



Investigating Fibroblast Activation State and Phenotype in Tumor Co-culture Models

Citation

Priess, Michelle Lynn. 2022. Investigating Fibroblast Activation State and Phenotype in Tumor Co-culture Models. Master's thesis, Harvard University Division of Continuing Education.

Link

<https://nrs.harvard.edu/URN-3:HUL.INSTREPOS:37371596>

Terms of use

This article was downloaded from Harvard University's DASH repository, and is made available under the terms and conditions applicable to Other Posted Material (LAA), as set forth at

<https://harvardwiki.atlassian.net/wiki/external/NGY5NDE4ZjgzNTc5NDQzMGIzZWZhMGFIOWI2M2EwYTg>

Accessibility

<https://accessibility.huit.harvard.edu/digital-accessibility-policy>

Share Your Story

The Harvard community has made this article openly available.

Please share how this access benefits you. [Submit a story](#)

Investigating Fibroblast Activation State and Phenotype in Tumor Co-culture Models

Michelle Priess

A Thesis in the Field of Biotechnology
for the Degree of Master of Liberal Arts in Extension Studies

Harvard University

May 2022

Abstract

Fibroblast cells in the tumor microenvironment play a role in cancer cell growth, progression of invasion, and secretion of extracellular matrix components, cytokines, and growth factors. Characterizing fibroblast subsets in the tumor microenvironment and their different activation states through the use of *in vitro* models may provide novel opportunities to identify fibroblast-specific anti-tumor therapies. This thesis work addresses the complexities involved in creating a physiologically relevant *in vitro* model by comparing expression patterns of five fibroblast activation markers (CD29, FAP, PDGFRb, Thy1.1 and Cav-1) in co-cultures with tumor cells in a typical two-dimensional cell culture method to a three-dimensional spheroid model. Markers of fibroblast activation had varying levels of expression in co-cultures with two different breast cancer lines, SKBR3 or MDA-MB-231, which may be reflected in the different morphology of the 3D spheroids. Fibroblasts grown in traditional 2D cultures appeared to be in a more uniformly activated state than 3D spheroid models, suggesting that 3D culture may be a better tool for identifying changes in the tumor microenvironment. The development and use of physiological *in vitro* models provide opportunities to study the complex roles played by fibroblasts in the tumor microenvironment as well as a system for validating potential targets for anti-tumor therapies.

Dedication

This thesis work is dedicated to my mother, Denise Priess. I am truly grateful for unconditional support throughout my entire education and career. As a recent breast cancer survivor, she is role model of courage and strength and an inspiration for the research I do.

Acknowledgments

Firstly, I would like to thank my thesis director, Monica Gostissa PhD, for her scientific mentorship throughout the thesis process. Without her support, guidance, and feedback this work would not have been possible. I would also like to thank Vikki Spaulding for supporting my career and scientific goals, as well as Kristen Leone and all members of my team at Jounce who helped with my technical skills. Thank you to Silva Krause PhD who was a role model for me as an early scientist, and Kristen Getchell who imparted her experience and advice going through the same degree and thesis process.

My current company, Jounce Therapeutics, provided the lab space and materials to run all of the experiments part of this thesis work. Both Jounce and my previous company, Momenta Pharmaceuticals, were extremely supportive through my degree process both scientifically and financially.

I would also like to thank all my advisors, professors, and fellow students at Harvard University over the past five years. Special thank you to my thesis research advisor Steven Denkin, thesis coordinator Gail Dourian, thesis format advisor Trudi Goldberg Pires, and academic advisor Joan Short.

Lastly I would like to thank my family and friends for their encouragement throughout this entire process. When completing a degree part time seemed daunting they always believed in me.

Table of Contents

Dedication	iv
Acknowledgments.....	v
List of Tables	viii
List of Figures	ix
Chapter I. Introduction.....	1
Cancer Associated Fibroblasts and the Tumor Microenvironment	1
Markers of Fibroblast Activation.....	4
Modeling the Tumor Microenvironment <i>In Vitro</i>	8
Fibroblast Phenotype Changes in 3D Culture.....	11
MDA-MB-231 and SKBR3 3D Breast Cancer Models.....	13
Chapter II. Materials and Methods	19
Cell lines	19
2D Cell Co-Culture.....	20
3D Cell Co-Culture.....	20
Preparing Cells for Flow Cytometry Analysis.....	22
Chapter III. Results	26
Gating Strategy	26
Marker Expression in Co-Cultured Fibroblasts	30
Fibroblast Marker Expression on Tumor Cells.....	36
Marker Expression on Fibroblast in Mono-culture.....	38

Chapter IV. Discussion	41
References	48

List of Tables

Table 1. Dilution Schedules for 3D Spheroid Formation	21
Table 2. Suggested Volumes of PBS and Organoid Harvesting Solution	23
Table 3. Flow cytometry panel for fibroblast marker expression.....	24
Table 4. Expression in 2D fibroblast mono-culture.....	39
Table 5. Averages of fibroblast maker expression in 2D mono-culture and co-culture with MDA-MB-231 or SKBR3.....	40

List of Figures

Figure 1. Depiction of the normal versus tumor microenvironment (from Tripathi et al., 2012).	3
Figure 2. Fibroblast Phenotype in 2D versus 3D Culture from Woodley et al., 2021	12
Figure 3. Spheroids formed by SKBR3 and NHDF at different seeding densities.....	14
Figure 4. Histology of SKBR3/NHDF spheroids	15
Figure 5. Fluorescent Images of Co-culture Spheroids.	16
Figure 6. Spheroids from tumor cell mono-cultures.....	18
Figure 7. Trevigen 3D Spheroid Assay Protocol	22
Figure 8. Gating strategy for MDA-MB-231/ NHDF co-cultures	28
Figure 9. Controls used for gating MDA-MB-231/ NHDF co-cultures	28
Figure 10. Gating strategy for SKBR3/ NHDF co-cultures.....	29
Figure 11. Controls used for gating SKBR3/ NHDF co-cultures.	29
Figure 12. CD29 expression in 2D and 3D NHDF co-culture with MDA-MB-231 and SKBR3 cells.....	31
Figure 13. FAP expression in 2D and 3D NHDF co-culture with MDA-MB-231 and SKBR3 cells.....	32
Figure 14. PDGFRb expression in 2D and 3D NHDF co-culture with MDA-MB-231 and SKBR3 cells.....	33
Figure 15. Thy1.1 expression in 2D and 3D NHDF co-culture with MDA-MB-231 and SKBR3 cells.....	34

Figure 16. Cav-1 expression in 2D and 3D NHDF co-culture with MDA-MB-231 and SKBR3 cells.....	35
Figure 17. Expression of fibroblast markers on tumor cells in 2D co-culture with NHDF.....	37
Figure 18. Expression of five fibroblast markers on tumor cells in 2D mono-culture.....	38

Chapter I.

Introduction

Cancer Associated Fibroblasts and the Tumor Microenvironment

The extracellular matrix (ECM) is the physical and biochemical framework that provides structural and biochemical support to the surrounding cells in a given tissue. The ECM is also responsible for regulating intercellular communication and maintaining normal homeostatic processes (Malik et al., 2015). The interstitial ECM makes up what is called the stroma, which is composed mostly of collagens, fibronectin, elastin, glycosaminoglycans, and proteoglycans. The stroma fills the space between organs and surrounds mesenchymal cells, which differentiate into many different cells of the body including connective tissue, blood, and smooth muscles (Kozlova et al., 2020). The most common type of mesenchymal cell in the stroma are fibroblasts, which synthesize ECM components, such as collagen, that maintain and provide a structural framework (Malik et al., 2015; Woodley et al., 2021). These fibroblast cells function to maintain physiological processes, such as tissue repair in response to injury, but can lead to tissue pathology associated with fibrosis and cancer progression due to stromagenesis (Rhee et al., 2009).

Stromagenesis is a dynamic process of ECM remodeling initiated by signaling from tumor cells and orchestrated largely by stromal fibroblasts, which creates an increasingly permissive environment for tumor growth and metastasis (Beacham and Cukierman, 2005). These fibroblasts deposit a buildup of factors such as collagen, fibronectin, growth factors, and other components that leads to the formation of a dense

stroma surrounding and protecting the tumor (Tripathi et al., 2012). Particularly in breast cancer, the progression from ductal carcinoma in situ (DCIS) to invasive ductal carcinoma (IDC) is believed to be actively driven by complex interactions with the surrounding microenvironment including signaling with various stromal fibroblasts (Sung et al., 2013).

Normal tissue associated fibroblasts (NAFs) serve a protective function and regulate the growth of normal epithelium in healthy tissue. Normal fibroblasts are activated and undergo phenotypic transition into cancer associated fibroblasts (CAFs), also known as myofibroblasts, upon stimulation with factors produced by tumor cells, such as transforming growth factor beta (TGF- β) (Yoshida et al., 2019). CAFs proliferate at a higher rate, secrete increased levels of ECM proteins, and express unique markers from normal fibroblasts (Sadlonova et al., 2009). These activated myofibroblasts can promote the growth of breast cancer cells (Sadlonova et al., 2009) and play a critical role in the progression of invasion via enhanced secretion of cytokines, growth factors, and proteases (Sung et al., 2013). Figure 1 below shows the role of signaling between cell types in the tumor microenvironment that contribute to cancer progression.

