated to photosensitizer resistance, since resistance to one photosensitizer does not correlate with resistance to others, so if one photosensitizer is ineffective then another is likely to work (Casas et al., 2011; Spring et al., 2015). This is likely because of the varied localizations of PS and ROS generations that can lead to cell death in multiple ways. Since cancer cells are already under oxidative stress, a strategy like PDT that dramatically increases ROS can overload ROS-resistance mechanisms, leading to rapid cell death (Casas et al., 2011). PDT can also directly target proteins in the Bcl-2 family,
thus preventing Bel-2 inhibition of Bad/Bax-mediated release of cytochrome c and caspase-9 activation (Figure 7) (Agarwal et al., 1991; Spring et al., 2015). ABC transporters such as ABCG2 recognize many photosensitizers, including BPD, so PDT may induce transporter photodamage that causes cells to retain more photosensitizer (Goler-Baron and Assaraf, 2012; Ishikawa et al., 2013; Spring et al., 2015). Overexpression of these transporters increases chemoresistance, but this is not necessarily the case for PDT resistance because of the potential for ABCG2 photodamage after photosensitizer light activation.

Additionally, PDT allows for better selectivity of treatment location than standard chemotherapy infusions, since PS localization and light activation provide two modes of selection. This can enable very high doses of PDT that do not damage a large area outside of the tumor, unlike high chemotherapy doses. PDT does little damage to collagen and elastin, thus preserving organ structure (Barr et al., 1987). So if a tumor is unresponsive to apoptotic signaling, cell death can still occur through necrosis or autophagy and higher PDT doses, or PS targeting to tumor cell membranes can also increase the likelihood of necrotic death over apoptotic death (Yoo and Ha, 2012). PDT leads to a faster and more direct apoptotic response than chemotherapy treatment, since it doesn’t depend on proliferation and DNA replication for its efficacy, and thus there are fewer chances for anti-apoptotic mechanisms to prevent cancer cell death.

Overall, pancreatic cancer has one of the worst prognoses and fewest effective treatment options. Even if a chemotherapy drug is able to target most epithelial tumor populations in pancreatic cancers, additional treatment strategies are needed to overcome
the mesenchymal and chemoresistant subpopulations associated with PDAC’s dismal survival rate. Motivated by this, I have explored using PDT as a treatment for chemoresistant and mesenchymal pancreatic cancer cells.
CHAPTER 2
MATERIALS AND METHODS

Cell Culture

PANC1, BxPC3, HPAF-II, and MRC-5 pancreatic cancer and fibroblast cell lines were grown in T-75 cell culture flasks based on ATCC guidelines with media changes 2-3 times per week. These cell lines were selected to represent a range of PDAC mutations and behaviors, with PANC1 derived from a poorly differentiated tumor, BxPC3 more moderately differentiated, and HPAF-II well-differentiated (Table 1). Additionally, based on analysis of gene expression in PDAC cell lines, PANC1 cells are classified as quasi-mesenchymal, while HPAF-II cells are described as a classical subtype expressing more epithelial genes (Collisson et al., 2011).

DMEM, RPMI, and MEM (HyClone; Logan, UT) were all supplemented with 10% heat-inactivated fetal bovine serum (HyClone; Logan, UT), 100 IU/mL penicillin and 1% streptomycin (HyClone; Logan, UT), and 0.5 µg/mL Amphotericin B (Corning; Corning, NY). Cell lines were passaged for a maximum of 25 times before discarding flasks and thawing stocks of the same cryogenically stored cell lines with earlier passage numbers.
Table 1. Characterization of pancreatic cancer cell lines. (Deer et al., 2010; Sipos et al., 2003)

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Source</th>
<th>Grade</th>
<th>Differentiation</th>
<th>Commonly Reported Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>KRAS</td>
</tr>
<tr>
<td>BxPC3</td>
<td>Primary Tumor</td>
<td>G2</td>
<td>Moderate to poor</td>
<td>WT</td>
</tr>
<tr>
<td>HPAF-II</td>
<td>Ascites</td>
<td>G2</td>
<td>Well</td>
<td>12 Asp</td>
</tr>
<tr>
<td>PANC1</td>
<td>Primary Tumor</td>
<td>G3</td>
<td>Poor</td>
<td>12 Asp</td>
</tr>
</tbody>
</table>

3D Cell Culture (Matrigel)

Growth factor reduced (GFR) Matrigel (Corning; Corning, NY) was thawed overnight on ice in a 4°C refrigerator. 230 µL of GFR Matrigel was pipetted in each well of a chilled 24-well black-walled plate and incubated at 37°C for 30 minutes to allow the GFR Matrigel to polymerize. After polymerization, the Matrigel beds were overlaid with pancreatic cancer cell lines at a concentration of 7,500 cells/mL in the appropriate media supplemented with 2% Matrigel. This diluted Matrigel in the media provides ECM signaling for spheroid cells without ECM bed contact (Lee et al., 2007). Cultures were then incubated at 37°C for up to 12 days to allow for the formation of 3D tumor spheroids. Throughout this process, the growing tumor nodules were imaged with the Zeiss Axio Observer.Z1 microscope to observe the growth rate and phenotypic characteristics of 3D-cultured PDAC cells.

Non-adherent Spheroid Growth and Transplantation

To prepare attachment-free multicellular spheroids, single cell suspensions of 1,000 cells/mL were added to the surface of agarose menisci (1% w/v protein electrophoresis grade agarose, Fisher BioReagents, Waltham, MA) formed previously in
96 well plates by dispensing heated agarose (50 µL/well) and allowing to gel at room temperature for 30 minutes. After 24 hours, multicellular aggregates were supplemented with complete medium containing 2% GFR Matrigel. After 12 days (with regular media additions), spheroids were transplanted into chilled 24 well plates (ibidi USA, Inc.; Madison, WI) containing either Matrigel or 1 mg/mL bovine type I collagen (COL1, Corning; Corning, NY) maintained in solution phase until spheroid was incorporated. Matrigel layers were initially formed as above and COL1 was prepared in 10x MEM (Sigma-Aldrich; St. Louis, MO) and sterile water, adjusted to a neutral pH with NaOH (Fisher Chemical; Pittsburgh, PA). Matrigel of COL1 gels were then set by incubation overnight at 37°C, embedding the spheroids. Transplanted spheroids were allowed to grow and invade into respective ECM microenvironments (with 500 µl DMEM/well) for 3 days prior to PDT or chemotherapy treatments.

**Cell Counting**

Cells were initially plated in 24-well plates at 7,500 cells/ml, over Matrigel beds for 3D cultures. Counts were done every 2-3 days. For monolayer counting, growth media in 3 wells was aspirated and replaced with DPBS (-Ca/-Mg) (HyClone; Logan, UT) for 10 minutes. DPBS was then aspirated, and cells were incubated with 0.05% trypsin (Gibco; Gaithersburg, MD) for 5 minutes at 37°C. Each well was then washed with complete growth media and pooled into a 15 ml centrifuge tube, pipetted repeatedly into a single-cell suspension, then counted on a hemocytometer. For 3D cultures, growth media in 3 wells was gently aspirated and replaced with 600 µl dispase (Corning;
Corning, NY) for 2 hours at 37°C. Each well was washed with complete media and then pooled into a 15 ml tube for resuspension and counting on a hemocytometer. Earlier versions of this protocol included centrifugation to remove dispase and allow for trypsin use in 3D culture, but this resulted in an approximately 25% cell loss due to incomplete pellet formation.

**Riboflavin Crosslinking of Collagen 1**

High-concentration rat-tail collagen 1 (Corning; Corning, NY) was prepared in ice cold sterile water, 10x MEM (Sigma-Aldrich; St. Louis, MO), NaOH (Fisher Chemical; Pittsburgh, PA), and 0.1% riboflavin-5-monophosphate (TCI America; Portland, OR) (from a 5 mM stock solution in PBS) to a final concentration of 3.7 mg/mL COL1. 240 µL COL1 was added to each well of a chilled 24-well plate (ibidi USA, Inc.; Madison, WI), 3 at a time, for crosslinking. The 24 well plate was placed 2.5 cm above a 450 nm LED (ThorLabs) set to a power of 420 mW/cm², with times based on fluence dose (2 seconds for 0.5 J/cm² or 35 seconds for 15 J/cm²). PANC1 spheroids, grown as detailed above, were immediately mixed into the crosslinked COL1 before it began gelling.

**Generation of Chemoresistant Sublines**

For chemoresistance induction, oxaliplatin was chosen because of its role in the FOLFIRINOX cocktail, and gemcitabine was chosen because it is also used in metastatic PDAC treatment, as discussed in the introduction (Conroy et al., 2011). Increasing
concentrations of oxaliplatin and/or gemcitabine were added to each cell type in regular media, until they retained a stable proliferative phenotype after multiple passages without chemotherapy drugs (approximately 20 passages total). Drug resistance was determined with dose response curves, and cell lines were defined as chemo-resistant when the IC50 value had increased significantly.

**Induction of Epithelial-Mesenchymal Transition**

To induce EMT with TGFβ (Gibco, Thermo-Fisher Scientific; Frederick, MD), 10 ng/mL TGFβ in 1% FBS DMEM was added to each cell line for 48 hours. Media removed from in vitro culturing of fibroblast cells (fibroblast conditioned media: FCM) contains growth factors including TGFβ and HGF (Ding et al., 2013; Lebret et al., 2007) that can induce EMT in cancer cell lines. To induce EMT with fibroblast-conditioned media (FCM), media from MRC-5 cells (fibroblasts) was collected and filtered to 0.2 microns to remove any cells or debris present, mixed with 50% regular media, then added to specified pancreatic cancer cell lines for 48 hours.

**Treatment with Chemotherapy Drugs**

Oxaliplatin (chosen as a major drug in the FOLFIRINOX cocktail (Conroy et al., 2011); Selleck Chemical; Houston, TX) was added to the media for each cell type at doses ranging from 0.1 µM to 500 µM for 48 hours (monolayer) or 72 hours (3D), as noted in figure legends. For gemcitabine treatments (Tocris; Bristol, UK), doses ranged from 0.1 µM to 1000 µM for 72 hours.
Treatment with Photodynamic Therapy

A 250 nM concentration of the photosensitizer BPD (benzoporphyrin derivative monoacid ring A; Sigma-Aldrich; St. Louis, MO), used in the Phase I/II clinical trial for pancreatic cancer PDT (Huggett et al., 2014) was diluted from a 500 µM stock solution in DMSO, added media for each cell type, incubated for 1 hour (or 2.5 hours in embedded 3D models to offset uptake through the ECM), then replaced with regular media. A 690 nm laser (Intense; New Brunswick, NJ) was used to excite the photosensitizer, with laser shutter timing controlled by THORLABS APT software (Figure 8). Doses ranged from 0.5 J/cm² to 50 J/cm² at a 50 mW/cm² fluence rate in monolayer and 100 mW/cm² in 3D cultures, and cell viability was determined 24 hours after treatment.

Figure 8. PDT apparatus. (1) THORLABS APT software to regulate the laser shutter. (2) Diode laser source with adjustable current/temperature. (3) Power/energy meter. (4) Manual solenoid shutter controller. (5) 690 nm laser with movable shutter. (6) Permeable plastic base for 24 or 96 well plates.
Viability Evaluation after Treatment

For monolayer treatments, viability was assessed via the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) at 490 nm absorbance in the BioTek® Epoch Microplate Spectrophotometer, according to Promega instructions. For 3D culture treatments, a customized MATLAB script (qVISTA), described by Celli et al. (2014), was used to determine nodule size and eccentricity over time, as well as the number of live and dead cells after treatment. Live signal was based on calcein AM fluorescence and dead signal was based on ethidium bromide fluorescence. Results of these colorimetric viability assays were validated through clonogenic survival assays, in specified experiments, following methods described in Franken et al. (2006).

Apoptosis and Necrosis Staining

To determine the ratio of necrotic and apoptotic cells after PDT, three live cell stains were used. FITC-labeled Annexin V detects apoptotic cells through binding to phosphatidylserine on the plasma membrane after loss of phospholipid asymmetry at early apoptotic stages, and cannot bind to normal living cells since it is unable to fully penetrate the phospholipid bilayer (Balaji et al., 2013). Since Annexin V can also bind to necrotic or dead cells, another stain is necessary to determine plasma membrane integrity, so propidium iodide was used to identify nuclei in necrotic cells (Bossy-Wetzel and Green, 2000). Additionally, Hoechst 33342 was used to label nuclei in all cells (live and dead) present in each sample (Atale et al., 2014).
After treatment, cells in monolayer or 3D cultures were washed once with 1x Annexin V binding buffer (Annexin V-FITC Fluorescence Microscopy Kit, BD Biosciences; San Jose, CA) after dilution from the 10x stock in PBS, then incubated for 30 minutes with Hoechst (33342, Trihydrochloride, Trihydrate, ThermoFisher Scientific; Waltham, MA), propidium iodide (BD Biosciences; San Jose, CA), and Annexin V-FITC (BD Biosciences; San Jose, CA) diluted in 1x Annexin V binding buffer. This was replaced with 1x Annexin V binding buffer before imaging using the same exposure times for all treatment groups and cell types on an automated Zeiss AxioObserver Z1 microscope. Unedited images were analyzed using a custom MATLAB script comparing the number of propidium iodide and Annexin V stained objects associated with nuclei.