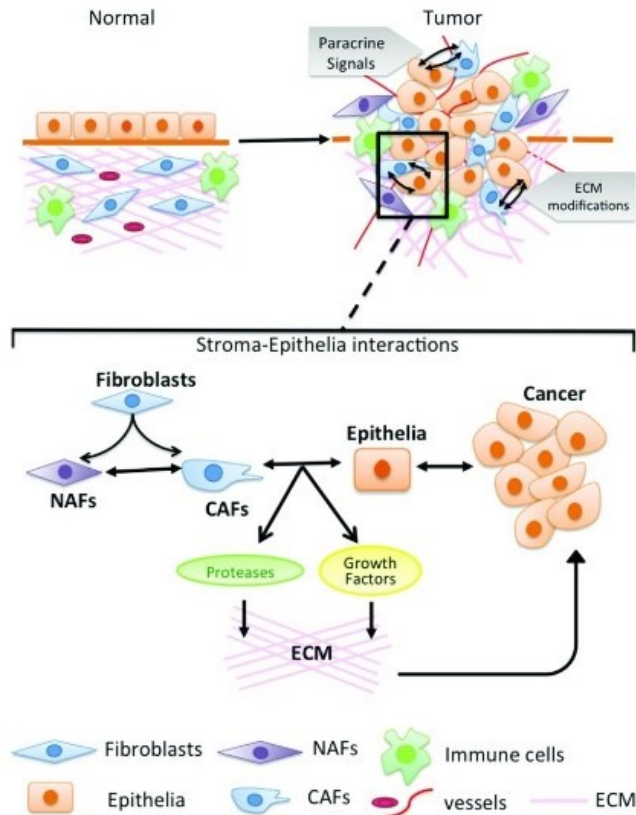


Figure 1. Depiction of the normal versus tumor microenvironment (from Tripathi et al., 2012).

Normal microenvironment contains fibroblasts, immune cells, blood vessels and epithelial cells. Normal fibroblasts (NAFs) are activated to cancer fibroblasts (CAFs) which cause remodeling of ECM by degradation and increased stiffness. The cross talk between normal fibroblasts, cancer fibroblasts and the epithelial cells leads to cancer progression.

Fibroblasts are especially predominant in the stroma of breast, lung, colorectal, prostate and pancreatic carcinomas creating a “desmoplastic” or “cold” microenvironment surrounding and protecting the tumor (Mahaidley and Mechta, 2020). This desmoplastic microenvironment results in less blood vessels for efficient penetration and delivery of drugs to the tumor as well as limited immune cell infiltration. This in turn causes increased resistance of the tumor to therapeutic intervention (Tripathi et al, 2012).

In these tumor types therefore, the development of new therapies targeting the stroma could have curative outcomes (Tripathi et al., 2012). Targeting immunosuppressive fibroblasts in the tumor microenvironment through specific markers of activation may also help to re-sensitize cancer cells to immune checkpoint therapies, such as PD-1 and CTLA-4 inhibitors, which activate immune cells to attack the tumor, as well as chemotherapies, making them potential options for combination treatments (Mahaidley and Mehta, 2020). Characterizing the expression of fibroblast specific markers through the use of *in vitro* tumor models may provide important information for identifying and studying potential cancer therapies specifically targeted to fibroblasts in the tumor microenvironment (Tripathi et al., 2012).

Markers of Fibroblast Activation

CD29, fibroblast activation protein (FAP), platelet-derived growth factor receptor beta (PDGFRb), and Thy1.1 have been well documented fibroblast activation markers that indicate their transition to CAFs (Mhaidly and Mehta 2020). Caveolin-1 (Cav-1) is expressed on the surface of fibroblasts and associated with a more quiescent state (Shen et al., 2015). Analysis of combinations of these markers have previously identified distinct CAF populations both *in vitro* and in human patients and linked particular CAF subsets with higher levels of activation markers to tumor growth and worse prognosis (Kieffer et al., 2020).

CD29, also known as integrin beta 1, is a cell surface receptor that mediates cell interaction with the extracellular matrix. CD29 forms integrin complexes that function as collagen receptors and regulates ECM stiffness and remodeling, facilitating cancer cell migration (Zeltz et al., 2020). Pelon et al. identified a subset of CAFs with high levels of

CD29 and α -SMA (smooth muscle actin, another common marker of fibroblast activation) that had higher motility and invasiveness into the surrounding matrix than other fibroblast subsets. They also found that the presence of these CD29 high fibroblasts in the tumor microenvironment of patients with breast cancer was associated with reduced overall survival (Pelon et al., 2020). Although CD29 is known to be involved in maintaining a CAF-like phenotype and is associated with worse prognosis in certain cancers, this protein is also expressed on multiple other cell types, including some tumor cells, so it is not a CAF-specific biomarker (Zeltz et al., 2020).

A more specific biomarker to identify CAF subsets is FAP, which is upregulated in activated fibroblasts and absent in quiescent fibroblasts (Lindner et al., 2019). FAP is a type II membrane-bound glycoprotein that is highly upregulated at sites of active tissue remodeling, including wound healing, fibrosis, and cancer (Zeltz et al., 2020). This protein is known to be immunosuppressive, involved in tumor angiogenesis, and associated with poor prognosis in cancer patients. Fibroblasts expressing FAP are broadly present in the microenvironment of a variety of tumors, including pancreas, breast, and lung, and thus could allow targeting of different tumor entities (Lindner et al., 2019). Preclinical studies with FAP targeting therapies have shown promising results in increasing fibroblast killing, chemotherapy penetration, suppression of metastasis, and in decreasing collagen expression (Lindner et al., 2019). Although a FAP-targeted antibody failed in a Phase 1 clinical trial for metastatic colorectal cancer, this is a good example of the potential benefits that a therapy targeted specifically to fibroblast cells within the tumor microenvironment could bring to patients (Zeltz et al., 2020).

PDGFRb, is the receptor for platelet-derived growth factor (PDGF), a mitogen and chemoattractant that plays a central role in modulating a broad range of cellular functions, including angiogenesis, cell migration, and ECM protein production in a variety of tissues (Hewitt et al., 2012). Activation of the PDGFRb receptor stimulates the recruitment of mesenchymal cells and is required for transition of fibroblasts to activated myofibroblasts (Agorku et al., 2019). The presence of PDGFRb-positive fibroblasts in the tumor microenvironment has been proven to correlate with cancer progression through multiple mechanisms, including inducing angiogenesis and immune evasion (Hu et al., 2021). PDGFRb positivity on fibroblasts is associated with shorter survival in patients with solid tumors, especially in non-small cell lung, breast, and pancreatic cancer. Hence, PDGFRb is a promising prognostic biomarker and a potential therapeutic strategy for patients with high expression of this receptor (Hu et al., 2021).

Thy1.1, also known as CD90.1, is another widely known protein for identifying fibroblasts from different species and tissues. This protein has been shown to mediate cell-cell interactions by binding to integrins in a variety of tissue contexts, including tumor tissue (Shliekelman et al., 2017). Agorku et al (2019) used the Thy1.1 protein to differentiate fibroblasts present in mouse tumors from other cell types and showed its correlation with increased proliferation. Only Thy1.1 positive fibroblasts are capable of myofibroblast differentiation after treatment with TGF- β (Koumas et al., 2003). In human lung cancers, a population of CAFs has been isolated that expresses Thy1.1, α -SMA, and FAP. These fibroblasts displayed a phenotype which is consistent with that of an activated myofibroblast, and elicited tumor metastasis and suppression of tumor-associated T-cell activation (Shliekelman et al., 2017). The presence of Thy-1.1-positive

fibroblasts in the tumor microenvironment was also shown to correlate with worse prognosis in patients with lung adenocarcinoma, making it a good biomarker candidate (Shliekelman et al., 2017).

Cav-1 is a scaffolding protein that is present on many different cell types, including fibroblasts, and has been shown to have inhibitory effects on the majority of interacting proteins (Haines et al., 2012). Shen et al. identified Cav-1 as an important stromal inhibitor of myofibroblast differentiation. Cav-1 acts to negatively regulate TGF- β -mediated activation of fibroblasts by inducing its internalization and subsequent degradation. Loss of Cav-1 expression is associated with increased deposition of ECM components and hyperactivation of TGF- β signaling pathway, potentially leading to a worse prognosis in cancer patients (Shen et al., 2014). Cav-1 expression may be downregulated in tumors via degradation and autophagy in a hypoxic and malnourished tumor microenvironment (Haines et al., 2012). This protein acts in the tumor microenvironment in contrast to the other four proteins described previously, which are upregulated in response to fibroblast activation, and can be used as marker of fibroblast quiescence.

As described above, fibroblasts are a very plastic cell type that can transition between different states of activation, both *in vivo* and when cultured in artificial *in vitro* systems. This thesis work will look at these five markers associated with activated fibroblasts to determine if normal human dermal fibroblasts in co-culture with tumor cells have transitioned to cancer associated fibroblasts in various *in vitro* culture conditions. All of these markers are present on the cell surface, allowing them to be stained with fluorescently conjugated antibodies and quantified by flow cytometry. Fibroblasts with

an activated phenotype would show higher expression of CD29, FAP, PDGFRb, and Thy1.1 and lower expression of Cav-1. Taken together, the expression of these five markers on fibroblasts will help to characterize their phenotype and level of activation. However, this is not an exhaustive list of biomarkers that can be used for CAF identification or biomarker analysis.

Modeling the Tumor Microenvironment *In Vitro*

Creating a physiological representation of the tumor microenvironment in a laboratory setting is critical to understanding the biology of different pathways driving tumor growth and testing the efficacy of potential therapeutics that could target this. Cell culture performed on traditional plastic or polystyrene limits studying tissue functions while also providing a physiologically irrelevant environment (Cummins et al., 2021). *In vitro* assays involving this two-dimensional (2D) monoculture often times do not reflect the complex extracellular matrix, chemical signaling, and cellular microenvironment of the tumor tissue, which may explain the failure of 2D models to predict clinical efficacy in many cases (Benton et al., 2015). The composition and structural properties of the ECM make it an important addition for studying morphology, gene expression, proliferation, invasion, and drug response for cancer cells grown *in vitro*. With the addition of an ECM gel, cells in culture are able to self-assemble into more physiological three-dimensional (3D) structures that display their invasive potential and correlate more highly with their gene expression profiles *in vivo* (Benton et al., 2015).