**Immunofluorescence**

Cells in optical-bottom multiwell plates were fixed with 4% formalin, incubated with 0.1% triton X-100 for 20 minutes, and washed three times with 0.1 M glycine. Monolayer cultures were refrigerated overnight (and 3D cultures at room temperature to prevent Matrigel liquefying) with primary antibodies against E-cadherin and vimentin (Cell Signaling EMT Duplex; Danvers, MA), Bcl-xL, or phalloidin (ThermoFisher Scientific Molecular Probes; Waltham, MA) as an F-actin stain. After washing with PBS, cells were incubated for 1 hour with mouse or rabbit Alexa Fluor secondary antibodies (Cell Signaling; Danvers, MA). Cells were mounted with ProLong Gold Antifade reagent with DAPI (ThermoFisher Scientific Molecular Probes; Waltham, MA) and imaged after 24 hours using the same exposure times for all treatment groups and cell types on an
automated Zeiss AxioObserver Z1 or LSM 880 confocal laser scanning microscope. Images were optimized for display in figures using the ImageJ Hi-Lo lookup table or ZEN software for 3D reconstructions. Unedited images were analyzed using fluorescent signal intensity for each protein normalized to the number of cells based on DAPI-stained nuclei.

**Western Blotting**

Cells grown on 60 mm plates were washed with ice cold PBS then lysed with cold RIPA (ThermoFisher Scientific; Waltham, MA) containing 1x Halt™ protease and phosphatase inhibitors (ThermoFisher Scientific; Waltham, MA). Collected lysates were centrifuged at 14000 g for 10 minutes, and aliquots were stored at -80°C until use. Protein concentration was quantified using the Pierce BCA protein assay (Thermo-Fisher Scientific; Waltham, MA). After SDS-PAGE separation, transfer to nitrocellulose, and blocking, membranes were incubated at 4°C overnight with antibodies against E-cadherin (BD Biosciences; San Jose, CA), vimentin (Sigma-Aldrich; St. Louis, MO), or Bcl-xL, with GAPDH as a loading control (Cell Signaling; Danvers, MA). After washing, membranes were incubated with HRP-linked rabbit or mouse secondary antibodies (Cell Signaling; Danvers, MA) for one hour. Chemiluminescent HRP substrate (Immobilon® Western HRP Substrate; EMD Millipore; Billerica, MA) was added prior to imaging with the C-DiGit® Blot Scanner (LI-COR; Lincoln, NE). Band density for each protein was normalized to the GAPDH loading control.
**Bulk Oscillatory Shear Rheology**

Figure 9A shows equations for the behavior of a purely elastic material (Hookean solid) where an applied shear stress produces a proportional shear strain and a purely viscous material (Newtonian fluid) where an applied shear stress produces a strain proportional to the rate of shearing. Oscillatory shear rheology can be used to characterize the viscosity and elasticity of relatively soft materials like the ECM, allowing for studies determining the effects of these properties on tumor behavior (Janmey et al., 2007). To do this, ECM samples are placed on a rheometer and a certain strain is applied, after which computer software is able to measure stress and calculate viscoelastic $G'$ and $G''$ storage and loss moduli (Figure 9B).

### Table: Elasticity and Viscosity

<table>
<thead>
<tr>
<th>(1) Hookean solid:</th>
<th>$\sigma = G'Y$</th>
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<tbody>
<tr>
<td>$\sigma$ = shear stress</td>
<td>$G$ = elastic shear modulus</td>
</tr>
<tr>
<td>(2) Newtonian fluid:</td>
<td>$\sigma = \eta \frac{dy}{dt}$</td>
</tr>
<tr>
<td>$\gamma$ = strain</td>
<td>$\eta$ = viscosity</td>
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### Figure 9. Rheology.

(A) Equations describing mechanical properties of ideal elastic or viscous materials. (B) Bulk oscillatory shear rheology equations for calculating viscoelastic material properties. (C) Particle-tracking microrheology equations for calculating viscoelastic material properties and comparing with bulk rheology results.
For mechanical characterization of ECM materials, 300 µL to 400 µL of ice cold ECM preparations identical to those used in cell cultures were pipetted onto the lower peltier plate (held to 4°C prior to contact with ECM) of a TA Instruments rheometer, Discovery HR-2 (New Castle, DE). While still in solution phase, a 40 mm parallel plate geometry was brought into contact with the ECM while examining the sample spreading to achieve optimal filling of the gap. Peltier plate temperature was then regulated to 37°C. After 45 minutes for gelation and equilibration, rheology measurements were performed. An initial oscillatory strain was conducted at low strain values to ensure linear response (both components of $G^*(\omega)$ complex modulus independent of applied strain). An appropriate strain value in the linear regime was selected for subsequent dynamic oscillatory shear measurements over a range of $1 < \omega < 100$ rad/s.

**Particle-Tracking Microrheology (PTMR)**

PTMR methods established by Mason and Weitz (Mason et al., 1997; Mason and Weitz, 1995) can be used to quantitatively monitor matrix degradation as a measure of invasiveness. In this process, Brownian motion of fluorescent tracer probes embedded in ECM products is used to determine the complex viscoelastic shear modulus, $G^*(\omega)$, at micron length scales using previously described methods (Jones et al., 2014) (Figure 9C). First, non-adherent spheroids are formed on 1% agarose beds, and then transferred into a 3D collagen or Matrigel matrix, prepared as described above, but containing embedded yellow-green fluorescent tracer probes (ThermoFisher Scientific Molecular Probes FluoSpheres; Waltham, MA). Video data (sequences of 800 frames) were obtained using
a Zeiss AxioCam HRM camera mounted on a Zeiss AxioObserver Z1 microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) at several points within the sample well, both adjacent to and far away from the tumor spheroid or nodules.

The Brownian motion of the probes in each video can be analyzed using the Generalized Stokes Einstein Relation (GSER) to estimate local viscoelastic response, $G^{*}(\omega)$, of the material through customized MATLAB routines adapted from open source code of Maria Kilfoil to determine the local rheological properties of the matrix at each sample point (see Figure 8C for microrheology equations). Changes in ECM stiffness are reported here as difference in the shear modulus (using the real component, $G'(\omega)$ of the complex modulus, at $\omega = 10$ rad/s) obtained from averages of all probe trajectories in all replicates between the noted comparative culture conditions after monitoring for 3 days.

**Invasion Assays**

Methods used were as described in Hall and Brooks (2014). 50 µL of GFR Matrigel, diluted by half in PBS, was added to the upper wells of a Corning 24-well transwell plate with an 8 µm pore size membrane (Corning, NY) and allowed to gel for an hour at 37°C. Serum starved cells (10,000 cells/mL) were added to the transwell over Matrigel beds, and FBS (10% in DMEM) was used as a chemoattractant in the lower wells. After 24 hours, the Matrigel and non-invaded cells were cleaned off the membrane, and invaded cells were fixed in 4% formalin for 20 minutes, then stained with 1% crystal violet (ThermoFisher Scientific; Waltham, MA) and counted on a light microscope.
Photosensitizer Uptake Measurements

Cells in 35mm dishes were incubated with 1 µM BPD (benzoporphyrin derivative monoacid ring A/verteporfin) for two hours. Following incubation, cells were washed with PBS twice and lysed using cold RIPA. BPD concentration in lysates was quantified with an Epoch fluorescent plate reader at 436 nm and 690 nm (nM BPD/mg total protein). Resulting BPD concentrations were normalized to protein concentrations in the lysates, determined using the Pierce BCA protein assay. Additionally, cells grown in Matrigel overlay cultures for 12 days were incubated with 1 µM BPD in media for 2 hours. BPD-media was then replaced with normal complete media, and the photosensitizer fluorescence was imaged using a custom benzoporphyrin filter cube (Chroma Technology Corporation; Bellows Falls, VT) on a Zeiss Axio Observer.Z1 microscope. Unedited images were analyzed in ImageJ to show BPD signal intensity per total tumor area.

Statistical Analysis

Two-tailed Student's t test was used to analyze normally distributed data. Results were considered significant if p <0.05 (*), <0.01 (**), <0.005 (***), or <0.001 (****), and n.s. is not significant. Error bars indicate SEM in all figures. Figures show representative data of at least 3 independent experiments, unless stated otherwise in figure legends.
CHAPTER 3

ECM REGULATION OF PDAC INVASION AND THERAPEUTIC RESPONSE

*Sections published in Cramer et al. (2017), Molecular Cancer Research

Motivated by the background in Chapter 1 above, I specifically examine response to BPD-PDT in contrast to oxaliplatin chemotherapy in PDAC spheroids transplanted into laminin-rich Matrigel and collagen 1 ECM environments that have differing physical and biological composition. These conditions were shown in studies with breast cancer models to constrain (Matrigel) and promote (collagen 1) invasion (Nguyen-Ngoc et al., 2012). As shown in the experimental schema (Figure 10), the use of previously established high content imaging for 3D tumor models (Celli et al., 2014) allows for co-registering treatment response with phenotypic parameters to examine differential response in ECM invading and non-invading PDAC populations.
Figure 10. Experimental design: influence of ECM on PDAC behavior and treatment response. (1) Initial formation of attachment-free spheroids on agarose beds for 12 days. (2) Transplantation and embedding of spheroids in rheologically characterized Matrigel or COL1 ECMs. (3) Longitudinal and terminal (immunofluorescence) imaging of growth and ECM invasion. (4) Treatment with chemotherapy (oxaliplatin) or PDT. (5) Imaging-based assessment of therapeutic response, co-registered with phenotype.

ECM Rheology and Regulation of PDAC Growth Behavior

Prior to treatment studies, I first characterized the growth and invasive behavior of PDAC 3D cultures with respect to ECM conditions with contrasting biological and biophysical properties (Figures 10 and 11). We selected reconstituted ECM materials modeling PDAC stroma rich in type I collagen (COL1), shown in previous reports to promote invasive behavior (Armstrong et al., 2004; Nguyen-Ngoc et al., 2012; Shields et al., 2011), contrasted with laminin-rich basement membrane (GFR Matrigel). PANC1 spheroids were initially grown in attachment-free conditions (agarose beds) for 12 days prior to transplantation into either ECM condition (Figures 10 and 11).
Figure 11. ECM composition and rigidity regulate PDAC tumor growth and invasive behavior. (A) Representative darkfield snapshots of PANC1 spheroids, one day following transplantation into COL1 or Matrigel ECM, showing extensive invasion into ECM in the former. (B) Analysis of ECM invasion with respect to radial distance from spheroid edge, after three days in each ECM (COL1: n=5; Matrigel: n=10). (C) Terminally fixed and stained COL1 and Matrigel PANC1 spheroids showing DAPI-stained nuclei and phalloidin-labeled F-actin. (D) For COL1 ECM, a representative confocal IF image showing increased mesenchymal markers in ECM infiltrating cells (lower right inset, increased vimentin and decreased E-cadherin) relative to inner spheroid populations with clear adherens junctions (upper right inset). (E) Bulk oscillatory shear rheology shows $G'$ (elastic/storage) and $G''$ (viscous/loss) moduli for both ECM materials used. Matrigel is a significantly stronger gel than the soft reconstituted COL1 used here, also likely contributing to increased motility in the latter [D. Jones Data].
After transplanting, growth behavior in each condition was monitored non-destructively via darkfield microscopy prior to terminal immunofluorescence analysis. For spheroids transplanted into COL1, within 24 hours the outer cells of the spheroid become more invasive and migrate into the ECM, while spheroids embedded in laminin-rich Matrigel do not exhibit significant invasive behavior (Figure 11A) though a budding pattern is consistently observed on spheroid surfaces. Darkfield image data was batch processed based using methods previously described (Celli et al., 2010) (see MATLAB code in Appendix B) to obtain relative size and position of invading populations and quantify overall extent of invasion by ECM microenvironment. After 3 days, both the total number of invading cells and invasion distance is significantly higher in COL1 (Figure 11B, red bars).

Spheroids fixed and stained after 3 days of growth in ECM show extensive F-actin staining (Figure 11C), increased vimentin, and loss of E-cadherin in invading cells (Figure 11D) in COL1, consistent with a more mesenchymal phenotype in highly motile ECM infiltrating cells. Conversely, cells in centers (Figure 11D, top right) exhibit markedly stronger honeycomb pattern E-cadherin staining characteristic of adherens junctions and epithelial phenotype. Figure 12 shows an additional example comparing EMT immunofluorescence staining for PANC1 spheroids in Matrigel or COL1, where those in Matrigel have a thin ring of vimentin around the outer edge and limited invasion into the ECM, and those in COL1 exhibit much more extensive invasion and vimentin staining. The invasive phenotype of PDAC cells observed here in COL1 ECM is consistent with previous reports showing increased EMT and invasion of both PDAC and
breast cancer cells in collagen-rich microenvironments (Nguyen-Ngoc et al., 2012; Shields et al., 2011; Shintani et al., 2006).