The use of three-dimensional tumor spheroid assays has recently become an emerging method to study the tumor microenvironment, because of the adaptability of these assays to study specific components of interest (Paramesh et al., 2018). Cells can be

seeded in a matrix, often consisting of ECM proteins, such as collagen, or a basement membrane-like matrix, such as Matrigel, that allow the formation of three-dimensional spheroid structures. During malignant progression *in vivo*, tumor cells acquire the ability to degrade the extracellular matrix and invade the surrounding tissue/spread to other organs (Vinci et al., 2015). This remodeling of the ECM can be recapitulated in 3D models as changes in spheroid morphology and phenotype. Tumor cells that are less malignant form 3D spheroids that grow in tighter clumps, whereas more highly malignant cells have the ability to degrade the surrounding matrix and display an invasive phenotype (Benton et al, 2015)

To mimic complexity of the tumor microenvironment and study the interaction between tumor cells and other cell types, fibroblasts, endothelial cells and/or immune cells may also be added to the 3D culture (Benton et al., 2015). 3D tumor and fibroblast co-culture methods provide a more physiological representation of the TME than typical 2D methods to study “cold” or stroma heavy tumors. These 3D models can be used to study the role of fibroblasts in tumor spheroids, specifically the expression of markers associated with the transition to cancer associated fibroblasts and the effect on phenotype of co-culture fibroblasts with tumor cells.

In work done at my previous company described by MacDonald et al (2019), pancreatic stellate cells, the most abundant type of fibroblast present in pancreatic cancer, were co-cultured with Aspc-1 pancreatic tumor cells in a 3D assay. The addition of fibroblast cells was crucial towards driving tumor cell invasion into the surrounding ECM as shown by the difference in spheroid phenotype as well as protein expression. This invasive pancreatic model was used to study the effect of a therapeutic candidate in

combination with chemotherapy on invasion and modulation of the tumor microenvironment, which was shown to be recapitulated in clinical trial patients with pancreatic cancer (MacDonald et al., 2019).

Sung et al (2013) showed that mammary fibroblasts cultured with breast cancer cells display different functional activity in 3D due to increased signaling, which in turn drives tumor cells to become more aggressive. Vinci et al (2015) developed 3D models of human glioblastoma and head and neck cancer with simple protocols that have allowed the quantification of the tumor cell invasion into the surrounding matrix over three days using a standard imaging technique. The model and protocol for this thesis work is very similar to the one described in Vinci et al. (2015), but uses the “Cultrex 3D Spheroid Invasion Assay” kit commercially available from Trevigen. This kit and protocol were also verified by Ravi et al (2014). Prior work in 3D spheroid systems has generally focused on the cancer or fibroblast cells alone and the role of fibroblast culture conditions on tumor-stromal cell interactions is still largely unknown (Sung et al., 2013).

Yakavets et al (2020) used 3D spheroids to create a breast cancer model consisting of MCF-7 breast cancer cells and MRC-5 fibroblasts to characterize the expression of α -SMA, a common marker of cancer associated fibroblasts, by immunohistochemistry. They also added TGF- β to the spheroid cultures to induce higher levels of fibroblast activation, proliferation, and migration to better study the subsequent effect on stromal components. The breast cancer model in this thesis uses similar methods and parameters as optimized in Yakavets et al (2020), including a 1:3 tumor to fibroblast ratio. Dolznig et al (2011) created a spheroid model consisting of a colon cancer line and normal colon fibroblasts embedded in collagen and analyzed for

expression of fibroblast activation markers through immunohistochemistry. They found that the normal fibroblasts grown in the 3D co-culture spheroids showed positive expression for PDGFRb, Thy1.1, FAP, and other markers closely resembling activated fibroblasts found *in vivo*.

Fibroblast/tumor co-culture 3D spheroids are an emerging way to study the tumor microenvironment, however there is little work directly comparing the effects of this method to traditional 2D culture methods (Beacham and Cukierman, 2005). Instead of using immunohistochemistry as a readout like many of these previous studies, the experiments in this thesis look at the expression of the CAF markers CD29, FAP, Thy1.1, PDGFRB, and Cav-1 using a flow cytometry method to compare tumor cells and fibroblasts co-cultured in 2D versus 3D.

Fibroblast Phenotype Changes in 3D Culture

Characterizing the differences in phenotype of fibroblasts grown in 2D versus 3D is important for understanding cell function and developing more accurate models of various diseases such as cancer and fibrosis (Cummins et al., 2021). The benefit of using a three-dimensional system where the fibroblasts are surrounded by an ECM is evident in their morphology, which is different from the flat, adherent morphology cells assume when moving on a solid 2D substrate (Vinci et al., 2015). The mechanical stretching of normal fibroblasts due to the stiff, plastic surface of 2D culture systems, coupled with the lack of ECM signals, induces phenotypic changes similar to those of CAFs (Mhaidly and Mehta, 2020). Smithmyer et al. (2019) found that fibroblasts grown in traditional 2D culture were more α -SMA positive and had higher rates of proliferation compared to those grown in 3D spheroid culture. 2D fibroblasts become more activated and

subsequently produce large quantities of matrix proteins and focal adhesions regardless of pathology or phenotype, as shown in Figure 2 (Cummins et al., 2021). The high activation of fibroblasts across 2D culture models makes them a non-ideal system to detect changes or predict therapeutic efficacy. Fibroblast phenotype and levels of activation are much more variable in 3D than 2D culture, which is more representative of their different activation states observed *in vivo*. This variability is due to the different mechanics of the gel they are suspended in, which can be experimentally manipulated to mimic the physiology of the *in vivo* tissue of interest. Figure 2C shows the difference in more relaxed or easily contractible gels where the fibroblasts remain quiescent versus more stiff and anchored gels where the cells become more activated (Woodley et al., 2021).

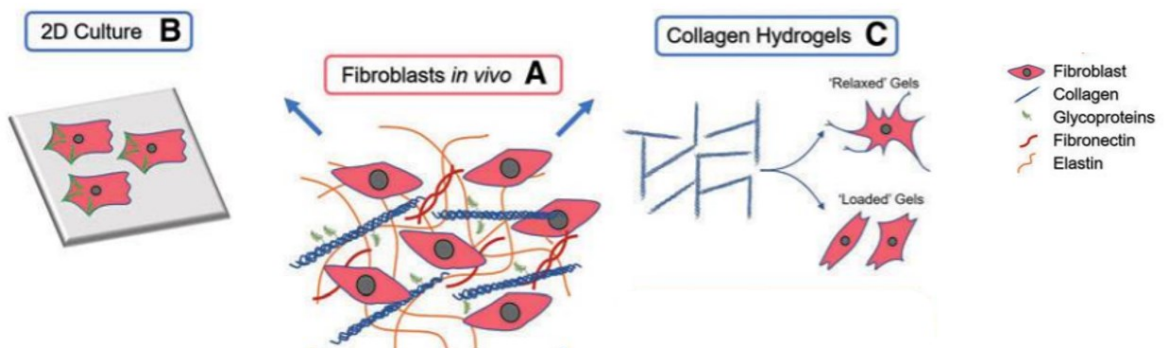


Figure 2. Fibroblast Phenotype in 2D versus 3D Culture from Woodley et al., 2021
A) Fibroblasts interact with components of the ECM *in vivo*. B) Stiff 2D culture is known to “activate” fibroblasts with the formation of α -SMA stress fibers (shown in green). C) Two distinct collagen hydrogel states, “relaxed” or “mechanically loaded”, yield distinct fibroblast phenotypes. Quiescent fibroblasts are dendritic in appearance, while stellate and bipolar fibroblasts were activated with α -SMA expression and high proliferation rates.

Moreover in 3D culture fibroblasts interact with proteins on all sides, as they would *in vivo* (Smithmyer et al., 2019), therefore fibroblasts grown in 3D culture may be more sensitive to activation agents, such as TGF- β , through complex signaling pathways (Beacher and Cukieron 2005). This also gives 3D culture models greater potential for identifying and studying efficacy of therapeutic interventions targeted to stromal fibroblasts. Less well studied is the change in fibroblast phenotype co-culture in a tumor spheroid model directly compared to a 2D counterpart.

MDA-MB-231 and SKBR3 3D Breast Cancer Models

In preliminary experiments, I decided to compare two different breast cancer cell lines that display different invasive properties *in vivo*. The MDA-MB-231 cell line was established from a pleural effusion of a 51-year-old Caucasian female with a metastatic mammary adenocarcinoma and is one of the most commonly used breast cancer cell lines (Holliday & Speirs, 2011). MDA-MB-231 cells are invasive *in vitro* and when implanted orthotopically produce xenografts that spontaneously metastasize to lymph nodes (Welsh, 2013). SKBR3 cells were derived from a pleural effusion originating in a 43-year-old Caucasian female with adenocarcinoma. These cells overexpress human epidermal growth factor receptor 2 (HER2), which plays an important role in the development and progression of certain aggressive types of breast cancer, however these SKBR3 are much less tumorigenic in mice than MDA-MD-231 cells (Holiday & Speirs, 2011).