**Figure 12. Collagen 1 promotes PDAC invasion and EMT.** Left: PANC1 spheroid embedded in Matrigel for 3 days, stained with DAPI (nuclei, blue) and EMT markers vimentin (red) and E-cadherin (green). Right: PANC1 spheroid embedded in COL1 for 3 days, showing more extensive invasion, with invasive cells staining strongly for vimentin. Scale bars are 200 µm.

The differences in PDAC motility in Matrigel and COL1 may be partly attributable to the contrasting mechanical properties of these materials. Identical preparations of both hydrogels as used for 3D cultures were characterized using bulk oscillatory shear rheology (Figure 11E). Both form viscoelastic gels with $G'$ (storage modulus) dominant over $G''$ (loss modulus). Though these are both soft gels, Matrigel is significantly stiffer with a $G'$ of approximately 90 Pa, within the range of previous reports (Paszek et al., 2005; Semler et al., 2000; Zaman et al., 2006) and roughly 20 times higher than that of the soft (1 mg/mL) COL1 hydrogel used here, which likely creates a more permissive environment for motility and invasion.
These results appear to be in contrast with other mechanobiology literature, where more invasion is seen in a stiffer ECM (Levental et al., 2009; Lu et al., 2012; Paszek et al., 2005). Here, however, cells are less invasive in the softer collagen ECM. This is likely because collagen 1 happens to activate invasive behavior independent of its rheological properties, and the Matrigel ECM, though somewhat stiffer, does not. Relative contributions of the biochemical and physical aspects of the ECM on invasion are discussed in more detail in Chapter 6, particularly in the context of collagen crosslinking.

**ECM Regulation of Treatment Response**

In the conditions established above, I examined differential response to PDT and chemotherapy in invading populations and the primary spheroid (Figure 13). I used oxaliplatin, a component of the multi-drug cocktail FOLFIRINOX, which has shown increased effectiveness over the standard gemcitabine treatment for PDAC (Conroy et al., 2011), for chemotherapy treatment. After spheroid transplantation into COL1 or Matrigel, cultures were treated with oxaliplatin chemotherapy or verteporfin PDT using the equivalent monolayer LD90 dose for each modality (Figure 13B, arrow). As shown in Figure 13 (A and C), oxaliplatin chemotherapy inhibits the growth of the primary spheroid to a greater extent than the equivalent PDT dose (see Appendix C for MATLAB code used to analyze viability in these experiments).

However, populations of invading cells (zoomed in regions in Figure 13A and graph in Figure 13D) exhibit the reverse trend, with no significant response to oxaliplatin
even at 500 µM but significantly higher sensitivity to PDT. The zoomed in regions of invading populations after oxaliplatin and PDT (Figure 13A, right panels) show large numbers of dead cells (red dots) following PDT, but little evidence of cell death following the chemotherapy treatment. These images were processed to quantitatively report the fraction of surviving ECM-infiltrating cells, outside the segmented primary nodule volume, for each treatment condition (Figure 13D). Furthermore, when viability of these ECM-invaders is plotted against invasion distance (Figure 13E), PDT is most effective on the leading cells with highest invasive velocity that have progressed more than 200 µm from the spheroid edge (Figure 13F).
Figure 13. ECM invading populations exhibit chemoresistance but enhanced sensitivity to PDT. (A) Representative PANC1 spheroids treated with either chemotherapy or PDT doses notes and stained with calcein (green) and ethidium bromide (red), showing viability of core spheroid cells and ECM infiltrating cells. Scale bars are 200 µm. (B) Dose response of PANC1 to oxaliplatin or PDT in monolayer used to inform dose selection for each therapy (shown above). (C) Dose response (normalized residual volume from image segmentation) for primary spheroids shows modest growth inhibition of primary nodule by oxaliplatin, but n.s. for PDT. (D) Dose response for invading cells (analysis applicable for COL1 only) shows no response to oxaliplatin, approximately 50% killing from PDT at 25 J/cm² (P <0.01). (E) Further analysis of individual invading cell viability with respect to radial distance from COL1 spheroid edge showing clear separation of PDT and chemotherapy response. (F) Breakdown of response in leading (d > 200 µm) and lagging invaders for chemotherapy and PDT shows further enhancement in PDT response for leading cells.
Mechanism of PDT-Induced Cell Death in 3D Culture

As a photosensitizer that localizes preferentially in mitochondria, BPD is expected to cause cancer cell death mainly through apoptosis. At higher PDT doses, however, necrotic cell death becomes more likely. Characteristics of necrosis are cytoplasmic swelling, plasma membrane disruptions, and ultimately rapid fragmentation of all cellular components. According to Plaetzer et al. (2002), high PDT doses can rapidly damage cellular components needed for maintaining enough ATP to carry out apoptosis.

Figure 14 shows PANC1 spheroids that have invaded into 1 mg/mL COL1 for 3 days. An untreated spheroid is shown at left, with most cells stained with Hoechst and still viable, except for the expected necrotic core. Three hours after PDT at 25 J/cm², a timepoint for determining early apoptotic cell death, the majority of cells invading into the COL1 are stained with annexin V (green), a sign of apoptosis, while those around the edge of the spheroid are stained with propidium iodide (red), a sign of necrotic death (Bossy-Wetzel and Green, 2000). At 24 hours post-PDT, most invading cells have died or are in the late stages of apoptosis, since propidium iodide stain has passed through the membranes.
Figure 14. Mechanism of PDT-induced cell death in 3D culture conditions. PANC1 spheroids grown for 3 days in 1 mg/mL COL1, then treated with 25 J/cm² BPD-PDT. Staining with Hoechst (live cells), propidium iodide (necrotic cells), and annexin V (apoptotic cells) was completed after 3 hours (middle panel) and 24 hours (right panel). An untreated spheroid is shown at left. Scale bars are 250 µm.

The bystander effect may account for some of the necrotic PDT response in regions where cells are concentrated, like at the edges of spheroids. According to Dahle et al. (1997), in the bystander effect, cells adjacent to those killed by direct PDT also respond in a chain reaction, with a higher degree of effect for necrotic cell death than apoptotic. The bystander effect is also seen in radiation treatments, where it is shown to be mediated by a combination of damage through ROS diffusion and intracellular communication via gap junctions (Castano et al., 2005; Shao et al., 2003).
CHAPTER 4

PDT RESPONSE IS INDEPENDENT OF EMT AND CHEMORESISTANCE STATUS IN MONOLAYER

*Sections published in Cramer et al. (2017), Molecular Cancer Research

PDAC cells invading into COL1 display a mesenchymal phenotype and reduced response to oxaliplatin chemotherapy, but a surprisingly enhanced PDT efficacy (Chapter 3, Figures 11-13). Therefore, I have approached this outcome from an alternative direction by directly producing PDAC cell lines with mesenchymal or chemoresistant phenotypes to determine if this also augments PDT response (Figure 15).

Figure 15. Experimental design: generation of mesenchymal or chemoresistant PDAC. Workflow for experiments with EMT induction or chemoresistant sublines (Figures 15 through 19). (1) Induce EMT with TGFβ or FCM, or produce chemoresistant PDAC sublines. (2) Characterize EMT status using quantitative immunofluorescence and western blotting. (3) Treat cell lines with BPD-PDT or oxaliplatin chemotherapy. (4) Evaluate treatment response via colorimetric MTS assays.
**TGFβ Induction of EMT**

Since TGFβ can induce EMT in pancreatic cancer cells, as described in Chapter 1, I used 10 ng/mL TGFβ to verify that TGFβ can induce EMT in serum-starved PANC1 cells. As shown in Figure 16A, activation of TGFβ in serum-starved PANC1 results in increased vimentin and decreased E-cadherin consistent with EMT and previous reports (Ellenrieder et al., 2001; Fuxe et al., 2010). PANC1 cells can be classified as a quasimesenchymal PDAC line (Collisson et al., 2011), so are expected to have much higher levels of mesenchymal gene expression compared to epithelial gene expression. The increase in vimentin and decrease in E-cadherin after PANC1 TGFβ incubation supports TGFβ-induced EMT induction for this pancreatic cancer cell line.

Having established EMT characteristics in TGFβ-treated pancreatic cancer cell lines, I determined if TGFβ EMT induction leads to resistance to chemotherapy in pancreatic cancer cells. To do this, I treated PANC1 cells with the chemotherapy drug oxaliplatin (a main component of the pancreatic cancer drug cocktail FOLFIRINOX). After a 48-hour TGFβ incubation, I treated PANC1 cells with oxaliplatin doses ranging from 0.1 µM to 500 µM for 48 hours. As expected, after oxaliplatin treatment, MTS evaluation of response shows that TGFβ-treated PANC1 cells remain significantly more viable at multiple oxaliplatin doses (Figure 16B). However, PDT response for PANC1 induced with TGFβ respond similarly to non EMT-induced PANC1 (Figure 16D). The same trend is evident in clonogenic survival assays (Figures 16C and 16E), which also show a significant enhancement in viability for PANC1 cells treated with TGFβ, but no
difference in PDT response. Some example phase-contrast images of PANC1 (+/- TGFβ) response to PDT are shown in Figure 17. The difference in phenotypes after TGFβ treatment can be seen clearly in the untreated cells (left panels), and some TGFβ-treated PANC1 still show an elongated morphology characteristic of EMT after the 2.5 J/cm² PDT dose (middle panels). At a 5 J/cm² PDT dose, a large amount of cell death is apparent in both cases (right panels), as quantified in Figure 16.
Figure 16. TGFβ-induced EMT decreases chemotherapy response but not PDT response. (A) 40x immunofluorescence images of PANC1 and PANC1 with 10 ng/mL TGFβ stained for vimentin (red), E-cadherin (green), and nuclei (blue). Scale bar is 50 µm. Quantification of immunofluorescence signal for E-cadherin (left) and vimentin (right) in PANC1 versus PANC1 with TGFβ is shown at far right. Fluorescence intensity is normalized to the number of nuclei (DAPI signal). (B) Surviving fraction of PANC1 and PANC1 + TGFβ after 48 hours of oxaliplatin treatment. (C) Clonogenic survival assay for PANC1 and PANC1 + TGFβ in the same oxaliplatin conditions. (D) Surviving fraction of PANC1 and PANC1 + TGFβ 24 hours after PDT. (E) Clonogenic survival assay for PANC1 and PANC1 + TGFβ after PDT.
Figure 17. TGFβ induced EMT does not affect response to PDT. Phase-contrast images of PANC1 cells in monolayer untreated (left), treated with 2.5 J/cm² BPD-PDT (middle), and 5 J/cm² BPD-PDT (right) 24 hours after treatment. Bottom panels show the same results for TGFβ treated PANC1 cells. In both cases, PDT causes increased stress and cell death as the dose increases. Scale bars are 50 µm.

Fibroblast Conditioned Media (FCM) Induction of EMT

Similar experiments were carried out using BxPC3 cells, which are SMAD4 deficient and do not have an activating KRAS mutation, both required for induction of EMT via TGFβ signaling (Ellenrieder et al., 2001; Subramanian et al., 2004). In addition to TGFβ signaling, EMT is also known to be driven by tumor-stroma crosstalk. As discussed above, communication between cancer cells and cancer associated fibroblasts, through HGF, hedgehog, and other signaling pathways, can lead to mesenchymal and invasive characteristics in cancer cells. Lebret et al. (2007) successfully induced EMT in a breast cancer model through the addition of conditioned media taken from fibroblast cells (fibroblast conditioned media—FCM).
Here, BxPC3 cells exposed to FCM collected from MRC5 cells (a fibroblast cell line) reveal increases in mesenchymal characteristics similar to those observed in TGFβ-treated PANC1, though without measurable loss of E-cadherin (Figure 18). Quantitative immunofluorescence for EMT markers BxPC-3 and BxPC-3 + FCM cells shows a significant increase in vimentin (Figure 18A). Immunofluorescence analysis did not show a significant difference in E-cadherin expression (Figure 18A). Similarly to PANC1 + TGFβ, BxPC3 + FCM show oxaliplatin resistance over a range of doses (Figure 16B and Figure 17B) but no difference in PDT response (Figure 16D and Figure 18C).
Figure 18. FCM-induced EMT in BxPC3 decreases chemotherapy response but not PDT response. (A) 40x immunofluorescence images of BxPC3 (left) and BxPC3 + FCM (right). BxPC3 + FCM show characteristic increased scattering and loss of adherens junctions in EMT-inducing conditions. Scale bar is 50 µm. Far right: Quantification of immunofluorescence signal for E-cadherin and vimentin in BxPC3 versus BxPC3 + FCM cell lines. Fluorescence intensity is normalized to the number of nuclei (DAPI signal). P-values are less than 0.05 for vimentin, but not significant for E-cadherin. (B) Comparison of the fraction of viable cells for a 48-hour oxaliplatin treatment after a 48-hour FCM incubation. (C) Comparison of BxPC3 and BxPC3 + FCM survival after PDT treatment.