In preliminary experiments, I optimized a 3D co-culture model of SKBR3 tumor cells with normal human dermal fibroblasts (NHDF). To ensure that the tumors were an

appropriate size and to avoid necrosis in the spheroid core, experiments were done to determine the final seeding density of 3,000 tumor cells and 9,000 fibroblast cells per well for this thesis. 3,000 tumor cells were determined to be optimal for maintaining an appropriate size range of the spheroids and the addition of fibroblasts did not drastically change the size of the spheroids only their shape. Several ratios of SKBR3 tumor cells to fibroblasts were also compared (Figure 3). The morphology changed as the amount of fibroblasts in the spheroid increased: the 1:3 ratio was determined to produce a more dramatic budding phenotype and chosen for all following experiments. The same 1:3 ratio was also used for the MDA-MB-231 co-cultures for consistency, and the additional fibroblasts did not change spheroid size or morphology in this case.

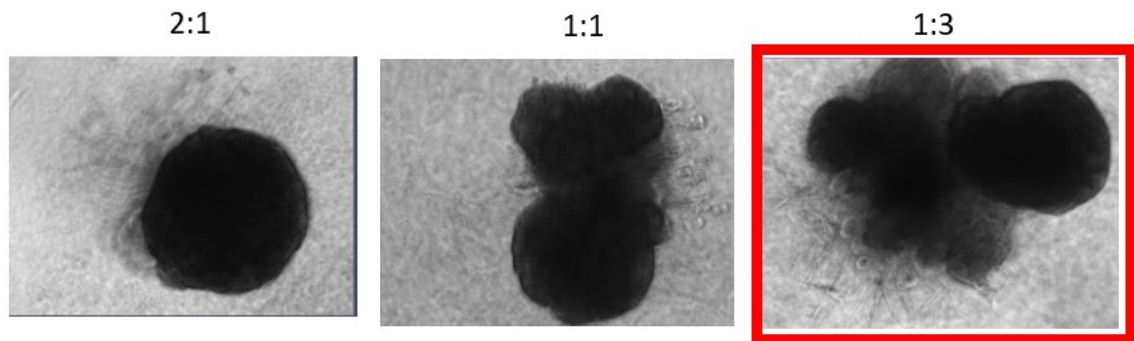


Figure 3. Spheroids formed by SKBR3 and NHDF at different seeding densities 3000 tumor cells were plated in each well with the ratios of tumor cells: fibroblasts are indicated above. Representative images are shown. The 1:3 ratio was chosen for follow-up experiments.

SKBR3/NHDF spheroids at the 3,000:9,000 seeding density were also analyzed by hematoxylin and eosin (H&E) staining (Figure 4A). Hematoxylin stains cell nuclei a purplish blue, and eosin stains the extracellular matrix and cytoplasm pink. The same spheroid was also stained with vimentin, an indicator of mesenchymal cells or fibroblasts, and cytokeratin, indicating epithelial or tumor tissue. This revealed that the fibroblasts were located mostly in the spheroid core and the tumor cells were located in the spheroid periphery. The size of the spheroids was estimated to be within the range recommended by the Trevigen kit (300-500 microns).

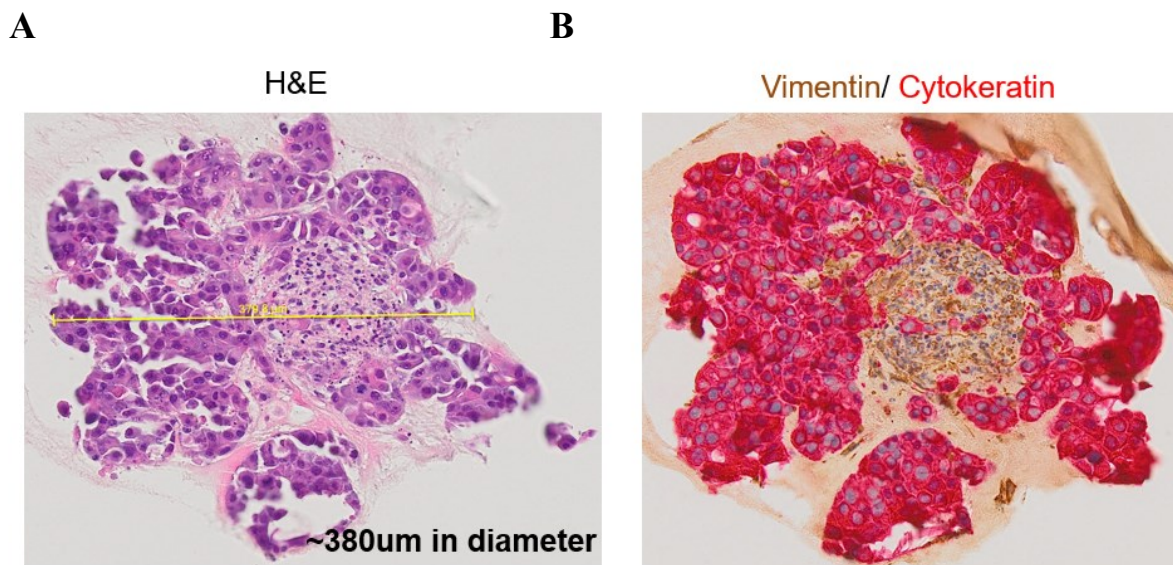


Figure 4. Histology of SKBR3/NHDF spheroids
A) H&E staining of spheroid at 3:000:9:000 tumor cell to fibroblast ratio. The diameter of this spheroid is 380 microns. B) The same spheroid stained with vimentin (brown) and cytokeratin (red).

To better compare the phenotype and location of fibroblasts within the SKBR3 or MDA-MB-231 spheroids, tumor cells were labelled with cell tracker blue and NHDF

tagged with fluorescent green fluorescent protein (GFP) and images were captured on a fluorescent microscope on Day 7 (Figure 5).

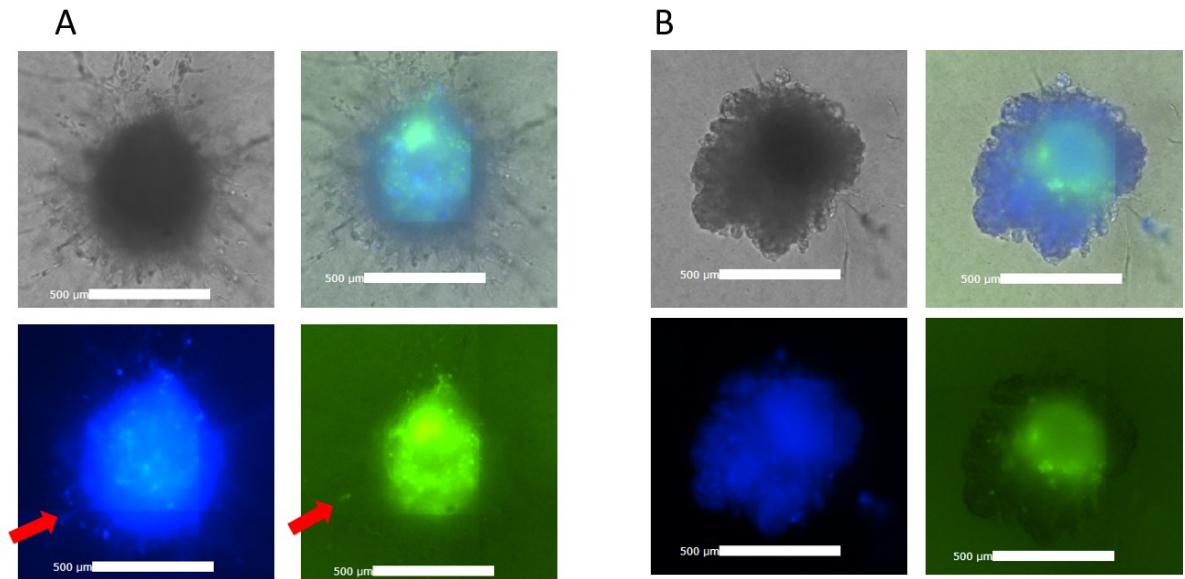


Figure 5. Fluorescent Images of Co-culture Spheroids. Representative images showing spheroids from NHDF and MDA-MB-231 (A) or SKBR3 (B) tumor cells. Tumor cells are labeled in blue (bottom left in each panel) and NHDF in green (bottom right). Phase contrast (left) and merged (right) images are shown on top. The red arrows indicate a “spike” where both MDA-MB-231 cells and fibroblasts invade into the extracellular matrix. The white bar indicates 500uM scale.

The location of the fibroblasts within the spheroid and the overall spheroid morphology was different between the two 3D co-culture models. In MDA-MB-231/NHDF spheroids, the fibroblasts are invading with the tumor cells into the surrounding matrix and have a “spiky” phenotype. In contrast, the fibroblasts in SKBR3/NHDF spheroids have a “budding” phenotype, where the buds are made up of

invading tumor cells only and the fibroblasts remain mostly in the spheroid core, as seen in the previous H&E staining.

Spheroids consisting of either MDA-MB-231 or SKBR3 tumor cells alone were also formed with a similar protocol and imaged (Figure 6). In the MDA-MB-231 only spheroids, the morphology is similar to the invasive “spiky” phenotype observed in the fibroblast co-cultures and the tumor cells are still growing into the extracellular matrix. This phenotype is similar to what is described in Han et al., (2010) and Ravi et al., (2014), where the morphology of 24 different breast cancer cell lines in 3D was studied and subtyped into groups, including the MDA-MB-231 “spiky” phenotype, based on gene expression profiles (Han et al, 2010). MDA-MB-231 breast cancer cells displaying the “spiky” phenotype are associated with more aggressive invasive behavior both *in vivo* and *ex vivo* (Han et al, 2010).