Additionally, PANC1+ FCM increases EMT marker expression and causes similar effects on oxaliplatin and PDT treatment response (Figure 19). Following the same protocol to induce EMT in this PDAC cell line, I added conditioned media from MRC5 cells to PANC1 cells, which also appears to induce EMT as it did for BxPC3 cells in Figure 18. Quantitative immunofluorescence for EMT markers in PANC1 and PANC1
+ FCM cells shows a significant increase in vimentin for those treated with FCM (Figure 19A). Immunofluorescence analysis did not show a significant difference in E-cadherin expression, however.

Since FCM, in addition to TGFβ, promotes EMT characteristics in pancreatic cancer cell lines (BxPC3 and PANC1), I validated that this EMT induction method similarly increased chemotherapy drug resistance. PANC1 cells grown in FCM show a decreased response to oxaliplatin treatment. For doses of 10 µM and 500 µM oxaliplatin, PANC1 cells with FCM retain significantly more viability after 48 hours of treatment (Figure 19B). When I grew PANC1 cells in FCM and treated them with PDT, the viability after treatment is not significantly different from PANC1 cells grown in regular DMEM (Figure 19C), a response similar to that seen in BxPC3 cells (Figure 18).
Figure 19. FCM-induced EMT in PANC1 decreases chemotherapy response but not PDT response. (A) EMT duplex IF for PANC1 and PANC1 + FCM shows increased vimentin after FCM treatment. (B) Oxaliplatin treatment response shows significantly increased viability in PANC1 + FCM (left) but no difference in PDT response (right).

Treatment experiments using direct generation of chemoresistant cell lines or EMT induction (using TGFβ or FCM) are consistent with the expectation that EMT populations are resistant to chemotherapy, but phenotype in monolayer apparently does not significantly affect PDT response. These results validate previous studies where a mesenchymal phenotype correlates with chemoresistance in a variety of cancers (Shah et al., 2007), as well as my experiments with TGFβ in the previous section, and lend more support to the hypothesis that EMT status has little effect on PDT response in monolayer.
Generation of Chemoresistant PDAC Sublines

Motivated by previous reports showing acquisition of increased invasion and EMT in chemoresistant cells (Shah et al., 2007), I generated and characterized drug-resistant PDAC sublines for further study. PANC1 and BxPC3 cells were exposed to oxaliplatin in increasing concentration over consecutive passages to establish stable, resistant sublines, PANC1OR and BxPC3OR, respectively (Figure 21). Generation of oxaliplatin resistance in PANC1 led to a decreased doubling time of 2.1 days versus 1.7 days in parent cells (Figure 20), also likely protective from classical chemotherapy drugs targeting replication.

Figure 20. Generating PANC1 oxaliplatin resistance decreases growth rate. (A) PANC1 cells grown in monolayer in a 24 well plate and periodically counted in triplicate have a doubling time of 1.7 days. (B) PANC1OR cells in identical conditions have a doubling time of 2.1 days.

As shown in Figure 21 (A, B, and C), PANC1OR cells express significantly more vimentin and significantly less E-cadherin based on quantitative immunofluorescence
verified by western blot, as well as the low cell-cell contact and spindle-shaped morphology characteristic of a mesenchymal phenotype. In additional experiments, a PANC1 subline resistant to both oxaliplatin and gemcitabine (PANC1ORGR) has similar EMT marker expression (Appendix A, Figure 43). The oxaliplatin-resistant BxPC3 line, BxPC3OR, also displays a partial increase in mesenchymal characteristics (Figure 21, A and B), where vimentin is increased but E-cadherin expression does not change significantly. While the total amount of E-cadherin remains the same after BxPC3 cells acquire chemoresistance, its cellular location seems different—borders of BxPC3 cells are clearly defined by E-cadherin expression, but its intensity is more dispersed in BxPC3OR cells (Figure 21A).
Figure 21. EMT characterization for oxaliplatin-resistant PDAC cell lines. (A) 40x immunofluorescence images of PANC1 and PANC1OR (top) and BxPC3 and BxPC3OR (bottom). (B) Quantification of immunofluorescence signal for E-cadherin (left) and vimentin (right) in PANC1 versus PANC1OR (top) and BxPC3 versus BxPC3OR (bottom) cell lines. Fluorescence intensity is normalized to the number of nuclei (blue signal). (C) Left: western blots of PANC1 and PANC1OR for vimentin, E-cadherin, and GAPDH. Right: quantification of westerns shown on left, with band density normalized to GAPDH.

Having determined that chemoresistant pancreatic cancer sublines have a mesenchymal phenotype, I designed experiments to validate that they are indeed significantly chemoresistant but not PDT-resistant. When I treated PANC1 and PANC1OR cell with various oxaliplatin doses for 48 hours, PANC1OR cells showed significantly increased viability at most doses (Figure 22A). Oxaliplatin resistant PANC1
cells (PANC1OR) have an IC50 for oxaliplatin of about 120 µM for a 48-hour treatment, while PANC1 cells have an IC50 of about 70 µM. Indeed, PANC1OR cells exhibit no oxaliplatin response until doses above 50 µM. BxPC3OR cells also respond significantly less to oxaliplatin treatment than BxPC3 (Figure 22C).

However, measurements of viability for each cell line are not significantly different 24 hours after a variety of PDT doses, except 10 J/cm² in BxPC3 cells (Figure 22, B and D). Overall, both methods of EMT induction (using TGFβ or FCM) as well as direct generation of chemoresistant pancreatic cancer sublines, validate that mesenchymal PDAC populations are resistant to chemotherapy drugs, but EMT status has little to no effect on PDT response. In monolayer, a mesenchymal phenotype alone is not sufficient to produce the PDT enhancement observed in ECM-invading spheroids in Chapter 3.
Figure 22. Comparison of oxaliplatin and PDT response in oxaliplatin-resistant PDAC cell lines. (A) Comparison of 48 hour oxaliplatin treatment in PANC1 and oxaliplatin-resistant PANC1 cells (PANC1OR). (B) Comparison of PANC1 and PANC1OR survival 24 hours after PDT treatment. (C) Comparison of 48 hour oxaliplatin treatment in BxPC3 and oxaliplatin-resistant BxPC3 cells (BxPC3OR). (D) Comparison of BxPC3 and BxPC3OR survival 24 hours after PDT treatment.
Mechanism of PDT-Induced Cell Death in Monolayer

In 3D cultures in Chapter 3, the majority of PDT-induced cell death at 25 J/cm$^2$ was apoptotic in ECM-invading cells compared to necrotic in cells near the spheroid edge (Figure 14). For monolayer cells, I compared PANC1 and PANC1OR PDT response at a range of PDT doses, similarly stained with Hoechst, propidium iodide, and Annexin V. At 3 hours post-PDT, the amount of necrosis (propidium iodide) versus apoptosis (Annexin) increases for both cell lines as the PDT dose increases (Figure 23). This result is in accordance with previous studies showing more necrosis at higher PDT doses (Kessel et al., 1995; Wyld et al., 2001). PANC1 cells, however, appear to die by a higher ratio of necrosis to apoptosis than PANC1OR cells, particularly at 10 J/cm$^2$. This may be because the drug-resistant cells have upregulated Bcl-xL expression, which PDT targets, causing apoptosis to occur more easily (Figure 31). Compared to the spheroid apoptosis versus necrosis images in Chapter 3, PANC1OR monolayer cells appear more similar in response to those that have invaded large distances along the ECM, and PANC1 monolayer cells are killed through a higher necrosis to apoptosis ratio more similar to those on the spheroid periphery.
Figure 23. Mechanism of PDT-induced cell death in monolayer. (A) PANC1 and PANC1OR monolayer cells treated with 10 J/cm² PDT and stained with Hoechst 33342 (blue), propidium iodide (red), and annexin V-FITC (green) 3 hours after PDT. Scale bars are 200 µm. (B) Quantified images showing the ratio of PI/Annexin V counts per nucleus at 3 hours after PDT doses ranging from 0 to 10 J/cm².

The mesenchymal populations of cancer cells shown in this chapter have expected chemoresistance, and alternatively, chemoresistant populations achieve a more mesenchymal phenotype. However, none of these monolayer experiments demonstrate the same profound enhancement of PDT response as shown with the presence of the ECM in Chapter 3. These results suggest that the observed increase in PDT efficacy in invasive populations requires interaction with ECM in a 3D environment.
CHAPTER 5
BEHAVIOR AND TREATMENT RESPONSE OF CHEMORESISTANT CELL LINES IN 3D

*Sections published in Cramer et al. (2017), Molecular Cancer Research

Having found phenotype-dependent treatment response related to ECM conditions, I further probed this in the inverse scenario, using genetically-matched PDAC cells with contrasting invasive potential, but placed in identical ECM microenvironments. Figure 24 shows a modified version of the experimental schema described earlier (Figure 15), with the addition of 3D models of chemoresistant cell growth and high-content image analysis similar to that used in Chapter 3.

All results reported in Chapter 4 are from monolayer in vitro experiments. As a way to better mimic potential in vivo response of PDT on mesenchymal and chemoresistant pancreatic cancer populations, I designed experiments using more sophisticated 3D tumor models. As discussed above in Chapter 1, 3D cultures exhibit different growth phenotypes and treatment response than monolayer cultures because of the increase in cell-cell and cell-ECM communication. Therefore, I analyzed differences in growth behavior and chemotherapy and PDT response in PANC1 and PANC1OR cells grown on GFR Matrigel beds in DMEM containing 2% Matrigel (Figure 24).
Figure 24. Experimental design: chemoresistant PDAC in 3D models. (1) Generate chemoresistant PDAC sublines. (2) Characterize EMT status using quantitative immunofluorescence and western blotting. (3) Treat cell lines with BPD-PDT or oxaliplatin chemotherapy in 3D and monolayer scenarios. (4) Evaluate treatment response via colorimetric MTS assays (monolayer) or high-content imaging analysis (3D).

3D Cultures of Chemoresistant PDAC Exhibit Increased Invasion

The 3D growth behaviors of parent and resistant sublines were evaluated using an established Matrigel overlay culture. Both cell lines form compact 3D nodules on the Matrigel beds, growing into a heterogeneous distribution of nodule sizes previously described as fitting a two-peaked Gaussian model (Celli et al., 2010) (Figure 25, day 6 through day 11). After this point, PANC1OR cells invade extensively into the surrounding ECM. Figure 25 shows a day 14 PANC1OR nodule (bottom right image), with cells moving off the edges and invading into the Matrigel, but PANC1 nodules at this time point have just continued to grow and merge, not invade into the ECM (bottom left image).
Figure 25. Growth of 3D PANC1 and chemoresistant PANC1 cultures. Images were taken over 14 days of growth. Images are 5x darkfield with 200 µm scale bars except PANC1OR day 14 is 10x with a 100 µm scale bar.

While both lines initially formed compact 3D nodules (Figure 25, day 6), at approximately day 12, PANC1OR cultures began exhibiting morphological changes and
invasion deep into ECM (Figures 25 and 26). Confocal imaging of DAPI/phalloidin stained PANC1 nodules displays a compact 3D structure and minimal invasion into Matrigel (dotted yellow line) (Figure 26A, upper panels). In contrast, PANC1OR cultures form large invasive protrusions, spreading over the surface and invading into the ECM bed (Figure 26A, lower panels) with a similar pattern of extensive invasion for the multidrug resistant 3D cultures (Appendix A, Figure 43). Additionally, immunofluorescence of EMT markers shows an increase in mesenchymal vimentin and a decrease in epithelial E-cadherin for PANC1OR 3D cultures, although IF processing methods disrupted some of the PANC1OR invasive phenotype (Figure 27). In support of this, an established transwell-insert invasion assay quantitatively shows enhanced invasion of PANC1OR through Matrigel ECM relative to PANC1 (Figure 26B).
Figure 26. 3D cultures of chemoresistant PDAC exhibit increased invasion. (A) DAPI/phalloidin-stained PANC1 and PANC1OR overlay cultures on Matrigel. Both lines initially form compact multicellular aggregates consistent with previous characterization, but resistant lines in 3D cultures spontaneously develop highly invasive phenotypes after 12 days in 3D culture (shown). The dotted yellow line indicates approximate Matrigel surface, showing invasion through the ECM bed by PANC1OR cultures. (B) Results of a transwell invasion assay characterizing increased invasive potential of PANC1OR through a Matrigel layer. (C) PTMR measurements show spatial variation in ECM compliance with lower stiffness in measurements obtained from upper focal planes (UFP) close to the 3D nodules on the surface, and stiffer ECM in lower focal planes (LFP). As expected, ECM degradation is more dramatic in drug resistant 3D cultures [D. Jones Data]. (D) Longitudinal analysis of PTMR measurements also shows increased rate of ECM degradation (decrease in rigidity) in the drug-resistant cultures, concomitant with remodeling to enable motility (P < 0.01) [D. Jones Data].
Chemoresistant PDAC Degrades the ECM: Particle-Tracking Microrheology

Particle-tracking microrheology (PTMR) can be used to monitor ECM degradation by 3D culture cell invasion, a process discussed in more detail in Chapter 2 above, and in Jones et al. (2014). During plating of 3D cultures, I embedded 1 μm fluorescent tracer probes into the Matrigel before allowing the gel to polymerize, and tracked the Brownian motion of the probes as the cultures grew and invaded. Figure 28
shows some example snapshots of the location of these probes in PANC1 or PANC1OR cultures. Interestingly, the distribution of these beads is visually different, with probes consistently packed around the PANC1 nodules and probes more dispersed in PANC1OR cultures (Figure 28A). The drug-resistant cultures have more heterogeneous growth behavior, however, and more tightly packed PTMR probes are present in nodules phenotypically similar to PANC1 (Figure 28A, arrow).

![Figure 28. Distribution of fluorescent probes used in PTMR.](image)

(A) 5x snapshots of fluorescent probes embedded in PANC1 (left) and PANC1OR (right) cultures. Scale bars are 200 µm. (B) 40x phase contrast (left) and fluorescent (right) images of the PANC1OR nodule indicated by the white arrow above.

In these conditions, a decrease in ECM rigidity is also observed concomitant with invasion. PTMR measurements show spatial variation in ECM compliance with lower stiffness measurements obtained from upper focal planes (UFP) close to 3D nodules on the surface, and stiffer ECM in lower focal planes (LFP) (Figure 26C). As expected,
ECM degradation is more dramatic in drug resistant 3D cultures. For PANC1OR, both the UFP and LFP measurements describe a softer ECM than the same regions in PANC1 cultures.

Additionally, analysis of tracer probe trajectories close to the interface of downward-progressing invaders shows an increase in thermally-driven movement, reporting a drop in stiffness ($G'(\omega)$) over a 24-hour period after 12 days of 3D growth in PANC1OR cultures compared to PANC1 (Figure 26D). While the laminin-rich basement membrane constrains invasion in the parent cell line, both in overlays and in the transplanted spheroids the chemoresistant subline with increased EMT is able to remodel ECM and enable 3D spreading and invasive motility, similar to what is observed in spheroids placed in soft COL1 (Figures 11, 12, 25-27).

**Chemotherapy and Photodynamic Therapy Response in 3D Cultures**

I then sought to determine whether differential response to PDT and chemotherapy in resistant and non-resistant lines in identical ECM would parallel results with the parent line in contrasting ECM environments shown in Chapter 3. Cancer cells grown in 3D with restored tumor-stroma interactions are less responsive to chemotherapy drugs than monolayer cultures (Celli et al., 2011). In addition to the biological effects of the ECM-tumor communication in decreasing treatment response, platinum agents like carboplatin or oxaliplatin poorly penetrate the center of large nodules (Alderden et al., 2007; Jung et al., 2012), likely more similar to in vivo treatment response. Therefore, it was important to evaluate PDT response in 3D models and validate monolayer results.
PANC1 and PANC1OR cultures were grown for 12 days (as described above) prior to intervention with oxaliplatin or BPD-PDT and terminal assessment via vital dye staining and quantitative treatment assessment (qVISTA) previously described (Celli et al., 2014). As expected, PANC1OR retain their resistance to oxaliplatin when grown in 3D cultures (Figure 29A). The volume of remaining nodules for PANC1OR did not decrease significantly, and remained significantly higher than PANC1 nodules at 50 μM oxaliplatin, while survival for PANC1 cells significantly decreased (Figure 29A).

However, PANC1OR cells exhibit significantly enhanced PDT response relative to PANC1 cells (Figure 29B, quantified graph; and Figure 29C, images). PANC1 cells show minimal PDT response in these 3D culture conditions, with about 90% viability, while PANC1OR cells here retain only about 40% survival at the same PDT dose (Figure 29B). Images of these treatments before qVISTA also make this differential response evident, since PANC1 images after PDT treatment have a thin ring of dead cells around nodules, but the more invasive PANC1OR images show mostly dead cells (Figure 29C). The red regions at the center of untreated nodules are necrotic cores, which are typical for large tumor nodules with limited oxygen and nutrient diffusion to the interior. Similarly, multidrug resistant PANC1ORGR cells exhibit enhanced PDT response relative to PANC1 cells when grown on Matrigel beds (Appendix A, Figure 41).

An additional example of PANC1 response to PDT is shown in Figure 30, where confocal slices of a nodule before treatment exhibit a necrotic core surrounded by live cells. After 25 J/cm² PDT, the nodule becomes disaggregated and is ringed by dead cells (Figure 30B), since BPD-PDT is most effective in the well-oxygenated tumor regions.
This is a common pattern in BPD-PDT—larger nodules (> 250 µm) of ovarian cancer also showed killing only on the nodule periphery (Jung et al., 2012).

It is not unexpected that chemoresistant cancer cells are responsive to PDT, particularly using verteporfin (BPD), which is known to be a potent mitochondrial inducer of apoptosis that bypasses mechanisms of drug resistance (Kessel and Luo, 1999). This is however, to the best of my knowledge, the first time that conditions have been identified in which drug resistant cells acquire enhanced sensitivity to PDT relative to their parent cells, using PDAC cells of common lineage, but contrasting chemosensitivity and invasive behavior. Yet this result is also consistent with the spheroid transplantation experiments (Chapter 3, Figures 10-13), in which ECM conditions that drive greater invasion are correlated with chemoresistance but enhanced response to PDT.
Figure 29. Chemoresistant PDAC displays enhanced PDT sensitivity in 3D culture. (A) Comparison of response to oxaliplatin in PANC1 and PANC1OR 3D cultures shows that resistance to low doses of oxaliplatin is preserved in 3D growth conditions, as expected. (B) PDT response of PANC1 and PANC1OR, shows dramatic increase in sensitivity in the latter. (C) Representative images of PDT response in PANC1 and PANC1OR 3D cultures stained with vital dyes calcein (live, green) and ethidium bromide (red, dead). Scale bars are 200 µm.
Figure 30. Region of 3D nodule cell death following PDT. (A) Confocal sections of an untreated PANC1 tumor nodule on a Matrigel bed, with live cells stained with calcein AM (green) and dead cells stained with ethidium bromide (red). (B) Similar sections of a PANC1 nodule after 25 J/cm² PDT, showing characteristic patterns of disaggregation and cell death at the outer surface. Scale bars are 200 μm.

Overall, both monolayer and 3D experiments with multiple EMT induction methods support my hypothesis that pancreatic cancer cells with a mesenchymal, chemoresistant, and invasive phenotype are still sensitive to PDT. Furthermore, this effect appears to be enhanced in more sophisticated and clinically relevant 3D tumor
models, since chemoresistant sublines grown on Matrigel overlay conditions are significantly more sensitive to PDT.

**Mechanistic Exploration of Enhanced PDT Response**

There are a number possible mechanisms for enhanced PDT effectiveness in chemoresistant and mesenchymal pancreatic cancer populations. It is reasonable that PDT remains effective in these populations where chemotherapy drugs do not, because the mechanisms of action are so different, as supported by previous studies (Celli et al., 2011). For example, slower cell division rates in drug resistant cells (Figure 20) may protect against chemotoxicity but not the more rapid caspase activation involved in PDT-induced cell death. The different growth phenotypes of these cell lines in Matrigel may also cause cells to be exposed to different light levels, since PANC1OR have more single cells invading below the ECM surface. However, the differences here are minimal and unlikely to be substantial compared to other mechanistic possibilities, since the shallow depth of Matrigel (< 1 mm) and sizes of nodules (< 1 mm) are both far less than the 690 nm light penetration through tissues (a few millimeters), as discussed in the Introduction. Some of the other ideas I have explored that influence PDT response include expression of apoptotic machinery, photosensitizer uptake rates, and availability of oxygen for photochemical reactions.
Anti-apoptotic protein expression

Chemoresistant pancreatic cancer cells often have higher levels of Bcl-2 family proteins (Yang et al., 2009a), and high Bcl-xL levels are protective against PDAC treatment with the chemotherapy drug gemcitabine (Bold et al., 1999; Schniewind et al., 2004b). As discussed in Chapter 1, PDT can directly target these anti-apoptotic proteins. I have evaluated the expression of the Bcl-2 family member Bcl-xL using immunofluorescence and western blotting. As revealed by quantitative analysis of immunofluorescence images in Figure 31 (A and B), PANC1OR cells express significantly more Bcl-xL. Additionally, a western blot analysis shows a slight increase in Bcl-xL in chemoresistant PANC1OR cells (Figure 31C). Therefore, although the higher expression of Bcl-xL is linked to chemoresistance, the protective effect of anti-apoptotic Bcl-xL expression may be bypassed, in this case, by rapid BPD-PDT induced apoptosis.
Figure 31. Bcl-xL expression is increased in oxaliplatin-resistant PANC1. (A) Bcl-xL immunofluorescence for PANC1 (top) and PANC1OR (bottom), with DAPI-stained nuclei shown in blue and Bcl-xL in red. (B) Quantified immunofluorescence, with fluorescent Bcl-xL signal normalized to the number of cells based on DAPI fluorescence. The p-value is less than 0.001. (C) Left: western blot for Bcl-xL in PANC1 and PANC1OR cells. Right: quantification of Bcl-xL western on left, normalized to the GAPDH band density.

A more complete analysis of apoptotic machinery in these cell lines before and after BPD-PDT would help to clarify these possibilities. While Bcl-xL can stabilize the mitochondrial transmembrane potential (anti-apoptotic), Bax is destabilizing and increases mitochondrial permeability (apoptosis-promoting) (Narita et al., 1998). The ratio of these proteins, therefore, is more predictive of apoptotic response after PDT (Kim et al., 1999; Mroz et al., 2011; Srivastava et al., 2001). Since ECM contacts can reduce
Bax activity and upregulate Bcl-xL (Gilmore et al., 2000; Martin and Vuori, 2004), it would be most useful to observe levels of these proteins in PANC1 and PANC1OR in 3D culture conditions.

_BPD uptake analysis_

A second mechanism that may influence PDT sensitivity in chemoresistant cell lines is BPD uptake. I would expect that since the growth phenotype of PANC1OR appears more diffuse than PANC1, these cells may more easily or more quickly absorb the photosensitizer. Using the same 3D culture conditions as those for treatment comparisons in Figure 29, I compared the uptake of BPD in PANC1 and PANC1OR. The amount of BPD uptake in each cell line is not significantly different (Figure 32, A and B), unlike differences in PDT response. Additionally, monolayer PANC1 and PANC1OR with the same BPD concentration, reported here in µM BPD/mg total protein, absorb approximately the same amount of BPD (Figure 32C). Neither measurement of BPD uptake shows differences between resistant and non-resistant sublines, suggesting that the observed increase in PDT sensitivity is a result of the biological response in these populations rather than simply the payload of singlet oxygen generated.
**Figure 32. BPD uptake in PANC1 and chemoresistant PANC1 cell lines.** (A) 5x images of PANC1 and PANC1OR spheroids incubated with 1 μM BPD for 1 hour. Scale bars are 200 μm. (B) BPD signal intensity of images normalized to total area, n = 5. (C) Amount of BPD per mg total protein for monolayer cells incubated with or without 1 μM BPD.

**Oxygen perfusion**

Since effective PDT requires the presence of sufficient oxygen, differences in nodule sizes between 3D cultures of PANC1 and PANC1OR cells may explain differential treatment response. I further analyzed experimental results from earlier in this chapter (Figure 29) to evaluate differences in viability after PDT based on 3D nodule volume. 3D cultures of tumor cells develop chemical gradients including oxygen.
variability (a hypoxic core), distribution of ATP, and distribution of glucose at a diameter of 200 µm or greater (Hirschhaeuser et al., 2010). PANC1 cells grow into large and compact nodules while PANC1OR cells detach and invade as the nodules grow, so PANC1 cells likely experience more hypoxia at the nodule center.