In the SKBR3 only spheroids, the morphology remains round throughout the seven day experiment and there is no “budding” invasion into the matrix as seen in the co-culture spheroids. This means that the addition of fibroblasts to the culture are responsible for the change in the phenotype of SKBR3 spheroids, perhaps due to differences in activation state between co-cultures. Analyzing the differences in marker expression on fibroblasts in co-culture with both types of cancer cells may help to explain the differences in their behavior.

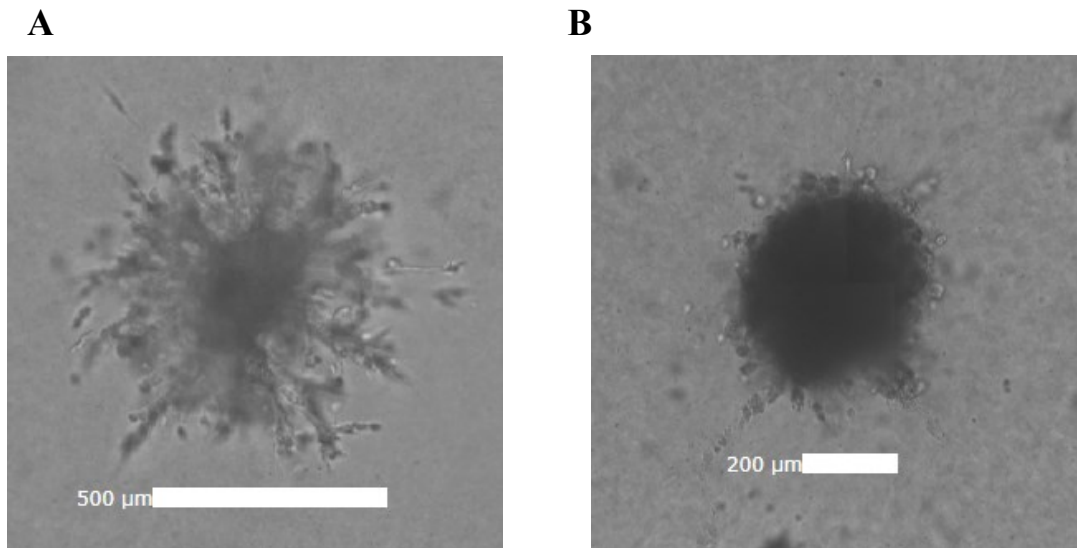


Figure 6. Spheroids from tumor cell mono-cultures. A) MDA-MB-231 tumor cell spheroids with “spiky” invasive phenotype. B) SKBR3 tumor cell spheroids with round phenotype. The white bars indicate 500um (A) or 200um (B) scale.

This thesis work looks specifically at the marker expression of fibroblasts co-cultured with SKBR3 and MDA-MB-231 cells and the resulting changes in phenotype and behavior. I compare fibroblasts and tumor cells grown in 3D spheroids to a 2D control to determine the effect of differences in culture method. There are still relatively few studies directly comparing 2D versus 3D *in vitro* systems and accurately modeling the tumor microenvironment may help to better predict clinical efficacy of potential therapeutics (Sung et al., 2013). Studying fibroblast marker expression and phenotype may also help inform cancer therapies that are targeted specifically to the fibroblasts in the tumor microenvironment.

Chapter II.

Materials and Methods

Different culture methods for the tumor and fibroblast cells were optimized in both two-dimensional plastic culture flasks and three-dimensional organoids surrounded by a collagen matrix. After culturing, the cells were stained with antibodies for several markers of fibroblast activation and analyzed by flow cytometry. This section will describe those methods and materials used for the thesis experiments.

Cell lines

GFP-expressing adult human dermal fibroblasts from normal tissue (NHDF) were purchased from Angio-Proteomie (#cAP-0008-adGFP) and cultured in Fibroblast Growth Medium also from Angio-Proteomie (# cAP-44). SKBR3 cells, commercially available from ATCC (HTB30), were grown in McCoy's media (ATCC 30-2007) with 10% fetal bovine serum (FBS). MDA-MB-231 cells, also available from ATCC (HTB-26), were grown using DMEM (ThermoFisher 72400146) with 10% FBS. All cell lines were cultured according to the manufacturer's instructions in 2D and expanded enough to perform co-culture experiments. To set up co-culture experiments, cells roughly 80% confluent were detached with Trypsin/EDTA solution, counted and assessed for viability on a Beckman ViCell counter. All cells used for experiments were >90% viable before proceeding to either 2D or 3D co-culture set up.

2D Cell Co-Culture

A six well plate was used to seed 2D co-cultures. The tumor to fibroblast cell ratio determined to be optimal during several pilot experiments that was used for all experiments was a 1:3 ratio for both MDA-MB-231 and SKBR3 plus NHDF co-cultures and are described in the next section. 100,000 SKBR3 or MDA-MB-231 plus 300,000 NHDF were added to each of the wells in the six well plate. The plates were incubated for seven days at 37 °C 5% CO₂ before the cells were harvested for flow cytometry analysis.

3D Cell Co-Culture

3D co-cultures were set up at the same time as the 2D cultures with the same cells. For 3D spheroid formation, the Cultrex 3D Spheroid Cell Invasion Assay (Trevigen Cat# 3500-096-K) was used. The 1:3 ratio of tumor cells to fibroblasts that was used for the 2D co-cultures was also used for the 3D culture spheroids.

On Day 0, a single cell suspension was prepared in the 10X Spheroid Formation ECM included in the kit, after being thawed on ice. Since plating a co-culture of cells, a 1:1 mix of the media for each respective cell line was used. See Table 1 below for calculations to prepare seeding mixture which included a 10% overage for multi-well dispensing.

Table 1. Dilution Schedules for 3D Spheroid Formation

Reagent	1 Well	24 Wells	96 Wells
10X Spheroid Formation ECM (4 °C)	5 µl	132 µl	528 µl
Tissue Culture Growth Medium (4 °C)	45-X µl	1188-X µl	4752-X µl
Cells	X µl	X µl	X µl
Total	50 µl	1320 µl	5280 µl

50 µl of the single cell suspension was dispensed in each well of the 3D Culture Qualified 96 Well Spheroid Formation Plate and centrifuged at 200 x g for 3 minutes at room temperature in a swinging bucket rotor. Half of the 96 well plate consisted of SKBR3/NHDF co-culture spheroids, and the other half of MDA-MB-231/NHDF spheroids. The plate was then incubated at 37 °C and 5% CO₂ in a tissue culture incubator for 72 hours to promote spheroid formation.

On Day 3, the invasion matrix was thawed and the 3D Culture 96 Well Spheroid Formation Plate placed on ice for 15 minutes to cool wells. While continuing to work on ice, 50 µl of the Invasion Matrix was added to each well of the plate being careful not to introduce any air bubbles. Plates were then centrifuged at 300 x g at 4°C for 5 minutes in a swinging bucket rotor to eliminate bubbles and position spheroids within the Invasion Matrix towards the middle of the well. The plate was transferred to a tissue culture incubator set at 37 °C and 5% CO₂ for one hour to promote gel formation of the Invasion Matrix. After one hour of incubation, 100 µL of mixed media was added to each of the wells, again being careful not to introduce any air bubbles in the wells. The plates were then placed in an incubator for the remaining four day duration of the assay. 2D monoculture of each cell type – MDA-MB-231, SKBR3, and NHDF were kept in culture

throughout the assay to be used as controls in the flow cytometry analysis. Figure 7 shows a schematic of the spheroid formation process.

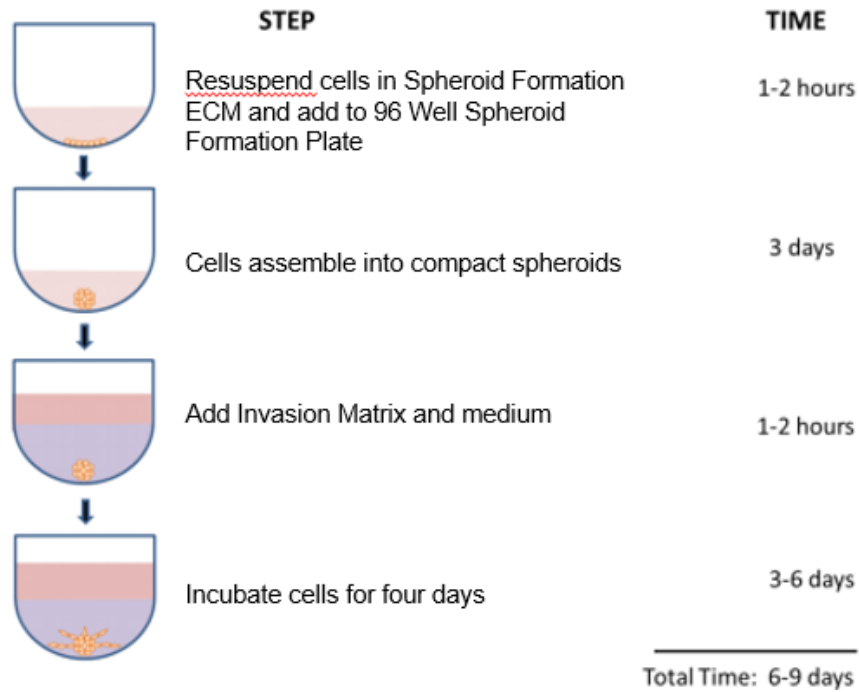


Figure 7. Trevigen 3D Spheroid Assay Protocol
Experiments in this thesis followed a 7 day protocol over which 3D spheroids were formed. Day 0 included plating the cells diluted in a spheroid formation ECM. Invasion matrix was added on Day 3 and incubated for an additional four days.

Preparing Cells for Flow Cytometry Analysis

On Day 7, the 2D cultures were harvested with Trypsin/EDTA as described above, and the 3D spheroids were harvested using Trevigen’s Cultrex Organoid Harvesting Solution (Cat #3700-100-01). The organoid harvesting solution provides a non-enzymatic method for depolymerizing extracellular matrix proteins to allow for

harvesting of intact organoids. Fibroblast marker expression was previously shown to be unaffected by treatment with this solution (data now shown). The following table is a guide for suggested working volumes for cold (4 °C) PBS and Organoid Harvesting Solution.

Table 2. Suggested Volumes of PBS and Organoid Harvesting Solution

Plate Type	Volume of Basement Membrane Matrix	Volume of PBS and Organoid Harvesting Solution
96 Well Plate	5 μ l	50 μ l
48 Well Plate	25 μ l	250 μ l
24 Well Plate	50 μ l	500 μ l

Working on ice, the cell culture media was aspirated, and each well was gently collected into a 15 mL tube, one for each type of co-culture. Each tube of spheroids was pelleted and washed with 10 volumes of cold PBS being careful not to disrupt basement membrane matrix containing organoids. The PBS was then carefully aspirated, and 10 volumes of cold Organoid Harvesting Solution was added to each of the tubes. The tube was then incubated at 4 °C for 60 minutes with moderate shaking. This incubation was complete when the basement membrane matrix dome was no longer visible and the organoids were seen floating around in the solution. After incubation, each tube was again spun down at 500 x g for 5 minutes at 4 °C in a swinging bucket rotor to pellet the cells, and the harvest buffer aspirated. The cells were washed again with cold (4 °C) PBS, and the centrifugation step repeated. The pellet was then re-suspended in FACS buffer (PBS, 5% FBS, 2 mM EDTA) to be used for flow analysis.

To prepare the cells for flow cytometry, they were first stained with the BV510 Zombie Aqua Viability Dye (Biolegend 423101). The dye was re-suspended in 100 μ L

DMSO, according to the manufacturer’s instructions, before being used. A 1:2,000 dilution of the viability dye was prepared in PBS. Cells were added to a 96 well plate, centrifuged, and excess liquid discarded. 200 uL of the viability stain solution was added to each well and incubated in the dark at 4°C for 30 minutes. The plate was then centrifuged and washed with FACs buffer. “Heat killed” positive controls were prepared by incubating MDA-MB-231 and SKBR3 at 70 °C for 10 minutes to induce cell death and ensure a strong positive signal for BV510. To reduce non-specific binding in the samples, a solution of true stain Fc block (Biolegend 422301) diluted 1:20 in FACS buffer was added to the cells in 100 uL volume. The plate was incubated for 30 minutes at 4°C.

A master mix of antibodies against the 5 fibroblast markers and labeled with different fluorophores was prepared using the dilutions in the table 3 below and added to each sample.

Table 3. Flow cytometry panel for fibroblast marker expression

Marker	Clone	Color	Dilution	Vendor and Catalogue Number
CD29	TS2/16	PECy7	100	Biolegend #303026
PDGFRb	28D4	BV805	50	BD #74900
Caveolin 1	4H312	PE	20	SC-70516 PE
FAP	427819	APC	50	R&D FAB3715A
Thy 1.1	5e10	AF700	100	Biolegend #328120
Zombie Aqua viability	N/A	BV510	1:2000	Biolegend #423101
GFP	N/A	FITC	N/A	N/A

“Fluorescence Minus One” (FMO) controls were prepared for each marker that included all of the antibodies except the one for the specific marker to ensure proper gating. After the antibodies were added to the samples, the plate was incubated at 4°C for 30 min in the dark then washed twice, re-suspended in 4% paraformaldehyde, and stored at 4°C protected from light until use for flow cytometry analysis.

Samples were run on a BD Fortessa instrument for data acquisition. Single stain controls were prepared for each color using beads to compensate overlap between each fluorescence channel. The single stained “heat killed” controls consisting of the tumor cells alone were used to compensate BV510. There are five channels from the antibodies listed in the table above plus the BV510 viability marker and the fluorescent GFP fibroblasts which were expressed in channel FITC. Flow cytometry data was analyzed using FlowJo and graphed using Prism.

Chapter III.

Results

Normal human dermal fibroblasts were co-cultured with either MDA-MB-231 or SKBR3 tumor cells, in both two- and three-dimensional co-culture models. Fibroblasts were then analyzed by flow cytometry for their expression of fibroblast activation markers including CD29, FAP, PDGFRb, Cav-1 and Thy1.1 (see table 3) to assess the effect of different culturing methods. This section describes the results of these experiments.

Gating Strategy

After the co-culture samples were collected, stained, and acquired on the flow cytometer, a gating strategy was first determined to distinguish different cell populations so that fibroblast markers could be assessed on cells of interest. The gating strategy was the same between the MDA-MB-231/ NHDF and SKBR3/ NHDF co-cultures, however the gates are slightly adjusted for differences in cell size and brightness. In the initial gating step, the main cell population was gated based on the size and granularity of the cells (forward scatter, FSC, versus side scatter, SSC, plot) to eliminate debris and small fragments. Then a single cell gate was applied to the population (FSC area versus height) to eliminate doublets and aggregates that could alter data. Dead cells were excluded by means of staining with the BV510 Zombie Aqua dye, which is non permeant to live cells but permeant to dead cells with compromised membranes. To more accurately draw the

viability gate, a “heat killed” cell control was used for both MDA-MB-231 and SKBR3 cells that had a distinct separation in the positive and negative BV510 signal. In 3D co-cultures with both types of tumor cells, viability was slightly lower than the corresponding 2D co-cultures. This is probably due to some necrosis within the core of the spheroid. This core necrosis is more reflective of tumors grown *in vivo* and is a key factor that influences response to therapy.

To differentiate fibroblasts from tumor cells in the co-culture samples, a gate was drawn using FITC positivity because the fibroblasts are tagged with GFP, which fluoresces in the FITC channel. Both MDA-MB-231 and SKBR3 cells alone with no fibroblasts added were run as a control to draw the FITC gate. Although fibroblasts were originally seeded at a much higher density than the tumor cells, their percentage in the culture at the end of seven days was much lower than the tumor cell percentage due to the slower growth rate of fibroblasts. The gating strategies for both the MDA-MB-231/NHDF and SKBR3/NHDF co-cultures in 2D and 3D are shown in the Figures 8 through 11 below.

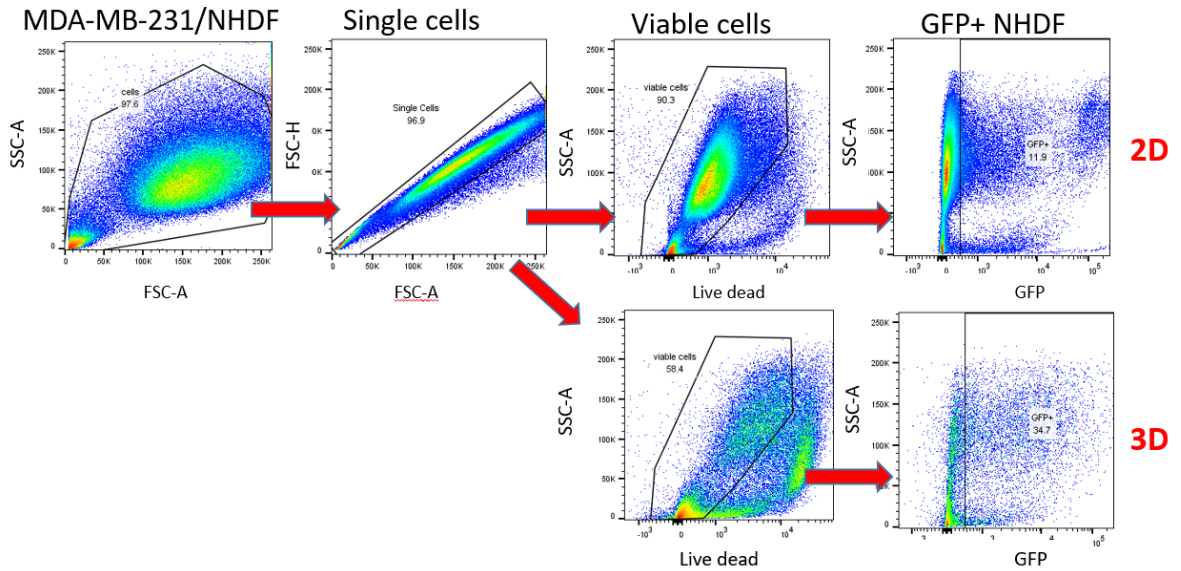


Figure 8. Gating strategy for MDA-MB-231/ NHDF co-cultures
 The MDA-MB-231/NHDF co-cultures in both 2D and 3D were first gated on the main cell population, followed by single cells and viable cells. The GFP+ fibroblasts were then gated out to look at their marker expression.

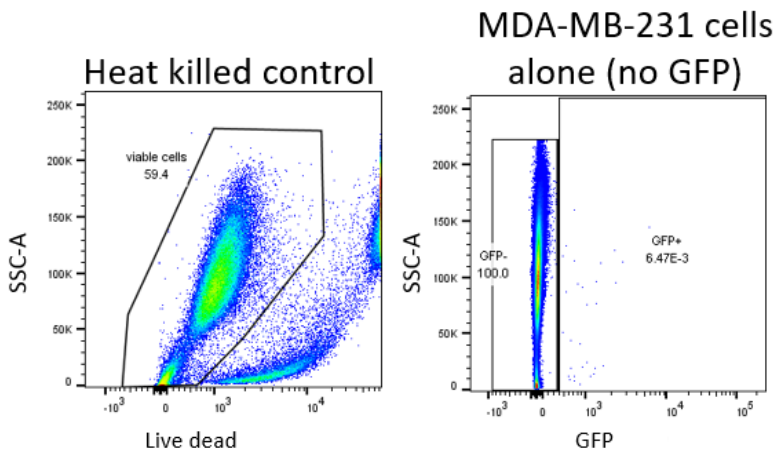


Figure 9. Controls used for gating MDA-MB-231/ NHDF co-cultures
 A “heat killed control” was used for the viability gate to distinguish the live and dead cells more clearly. The tumor cells alone were used to determine the GFP+ gate.