Figure 33A shows individual PANC1 or PANC1OR nodules from 9 image fields sorted by volume and viability. As the PDT dose increases (Figure 33A, right plot), the cluster of PANC1OR nodules becomes visually separate from the cluster of PANC1 nodules, supporting the average viability decreases shown earlier in Figure 29. In the untreated conditions, PANC1 nodule sizes follow a classic bimodal Gaussian distribution pattern (Celli et al., 2011). However, the frequency of PANC1OR nodule volumes peaks at smaller volumes and tails off at higher volumes (Figure 33B). When comparing the larger half of nodules (greater than 500,000 µm³) to the smaller half, PANC1OR nodules respond significantly better to PDT at all size ranges (Figure 33C). Therefore, no matter the nodule size, the chemoresistant cells are more responsive to PDT, suggesting that oxygenation alone is not the basis for increased PDT efficacy in this population.
Figure 33. PANC1OR remain more responsive to PDT at all nodule sizes. (A) PANC1 nodules are plotted in black and PANC1OR nodules are plotted in red according to volume and viability with untreated PANC1 and PANC1OR nodules (left), those treated with 10 J/cm² PDT (middle) and those treated with 25 J/cm² PDT (right). (B) Frequency histogram of untreated nodules showing bimodal Gaussian distribution for PANC1 and a peak at smaller volumes for PANC1OR. (C) Viability of PANC1 and PANC1OR nodules with volumes less than 500,000 µm³ (left) and greater than 500,000 µm³ (right). Both sets show statistically significant decreases in viability between cell lines after 25 J/cm² PDT.

However, this analysis is only a surrogate for oxygen availability, and may not reflect its true values. These volume measurements are based on nodule area but the depth of the PANC1 and PANC1OR nodules could be different, thus making some more ellipsoidal. Additionally, PANC1 cells may be more compact than the PANC1OR, a metric not explored in this volumetric analysis. Compared to a compact spherical nodule,
a more diffuse elliptical nodule has a less dramatic hypoxic gradient (Leung et al., 2015), which would affect oxygen diffusion and consumption patterns during PDT. Therefore, it would be essential to measure oxygenation availability using oxygen electrodes (Nichols and Foster, 1994) or markers of oxygenation like 2-nitroimidazole pimodinazole (Evans, 2015; Klein et al., 2012) to be confident in the influence of oxygen on the PDT response for these cell lines.
CHAPTER 6

DISENTANGLING THE BIOCHEMICAL AND PHYSICAL ROLES OF THE ECM


ECM Combination Cultures

I recently showed that in PDAC 3D cultures transplanted into ECM conditions known to promote invasive behavior, the invading tumor cells become chemoresistant, as expected, but exhibited an increased sensitivity to PDT (Cramer et al., 2017). However, the relationship between biological composition and physical characteristics of the ECM, as regulators of PDT response, is still unclear. To examine this, I grew PDAC spheroids in combinations of a soft, invasion-promoting ECM protein (type 1 collagen) and a stiffer, invasion-constraining basement membrane product (laminin-rich Matrigel) (Nguyen-Ngoc et al., 2012). Here, I compare treatment response of embedded spheroids to oxaliplatin, a component of the FOLFIRINOX chemotherapy cocktail that showed a significant survival benefit over standard gemcitabine for PDAC (Conroy et al., 2011), and verteporfin PDT, the photosensitizer used in PDT clinical trials mentioned above (Huggett et al., 2014).
Rheological characteristics of the ECM models

Prior to embedding spheroids into 3D cell culture models containing combinations of COL1 and Matrigel, I characterized rheological properties of each ECM component using bulk oscillatory shear rheology measurements (Figure 34). The 50% Matrigel used here is a soft gel with a $G'$ storage modulus of less than 50 Pa, half the stiffness shown in previous reports (Cramer et al., 2017; Paszek et al., 2005; Semler et al., 2000; Zaman et al., 2006), as expected. The COL1 hydrogel at a concentration of 1 mg/mL is about 10 times softer than this (Figure 11E; (Cramer et al., 2017)). As shown in Figure 34, the $G'$ viscoelastic storage modulus increases significantly as more COL1 is added to the ECM mixture.
Figure 34. Bulk oscillatory shear rheology measurements of the storage modulus ($G'$) for 50% Matrigel compared to Matrigel and collagen 1 mixtures. 50% M, n=3; M + C (1 mg/mL), n=3; M + C (2.5 mg/mL), n=3; M + C (4 mg/mL), n=6. [H. El-Hamidi data]

**Effects of ECM composition on PDAC growth behavior and invasive potential**

PANC1 spheroids were grown on attachment-free agarose beds for 12 days prior to embedding in ECM combinations described above. Spheroids embedded in 50% Matrigel exhibit little to no invasion (Figure 35A, top right image; and Figures 35B and 35C, left column), comparable to my previous report describing invasive behavior of PANC1 spheroids embedded in 100% Matrigel (Cramer et al., 2017), and Chapter 3 above.

As the ratio of COL1 to Matrigel increases in the ECM mixtures, more PANC1 cells break off the main spheroid and invade into the surrounding ECM (Figure 35A, left
panels). This is quantified at right, where the average number of invading cells per spheroid increases significantly (Figure 35B), with the greatest number of invading cells present in COL1 only (1 mg/mL) ECM environments, which is also the softest ECM condition described here. Despite previous reports showing increased invasive behavior in stiffer microenvironments (Levental et al., 2009; Lu et al., 2012; Paszek et al., 2005), I have found that the invasion-promoting effects of COL1 (or invasion-inhibiting characteristics of Matrigel) overcome potential effects of increasing ECM stiffness as a stimulant of PDAC invasive behavior.

Additionally, when the average distance of all cells invading into the ECM is quantified, the presence of COL1 is correlated with an invasion distance of about 200 µm regardless of COL1 concentration (Figure 35C). This is significantly farther than the minimal distance traveled by the few invading cells in 50% Matrigel (Figure 35C, left column). Thus, interaction of the PANC1 cells with any amount of COL1 appears to encourage their migration along the COL1 ECM fibers.
**Figure 35. Growth behavior of spheroids in varying ECM compositions.** (A) 5x images of PANC1 spheroids in Matrigel (M) and collagen 1 (COL1) combinations after 6 days of growth, stained with calcein AM (green, live cells) and ethidium bromide (red, dead cells). Scale bars are 250 µm. (B) Average number of invading cells for each ECM condition. (C) Average distance traveled for invading cells in each ECM condition, from the edge of each spheroid. For both (B) and (C), M: n=4; M+C (1mg/mL): n=7; M+C (2.5mg/mL): n=6; M+C (4mg/mL): n=8; COL1 (1mg/mL): n=4.

**ECM composition impacts PDAC response to therapeutic interventions**

In the conditions established above, I compared differential responses of invading cells and main PDAC spheroids to oxaliplatin chemotherapy and verteporfin PDT at equivalent monolayer LD90 doses previously calculated (Chapter 3, Figure 13; Cramer et al., 2017). Images taken after live-dead staining like those shown above (Figure 35A) were quantitatively processed using previously described methods to report ratios of live
(green) to dead (red) cells in the main spheroid and invading cells (Celli et al., 2014). The red staining observed in the center of each spheroid shows a necrotic core standard for large tumor nodules with limited oxygen and nutrient diffusion to the interior.

Figure 36. Treatment response of invading cells and primary spheroids in each ECM condition. (A) Surviving fraction of invading cells treated with 25 J/cm² PDT or 500 µM oxaliplatin, normalized to untreated controls. (B) Main spheroid volume remaining after treatment with 25 J/cm² PDT or 500 µM oxaliplatin, normalized to the live volume of untreated control spheroids.
Oxaliplatin appears to significantly decrease the live spheroid volume remaining after treatment for 3 days (Figure 36B), but has almost no effect on invading cells (Figure 36A). Since there are so few invading cells in 50% Matrigel only conditions, these are not shown in Figure 36A. While oxaliplatin seems to be significantly more effective for invading cells in one condition (M + 4 mg/mL COL1), the decrease in viability is minimal.

PDT, however, shows the opposite trend to chemotherapy treatment. At 25 J/cm², PDT is minimally effective on the main spheroid, decreasing the live volume by at most 25% (Figure 36B). Importantly, however, in ECM conditions that promote the most invasion (1 mg/mL COL1 only), PDT efficacy is significantly enhanced compared to conditions with less invasion (Figure 36A).

In these conditions, it appears that the biochemical composition of the ECM (the invasive-promoting effects of COL1) is more influential than its physical characteristics, since the most invasion occurred at the highest ratio of COL1 to Matrigel, even though this was also the least rigid ECM combination examined. Importantly, these results support previous studies showing PDT efficacy in conditions that promote metastasis and chemoresistance (Celli et al., 2011; Cramer et al., 2017), and also motivate the need for developing additional 3D models to further disentangle the intricate interplay of ECM stiffness and protein composition with PDAC behavior and PDT response.
Collagen I Crosslinking

ECM combination experiments in the previous section prompted me to explore additional experimental methods to help clarify effects of ECM rigidity by modifying ECM stiffness while minimizing changes in biochemical composition. Riboflavin-UVA induced collagen crosslinking has been shown to increase corneal rigidity in ophthalmologic scenarios (Snibson, 2010; Wollensak et al., 2003) and to increase dentin collagen matrix stability in dental bonding (Brennan-Pierce et al., 2014; Cova et al., 2011). Similarly to PDT, riboflavin (RF) excitement (with a peak absorption at 370 nm), into the triplet state generates singlet oxygen and superoxide radicals which form covalent bonds between the collagen molecules through oxidative deamination (McCall et al., 2010; Snibson, 2010). Based on this information, I have designed additional 3D culture models to help disentangle the biochemical and physical role of ECM components in PDAC invasive potential and treatment response (Figure 37).
Figure 37. Experimental design: riboflavin-induced collagen crosslinking. 1: Mix RF with COL1 mixture and expose to light to induce a low (0.5 J/cm²) or high (15 J/cm²) amount of crosslinking. 2: Embed spheroids and wait 3 days for invasion. 3: Treat both crosslinking condition spheroids with PDT or oxaliplatin chemotherapy. 4: High-throughput quantitative image analysis.

Rheological analysis and invasive potential

Bulk rheological measurements of COL1 after RF-induced crosslinking show an increase in stiffness as fluence increases, up to about 25 J/cm² (Figure 38A). Above this light dose there is a dramatic drop in stiffness, indicating sufficient ROS production to destroy collagen structure instead of crosslinking. Spheroids were also implanted into these crosslinked COL1 gels, and imaged over 6 days. The velocity (µm per day) of cells invading into COL1 decreases as the amount of crosslinking and stiffness increases (Figure 38B).

In vivo and in vitro, lysyl oxidase-mediated collagen crosslinking and stiffening promotes the development of focal adhesions and drives breast cancer invasion (Levental et al., 2009). However, based on the bulk rheology measurements shown here, increases
in stiffness in an already invasion-promoting ECM only decrease the velocity of invading cells (Figure 36B). Additionally, Figure 36C shows images of PANC1 spheroids embedded in non-crosslinked (top) and photocrosslinked (bottom) COL1 gels after 3 days of invasion, stained for EMT markers. These images demonstrate the decrease in potential invasive velocity quantified in Figure 31B, as well as support data in previous experiments describing the mesenchymal phenotype of invasive PDAC.

Results here support the likelihood of a biphasic invasion response to ECM stiffness. This has been modeled by Lang et al. (2015) and Ahmadzadeh et al. (2017), where increasing stiffness also increases invasion, but only up to the point where the rigidity of tightly-packed ECM structures becomes physically limiting. In above experiments combining ECM types and crosslinking collagen, rigidity certainly increases, although this is confounded by the differences in biochemical composition in admixtures as previously noted. The presence of collagen 1 likely effects a biochemical promotion of invasion at any concentration, but cells achieve greater motility in the gels with less crosslinking.

In addition to rigidity increases, combining ECMs and crosslinking collagen also change the sizes of pores in the ECM that cells can easily travel through. Other studies determining invasion speed of glioma cells in collagen 1 (Yang et al., 2010) and movement of MDA-MB-231 cells in collagen 1 (Carey et al., 2012) have shown that ECM pore size is another factor influencing the invasive potential of cancer cells, a variable sometimes more influential than bulk ECM stiffness. In vivo, the structure of the ECM and sizes of pores between the protein fibers can be quite variable (between
tumors) and heterogeneous (within tumors) (Keely and Nain, 2015). In PDAC tumors, because of their extensive desmoplasia, the tendency is likely to trend to tighter ECM networks with smaller pore sizes, although the amount of fibrosis in general also varies (Erkan et al., 2012b).