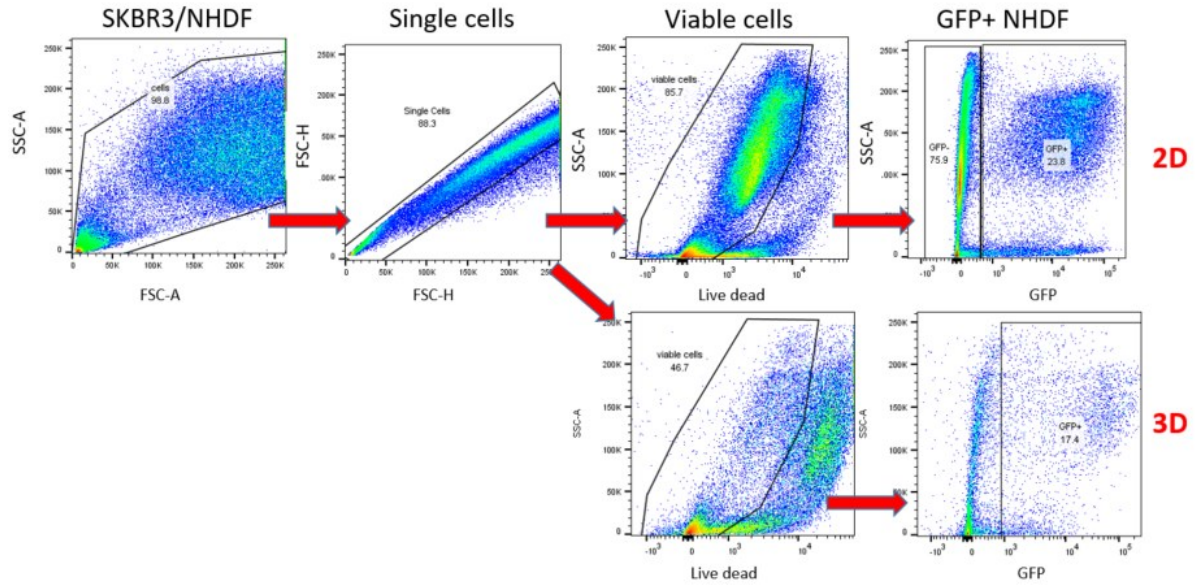


Figure 10. Gating strategy for SKBR3/ NHDF co-cultures
 The SKBR3/NHDF co-cultures in both 2D and 3D were first gated on the main cell population, followed by single cells and viable cells. The GFP+ fibroblasts were then gated out to look at their marker expression.

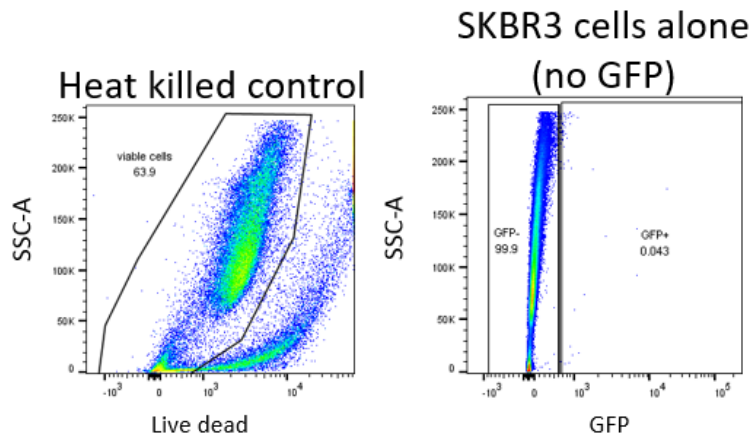


Figure 11. Controls used for gating SKBR3/ NHDF co-cultures.
 A “heat killed control” was used for the viability gate to distinguish the live and dead cells more clearly. The tumor cells alone were used to determine the GFP+ gate.

Marker Expression in Co-Cultured Fibroblasts

Positivity to each of the five markers – CD29, FAP, PDGFRb, Cav-1, and Thy1.1 – was analyzed on the GFP-positive fibroblasts determined using the gating strategy described previously. For each of the markers, an FMO control containing labeled antibodies for all markers except the one of interest was used to account for any background fluorescence and identify where the appropriate positive gate for that marker should be set. Separate FMO controls were used for the MDA-MB-231 and SKBR3 co-cultures.

Figure 12 shows the CD29 expression in GFP-positive fibroblasts from both MDA-MB-231/ NHDF co-cultures (Figure 12A) and SKBR3/ NHDF (Figure 12B). The data shows that CD29 is expressed in the majority of fibroblasts in 2D and 3D co-culture with both MDA and SKBR3, with slightly higher expression in the 2D MDA-MB-231 co-culture. Percentages of CD29-positive fibroblasts were similar in both 2D and 3D SKBR3 co-cultures.

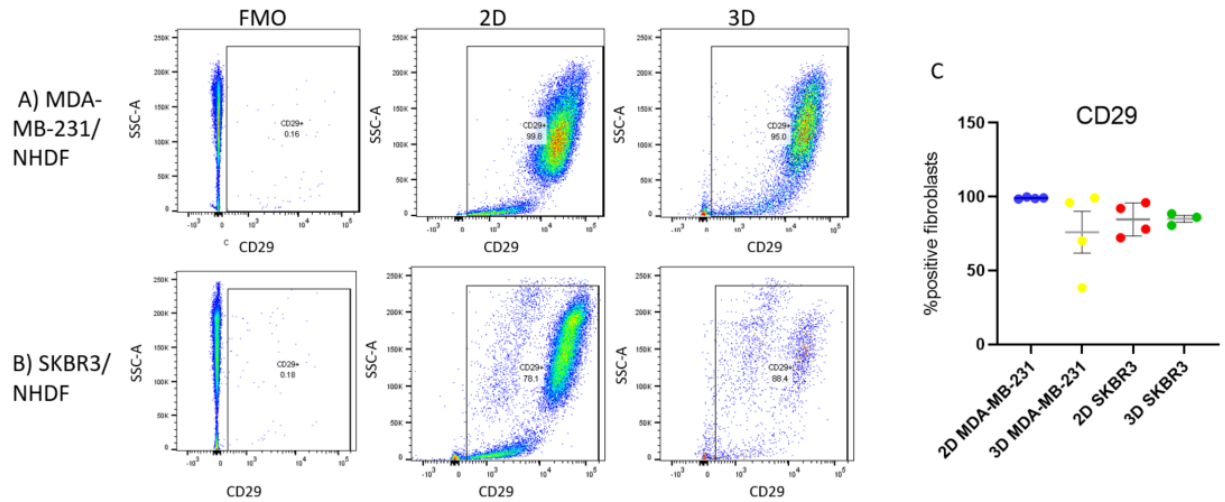


Figure 12. CD29 expression in 2D and 3D NHDF co-culture with MDA-MB-231 and SKBR3 cells.

A and B) Representative flow cytometry plots showing CD29 expression in NHDF from MDA-MB-231 (A) or SKBR3 (B) co-culture in 2D and 3D. CD29-positive cells were gated using the FMO control shown on the left. C) Graph showing data from four experimental repeats with standard error, with the exception of the 3D SKBR3/ NHDF co-culture, which shows data from three experimental repeats.

Figure 13 shows FAP expression in the GFP-positive fibroblasts from the four experimental co-culture conditions. Fibroblasts showed FAP expression in the 2D co-cultures with both types of tumor cells, however percentage of expression was overall higher in SKBR3 co-cultures. Overall, percentage of FAP-expressing NHDFs was higher in 2D than 3D culture. In 3D culture, FAP expression was detected only on fibroblasts from SKBR3 co-culture, while fibroblasts from MDA-MB-231 co-culture showed no FAP signal.

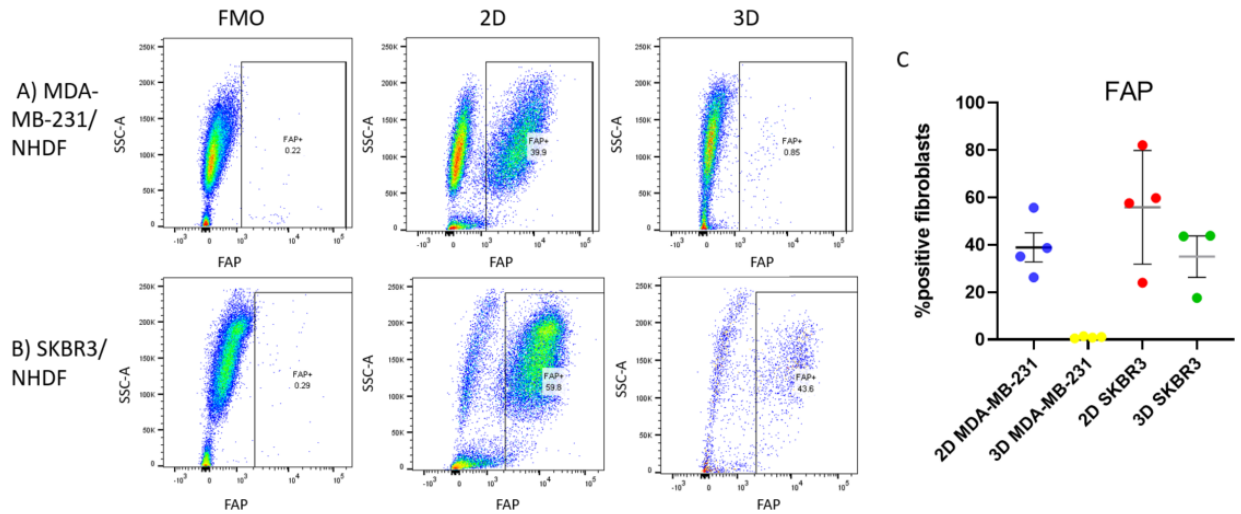


Figure 13. FAP expression in 2D and 3D NHDF co-culture with MDA-MB-231 and SKBR3 cells.