Figure 38. COL1 crosslinking increases COL1 stiffness and decreases PDAC invasion. (A) RF-crosslinked COL1 (3.7 mg/mL) G’ storage modulus increases up to a fluence of about 25 J/cm² [R. Jafari Data]. (B) The velocity of PANC1 cells invading from a spheroid into the COL1 matrix decreases as the fluence dose promoting RF-induced crosslinking increases [R. Jafari Data]. (C) 5x images of PANC1 spheroids embedded in COL1 (no crosslinking) (top) or COL1 (15 J/cm² crosslinking) (bottom), stained with DAPI, vimentin, and E-cadherin.
Chemotherapy and PDT response

In earlier experiments comparing a stiffer, invasion-constraining ECM to a softer, invasion-promoting ECM, PDT was significantly more effective than oxaliplatin in invading PDAC populations (Chapter 3). Using COL1 photocrosslinking, I can compare treatment response in ECM conditions with different stiffness but that remain biochemically similar. PANC1 spheroids permitted to invade for 3 days into minimally crosslinked (0.5 J/cm²) or highly crosslinked (25 J/cm²) COL1 show no difference in PDT or oxaliplatin response (Figure 39, Figure 40 A and B). PDT remains more effective on invasive cells (Figures 39 and 40A) and oxaliplatin remains more effective on the spheroid volume (Figures 90 and 40B). While there appear to be no significant responses to stiffness in terms of therapeutic response, these results do importantly validate those observed in previous chapters, that PDT targets populations of drug resistant cells emerging from stromal interactions, specifically ECM cues.
Figure 39. RF-induced COL1 crosslinking limits PDAC invasion but does not influence treatment response. Images of PANC1 spheroids allowed to invade for 3 days in slightly crosslinked (left) or more crosslinked (right) COL1. Untreated spheroids (top) show less invasion with 15 J/cm$^2$ crosslinking. Images of treated spheroids show extensive death after PDT in invading cells (middle panels) and decreases in cell proliferation after oxaliplatin treatment (bottom panels). Scale bars are 200 µm.
Figure 40. RF crosslinking to increase COL1 stiffness has no effect on chemotherapy or PDT response. (A) Viability of PANC1 cells invading from a spheroid in 0.5 J/cm² (n=3) or 15 J/cm² (n=4) crosslinked COL1 (3.7 mg/mL). P-values are n.s. (B) PANC1 spheroid volume remaining after treatment with PDT or oxaliplatin. P-values are n.s.
CHAPTER 7
CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Tumors are more than a collection of mutated, rapidly growing, and unruly cells, since the surrounding microenvironment (both physical and biological) is also an enormously influential regulator of cancer growth, metastasis, and therapeutic response. Since chemotherapy and PDT induce cell death through different mechanisms, it is important to determine which cancer cell populations, in which sort of environmental context, will be most responsive to each treatment option.

Both 3D tumor model experimental designs, manipulating the ECM environment of a given cell type (Chapters 3 and 6) and manipulating the biology of a parent cell type in a given ECM (Chapter 4), show that highly motile ECM invading cells are markedly more susceptible to PDT. Noting that in the conditions where enhanced response to PDT is observed there is also acquisition of a mesenchymal phenotype, I also examined whether the direct biochemical induction of this phenotypic transition would have a similar effect on differential sensitivity to chemotherapy and PDT.
Results of experiments described above using multiple cell lines (PANC1 and BxPC3) and EMT induction methods (TGFβ or FCM), as well as creating chemoresistant sublines, support my hypothesis that PDT effectively targets chemoresistant and mesenchymal pancreatic cancer populations. However, this effect is only significantly enhanced in more sophisticated and clinically relevant 3D pancreatic cancer models. These results suggest that the observed increase in PDT efficacy in invasive populations requires interaction with ECM in a 3D environment, although various exploratory studies of the mechanisms promoting this effect were inconclusive (Chapter 5).

This finding motivates further mechanistic exploration to identify biological changes in these cells (e.g. altered integrin signaling) that could be connected with susceptibility to PDT (e.g. oxidative stress response). Additionally, resistance to cisplatin in ovarian cancer cells is associated with elevated glutathione (GSH) levels to help cope with increase oxidative stress caused by chemotherapeutic agents (Godwin et al., 1992; Traverso et al., 2013). However, invasive cancer cells alter metabolic pathways as they become more motile, acquiring high energy requirements (Yang et al., 2014), which may deplete glutathione and deprive the most invasive cells some antioxidant protection. Indeed, Fujita et al. have reported that invading PDAC cells (specifically PANC1) consume GSH to overcome oxidative stress that occurs in the migration process (Fujita et al., 2017). ROS generation via PDT further depletes these GSH stores (Magi et al., 2004) and may disturb the delicate redox balance in chemoresistant cell lines, encouraging an apoptotic response.
While the 3D models used here simulate in vivo scenarios more closely than monolayer culture methods, some limitations to the model include the lack of immune system interaction, connections with stromal signaling partners like fibroblasts, lack of vasculature, and less stiffness/pressure. The stiffest ECM material used here, crosslinked COL1, has an elastic modulus of only about 140 Pa, an order of magnitude softer than in vivo tumor tissues, which can range from 1-35 kPa (Yu et al., 2011). Despite this, the above studies entailed development of new tumor modeling and imaging frameworks for biophysically-informed therapeutic evaluation. These approaches enable not only direct co-registration of cell motility and treatment response, but also lend themselves to broader applicability to other cancer therapeutics going forward.

Collectively, results of all these experiments show that PDT targets invasive and chemoresistant PDAC populations associated with the aggressive metastatic spread and the notoriously poor outcomes for this disease. Combined with recent clinical studies establishing safety and feasibility of therapeutic light delivery to the pancreas (Huggett et al., 2014), these results suggest the promise of PDT for targeting invasive and drug resistant PDAC. Within the context of optimizing clinical PDT for PDAC, these insights could help to inform treatment design, specifically targeting invading fields containing early metastatic cells that would otherwise escape chemotherapy. Implementing this knowledge clinically would entail interstitial fiber optic targeting of light nearer to the edges of the tumor, where PDT is most effective, instead of the highest dose centered in the tumor core. Additionally, this therapeutic process will be more useful after more progress is made in earlier PDAC diagnosis. For example, identification of plasma
biomarkers (Balasenthil et al., 2017) would dramatically increase survival, since 5-year survival rates after node- and margin-negative tumor resection are over 40% (Cameron et al., 2006).

The insight that the primary proliferating spheroid mass in 3D cultures is more susceptible to chemotherapy (at least with oxaliplatin) indicates that a combination of a chemotherapy drug and PDT would achieve more complete response, a theme that echoes numerous earlier studies on PDT-based combinations (del Carmen et al., 2005; Ferrario et al., 2002; Gomer et al., 2006; Kosharskyy et al., 2006; Rizvi et al., 2010). Chemoresistant and invasive pancreatic cancer cells are exactly the populations associated with the worst prognoses and most challenging to target with other therapeutics, so their enhanced PDT response in these experiments is particularly promising for development of future PDAC treatment planning. After additional pre-clinical and clinical trials, PDT will likely have support as an effective strategy for targeting regional and locally advanced disease and in combination with chemotherapy options for distant metastases.

**Future Directions**

*ECM microstructure analysis*

Bulk ECM stiffness, while clearly influential in tumor behavior, does not necessarily reflect the local environment encountered by an individual cancer cell. While COL1 crosslinking completed in Chapter 6 increased total ECM stiffness, based on bulk rheology measurements, this did not appear to influence the average treatment response
of PDAC cells. It may be more interesting to correlate the behavior of an individual invading cell with its local ECM microenvironment, as noted by Carey et al. (2012) and Pathak and Kumar (2011). To do this, a combination of PTMR measurements and collagen matrix structure imaging could match local ECM alignment, organization, and stiffness to an individual cell’s invasive capacity and susceptibility to treatment. For example, second harmonic generation (SHG) imaging can be used to observe the organization of structural proteins, particularly fibrillar collagen, over time (Brown et al., 2003; Campagnola et al., 2002; Provenzano et al., 2006). SHG imaging would allow construction of a map of the structure of collagen fibers and pore sizes between them that could lead to a better understanding of factors influencing cell motility and treatment response.

Determining differential integrin expression

Integrins are two-subunit proteins that mediate cell-ECM interactions, influencing cell motility, adhesion, survival, and chemoresistance (Aoudjit and Vuori, 2012; Desgroisellier and Cheresh, 2010; Huveneers and Danen, 2009). Expression of certain integrins, such as α5β1 and α5β3, is highly upregulated in cancer cells compared to normal epithelial tissue (Desgroisellier and Cheresh, 2010). BPD-PDT disrupts cell-ECM interactions, decreasing the number of ovarian cancer β1 integrin focal adhesions and causing decreased adhesion to variety of ECM proteins (Runnels et al., 1999). Therefore, it would be useful to correlate the invasive phenotype of PDAC cells with fluctuations in integrin expression, as these are involved in cell-ECM interactions and likely change.
after exposure to PDT. Immunofluorescence and western blotting could be used to track changes in integrin expression (including α5β1 and α5β3) as 3D cultures of PANC1 and PANC1OR cells grow, and also determine if PDT alters integrin expression in each of these cell lines.

*Cancer stem cell analysis*

As discussed in Chapter 1, chemoresistant cells are likely to have more cancer stem cell characteristics and express pancreatic cancer CSC markers. Steps to explore CSC markers in PDAC may include analyzing the expression of CD44, a pancreatic cancer stem cell marker, in parent and drug-resistant cell lines. The EMT-inducer ZEB1 also helps to maintain a stem cell phenotype (Wellner et al., 2009), so it would be useful to evaluate differential expression of ZEB1 in parent and drug-resistant PDAC cell lines. Chemoresistant pancreatic cancer cells and cancer stem cells are also more likely to have high ROS defenses, but PDT may be able to increase ROS enough to overcome CSC ROS defenses. A number of small-molecule fluorescent probes are available for ROS imaging, including dihydroethidium (DHE), so it would be interesting to compare ROS expression and generation during PDT to the population of CSC present in parent and drug-resistant cultures. Western blotting and immunofluorescence could be used to evaluate levels of pancreatic cancer CSC markers like ZEB1 and CD44 in parent and chemoresistant cell lines.
Monitoring invasive progression of drug-resistant sublines in an orthotopic PDAC xenograft

In order to further validate conclusions about the invasive potential of drug-resistant PDAC sublines, I could complete in vivo invasion assays using an active approved animal protocol. These methods would be based on a previously described orthotopic xenograft pancreatic cancer model (Samkoe et al., 2010). For orthotopic tumor implantation in hairless nude mice, a suspension of 1x10^6 PANC1 or drug resistant PANC1 cells suspended in 50% Matrigel DPBS would be injected into each pancreas following analgesia/anesthesia. Tumor growth would be monitored using caliper measurements every 3 days, and mice would be sacrificed at 10 weeks for analysis of tumors and metastases (Bose et al., 2011). To analyze the invasive and metastatic potential of implanted cell lines, RT-PCR could be completed. Since the cell lines implanted in mice, in these case, are human cell lines, the amount of expression of a human housekeeping gene (such as GAPDH) in common metastatic sites (liver, bone marrow, lungs, etc.) describes the amount of metastasis. These results would determine if the invasive phenotype is maintained in vivo. To build on these in vivo invasion assays of PANC1 and PANC1OR, it would also be interesting to determine of the differential PDT response observed in these cell lines in 3D culture is maintained in a mouse model.
APPENDICES

APPENDIX A: ADDITIONAL CHEMORESISTANCE GENERATION AND EMT INDUCTION EXPERIMENTS

*Some sections are supplemental figures in Cramer et al. (2017), Molecular Cancer Research

**HPAF-II Gemcitabine Resistance**

A stably proliferating gemcitabine-resistant BxPC3 cell line can be selected from 40 nM gemcitabine treatment for 2 months (Wellner et al., 2009), so this was a reasonable starting concentration for development of additional gemcitabine-resistant cell lines. HPAF-II is a well-differentiated pancreatic cancer cell line with epithelial characteristics similar to BxPC3 (See Table 1, in Chapter 2). Gemcitabine additions to the media for HPAF-II cells were slowly increased up to 100 nm gemcitabine over a period of months.

Figure 41 (left) confirms that growing HPAF-II cells in low doses of gemcitabine over time produced subline (HPAF-II-GR) significantly less responsive to gemcitabine treatments at a variety of doses. However, while this cell line appears less organized and with less well-defined E-cadherin borders between cells, there is no evidence of increased vimentin expression. Similar attempts to produce a stably proliferating gemcitabine resistant BxPC3 subline were unfortunately and surprisingly unsuccessful.
Figure 41. Generation of HPAF-II cell line resistant to gemcitabine (HPAF-II-GR). Left: Surviving fraction of HPAF-II and HPAF-II-GR after 72 hours of gemcitabine treatment, analyzed via MTS. Right: Immunofluorescence images of HPAF-II (top) and HPAF-II-GR (bottom) stained for nuclei (blue), E-cadherin (green), and vimentin (red).

**HPAF-II + TGFβ EMT Induction**

Similarly to PANC1 induction of EMT using 10 ng/ml TGFβ treatment, I also attempted this in the HPAF-II cell line. Figure 42 (left) shows an image of the HPAF-II cell line growing in regular media, with well-defined boundaries between cells. After treatment with TGFβ for two days, the cells do exhibit a less well-organized epithelial growth pattern with some instances of a more mesenchymal phenotype (Figure 42, right).
However, the major difference here appears to be the growth rate, as there are about half as many cells in the TGFβ-treated HPAF-II cultures.

![Image](image1.png)

**Figure 42. Induction of EMT in HPAF-II cells with TGFβ.** Left: phase-contrast image of HPAF-II cells in monolayer. Right: phase-contrast image of the HPAF-II phenotype after 2 days growing in media containing 10 ng/ml TGFβ.

**PANC1 Gemcitabine Resistance**

While I was unable to produce a stably proliferating gemcitabine-resistant PANC1 cell line, I did select for PANC1OR cells that were also resistant to gemcitabine, creating the multidrug resistant PANC1ORGR. These have a similar phenotype to that seen in PANC1OR, with less E-cadherin and more vimentin than the parent PANC1 cell line, based on quantitative immunofluorescence and western blotting analysis (Figure 43 A and B).
Figure 43. The multi-drug resistant PANC1ORGR is more mesenchymal and more sensitive to PDT than PANC1. (A) EMT duplex IF for PANC1 and PANC1ORGR showing increased vimentin (red) and decreased E-cadherin (green). (B) Western blot for E-cadherin and vimentin in PANC1 and PANC1ORGR normalized to GAPDH. (C) PANC1ORGR confocal image of DAPI-stained nuclei and phalloidin after 12 days of growth on Matrigel. (D) PDT response for PANC1 and PANC1ORGR grown on Matrigel beds and quantified at right.

When plating this cell line on 3D, it becomes more invasive over time. A 3D nodule is shown in Figure 43C, exhibiting similar growth behavior to PANC1OR cells in Chapter 5. Additionally, treating PANC1ORGR nodules with PDT shows a clear visual difference in response, where a PDT dose of 10 J/cm² causes significantly more cell death than PANC1 nodules (Figure 43D, images). Quantification of viability for 10 J/cm² and 25 J/cm² supports this observation (Figure 43D, graph).
Mathematical modeling of tumor growth is clinically useful, as it allows for determining what stage of growth a tumor has reached and what therapies might be most useful at that stage or growth rate, as well as evaluating how these change in response to treatment. In vivo, tumors form and grow in confined space with limited nutrient availability, so initial exponential growth slows as tumors become larger. Various mathematical descriptions of this process include combinations of linear, exponential, cube root, and logarithmic models. A more suitable model to describe the growth of a confined population is Gompertzian, as used by Laird (1964). This has the following form, where \( W \) is the tumor size at time \( t \), \( W_0 \) is the initial tumor size, and other variables are constants:

\[
\frac{W}{W_0} = e^{A(1-e^{-at})}
\]

In vitro cell cultures are limited by space more than nutrients, as their growth media is regularly refreshed, but it would still make sense for an asymptotic Gompertzian equation to model their growth. To determine if this was the case, I collected growth rate data for 3D Matrigel and monolayer PANC1 cultures by periodically counting cells in cultures up to about 25 days of growth. Surprisingly, at least in this timeframe, the rate of growth did not appear to plateau (Figure 44). Even in monolayer, PANC1 cells seem to have lost contact inhibition, and continue to grow in piles on top of each other as long as nutrients are consistently replenished (Figure 44, right image).
**Figure 44. Growth rates of PANC1 in 3D and monolayer.** Cells were initially plated at 7,500 per well, and allowed to grow for over three weeks with regular media changes. 5x darkfield images show representative growth at day 17 in 3D (left) and monolayer (right). Scale bars are 200 µm. The graph below shows three separate experiments involving cell counts in triplicate, including various outliers.
APPENDIX D: MATLAB CODE

Darkfield Invaders

% script to obtain number of invading cells and position with respect to
% primary spheroid in 3D spheroid collagen/matrigel transplants

PATH='C:\Users\Michael\Dropbox\LAB DATA\Gwen\PANC1 collagen matrigel
spheroid chemo PDT tx\2016-2-20 PANC1 col mat spheroid PDT Ox\Day 1 images';
Filename='2016-2-17 PANC1 spheroids collagen matrigel Day 1.czi';

img = bfopen(strcat(PATH, Filename));
[s1,s2] = size(img);
micperpix=1.02; % for AxioObserver 5X objective and 0.63x camera adapter
minsize=50;
params= [];
summaryarray= [];
GAUSSIAN_FILTER1 = fspecial('gaussian', [3,3], .75);

for i=1:s1
    field=img(Chivukula et al., 2015);
    pixels=filter2(GAUSSIAN_FILTER1, field(Chivukula et al.));
    %figure; imagesc(pixels);
    pixels=double(pixels./max(max(pixels)));
    pixelsBW=im2bw(pixels, graythresh(pixels));
    pixelsBW=imfill(pixelsBW, 'holes'); % fill in holes so that objects are solid (not
donuts)
    pixelsBW=imclearborder(pixelsBW);
    %pixelsBW=imdilate(pixelsBW, strel('disk', 5));
imshow(pixelsBW);
imwrite(pixelsBW, strcat(PATH, 'field_', num2str(i), '.tif'));
[labeled, N] = bwlabel(pixelsBW);
objectdata = regionprops(labeled, 'Area', 'Centroid');
params(:,1)=objectdata.Area.*micperpix^2;
for j=1:N
    a=objectdata(j,1).Centroid;
    params(j,2)=a(1)*micperpix;
    params(j,3)=a(2)*micperpix;

end
outliers=params(:,1)<minsize;
params(any(outliers,2),:) = [];
[A, I] = max(params(:,1)); % find indices, I of largest object (area A), assume its the primary spheroid
if numel(A) == 0
  params(1,4:7)= NaN;
dlmwrite(strcat(PATH, 'field_', num2str(i), 'data.txt'), params, 'delimiter', '	')
else
  r=(A(1)/pi)^0.5; %effective radius for equivalent circular primary nodule
  Xpos=params(I(1), 2); % get x and y positions of big spheroid
  Ypos=params(I(1), 3);
  params(I(1),:)=[]; %clear the data for the primary spheroid (it's not an "invader")
  newsize=size(params); %number of invaders after size cutoff and omitting primary nodule
  for k=1:newsize(1)
    params(k,4)=params(k,2)-Xpos; % x- position with origin at big spheroid
    params(k,5)=params(k,3)-Ypos; % y-position
    params(k,6)=(params(k,4)^2 + params(k,5)^2)^0.5; % distance to center of big spheroid
    params(k,7)=params(k,6)-r; %approx distance to edge of big spheroid
  end
dlmwrite(strcat(PATH, 'field_', num2str(i), 'data.txt'), params, 'delimiter', '	')
end
newsize=size(params);
summaryarray(i,1)=i; %summaryarray - 1st column is field number
summaryarray(i,2) = newsize(1); %2nd column is number of invaders
if numel(params) > 0
  summaryarray(i,3) = mean(params(:,7)); % average distance of invading cells from spheroid edge
  summaryarray(i,4) = max(params(:,7)); % max invading cell distance
else
  summaryarray(i,3)= NaN;
  summaryarray(i,4)= NaN;
end

clear objectdata params outliers A I N r newsize
end
dlmwrite(strcat(PATH, 'summaryfile.txt'), summaryarray, 'delimiter', '	')
clear summaryarray
Vital Dye Invaders with Volumes

% script to obtain number of invading cells and position with respect to % primary spheroid in 3D spheroid collagen/matrigel transplants
% mostly the same as DFinvaders.m, but expects individual files rather than % a single composite multi-field czi
% Now can use czi file

PATH='C:\Users\CBPM\Dropbox\LAB DATA\Gwen\Collagen riboflavin crosslinking\2017-1-25 col1 xlink pdt ox';
outputfolder = strcat(PATH, 'OUTPUT');
mkdir(outputfolder);
%save(fullfile(outputfolder));
%files=dir(strcat(PATH, '*.*.czi'));
%[s1,s2]=size(files); 
Filename='2017-1-25 panc1 col xlink pdt ox lower power.czi';

img = bfopen(strcat(PATH, Filename));
[s1,s2] = size(img);
summaryarray=zeros(s1,6);
micperpix=1.02; % for AxioObserver 5X objective and 0.63x camera adapter (scanned resolution)
minsize=50;
params= [];
summaryarray=[];

GAUSSIAN_FILTER1 = fspecial('gaussian', [3,3], .75);

for i=1:s1
    %if files(i).isdir == 0
        %img=bfopen(strcat(PATH,files(i).name));
        field=img(Siegel et al., 2014);
        livepixels=filter2(GAUSSIAN_FILTER1, field{2,1}*);
        deadpixels=filter2(GAUSSIAN_FILTER1, field{1,1})*);
    %figure; imagesc(pixels);
        livepixels=double(livepixels./max(max(livepixels)));
        livepixelsBW=im2bw(livepixels, graythresh(livepixels));
        livepixelsBW=imfill(livepixelsBW, 'holes'); % fill in holes so that objects are solid (not donuts)
        %pixelsBW=imdilate(pixelsBW, strel('disk', 5));
        deadpixels=double(deadpixels./max(max(deadpixels)));
        deadpixelsBW=im2bw(deadpixels, graythresh(deadpixels));
        %summaryarray=[...];
    end
end
deadpixelsBW = imclearborder(deadpixelsBW);
totalmask = livepixelsBW + deadpixelsBW;
imshow(totalmask);
imwrite(totalmask, strcat(outputfolder, '\field_', num2str(i), 'BWimg.tif'));
[labeled, N] = bwlabel(totalmask);
objectdata = regionprops(labeled, 'Area', 'Centroid', 'PixelList', 'Perimeter');
params(:,1) = [objectdata.Area]*micperpix^2;
params(:,10) = [objectdata.Perimeter]*micperpix;
for j = 1:N
    a = objectdata(j,1).Centroid;
    params(j,2) = a(1)*micperpix;
    params(j,3) = a(2)*micperpix;
end

[A, I] = max(params(:,1)); % find indices, I of largest object (area A), assume its the primary spheroid
if numel(A) == 0
    params(1,4:10) = NaN;
dlmwrite(strcat(outputfolder, '\field_', num2str(i), 'data.txt'), params, 'delimiter', '	')
else
    r = (A(1)/pi)^0.5; % effective radius for equivalent circular primary nodule
    Xpos = params(I(1), 2); % get x and y positions of big spheroid
    Ypos = params(I(1), 3);
    for k = 1:N
        params(k,4) = params(k,2) - Xpos; % x-position with origin at big spheroid
        params(k,5) = params(k,3) - Ypos; % y-position
        params(k,6) = (params(k,4)^2 + params(k,5)^2)^0.5; % distance to center of big spheroid
        params(k,7) = params(k,6) - r; % approx distance to edge of big spheroid
    end
    % now and add new columns for the live
    % signal and the dead signal of each object (for now, these values
    % are raw intensities with no background subtraction)
nodulepixels = [objectdata(k).PixelList];
pixelDimensions = size(nodulepixels);
noduleMask = zeros(size(totalmask));
for l = 1:pixelDimensions(1)
noduleMask(nodulepixels(l,2), nodulepixels(l,1)) = 1;
end
liveNodule = livepixels.*noduleMask;
deadNodule = deadpixels.*noduleMask;
params(k,8) = mean2(liveNodule);
params(k,9) = mean2(deadNodule);
end

% get rid of artefacts and the primary nodule (not an "invader")
% params(I(1),:)=[]; % clear the data for the primary spheroid (it's not an "invader")
perimeter = params(I(1), 10); % note perimeter of largest nodule
outliers=params(:,1)<minsize;
params(any(outliers,2),:) = [];

newsize=size(params); % number of invaders after size cutoff and omitting primary nodule

% now write the individual output file
dlmwrite(strcat(outputfolder, 'field_', num2str(i), 'data.txt'), params, 'delimiter', '	')
end

% populate a summary array
summaryarray(i,1)=i; % summaryarray - 1st column is field number
summaryarray(i,2) = newsize(1); % 2nd column is number of invaders
if numel(params) > 0
    summaryarray(i,3) = mean(params(:,7)); % average distance of invading cells from spheroid edge
    summaryarray(i,4) = max(params(:,7)); % max invading cell distance
    summaryarray(i,5) = mean(params(:,8)); % average live signal of all invading cells
    summaryarray(i,6) = mean(params(:,9)); % average dead signal of all invading cells
    summaryarray(i,7) = A; % col 7 is area of largest object (primary spheroid)
    summaryarray(i,8) = perimeter; % perimeter of largest object (primary spheroid)
else
    summaryarray(i,3)= NaN;
    summaryarray(i,4)= NaN;
    summaryarray(i,5)= NaN;
    summaryarray(i,6)= NaN;
    summaryarray(i,7)= NaN;
    summaryarray(i,8)= NaN;
end

clear field livepixels deadpixels livepixelsBW deadpixelsBW totalmask noduleMask liveNodule deadNodule objectdata params outliers A I N r newsize
end
% end
dlmwrite(strcat(outputfolder, 'summaryfile.txt'), summaryarray, 'delimiter', '	')
clear summaryarray
REFERENCES


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