A and B) Representative flow cytometry plots showing FAP expression in NHDF from MDA-MB-231 (A) or SKBR3 (B) co-culture in 2D and 3D. FAP-positive cells were gated using the FMO control shown on the left. C) Graph showing data from four experimental repeats with standard error, with the exception of the 3D SKBR3/ NHDF co-culture, which shows data from three experimental repeats.

PDGFRb was gated similarly to the markers described previously and expression data shown in Figure 14. PDGFRb was expressed on NHDF cells grown in both 2D co-cultures, with higher frequency again observed in SKBR3 co-cultures. Fibroblasts in 3D co-culture with MDA-MB-231 cells did not show any PDGFRb expression, while fibroblasts in 3D co-culture with SKBR3 expressed PDGFRb although at a lower percentage as the respective 2D co-culture.

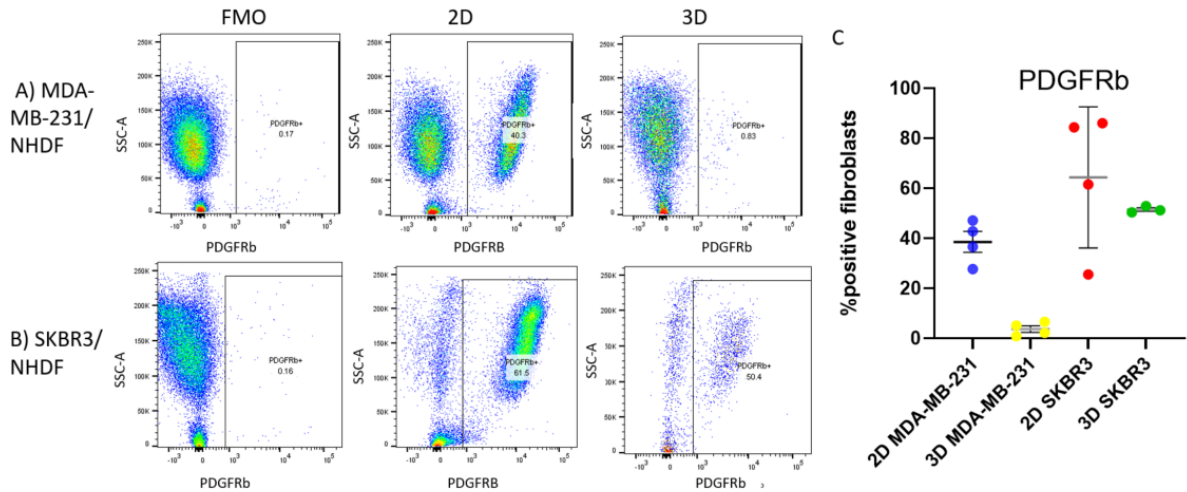


Figure 14. PDGFRb expression in 2D and 3D NHDF co-culture with MDA-MB-231 and SKBR3 cells.

A and B) Representative flow cytometry plots showing PDGFRb expression in NHDF from MDA-MB-231 (A) or SKBR3 (B) co-culture in 2D and 3D. PDGFRb-positive cells were gated using the FMO control shown on the left. C) Graph showing data from four experimental repeats with standard error, with the exception of the 3D SKBR3/NHDF co-culture, which shows data from three experimental repeats.

Thy1.1 expression on GFP-positive NHDF from 2D and 3D co-cultures with MDA-MB-231 (Figure 15A) and SKBR3 (Figure 15B) tumor cells was analyzed. Fibroblasts from both 2D co-cultures showed a distinct Thy1.1 positive population that had a greater percentage than the corresponding 3D spheroids. Only fibroblasts co-cultured with SKBR3 cells but not with MDA-MB-231 cells showed positivity from 3D spheroids. Fibroblasts grown in SKBR3 co-cultures showed greater frequency of Thy1.1 positivity than those grown in MDA-MB-231 co-cultures.

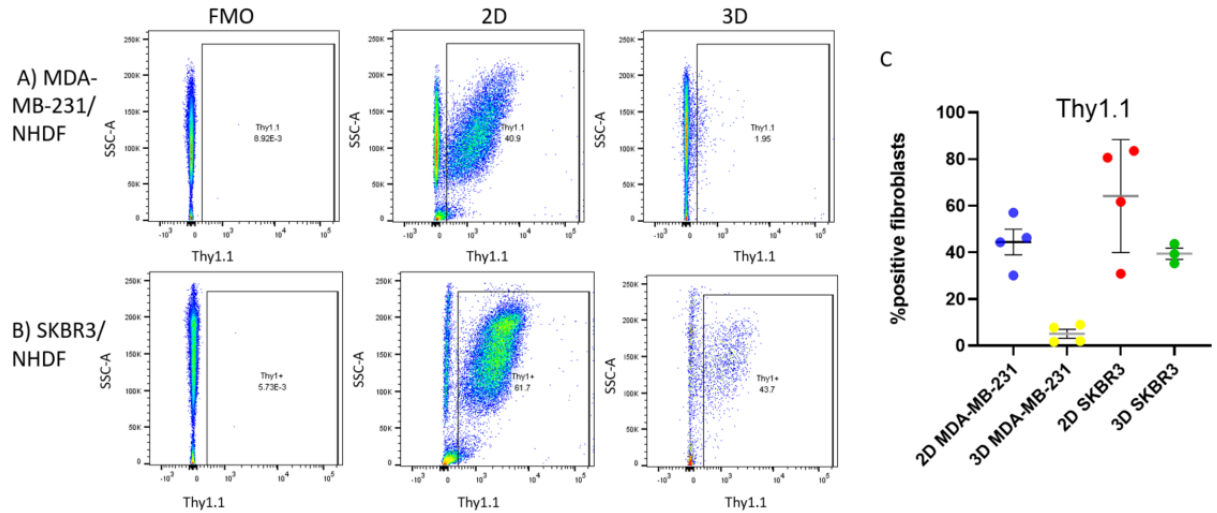


Figure 15. Thy1.1 expression in 2D and 3D NHDF co-culture with MDA-MB-231 and SKBR3 cells.

A and B) Representative flow cytometry plots showing Thy1.1 expression in NHDF from MDA-MB-231 (A) or SKBR3 (B) co-culture in 2D and 3D. Thy1.1-positive cells were gated using the FMO control shown on the left. C) Graph showing data from four experimental repeats with standard error, with the exception of the 3D SKBR3/NHDF co-culture, which shows data from three experimental repeats.

Lastly, Cav-1 PE expression was assessed on GFP-positive fibroblasts from 2D and 3D MDA-MB-231 co-cultures (Figure 16A) and from 2D and 3D SKBR3 co-cultures (Figure 16B). Cav-1 was expressed in fibroblasts from all co-cultures, but percentage of positive cells was slightly higher in the 3D co-cultures than their respective 2D co-cultures. Both types of tumor cells co-culture showed similar percentages of Cav-1 positive fibroblasts.

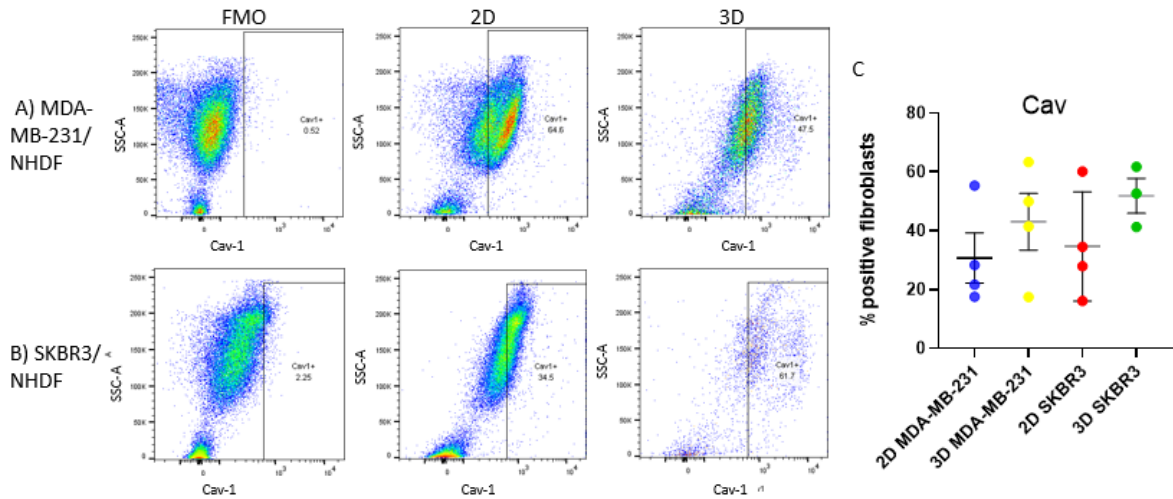


Figure 16. Cav-1 expression in 2D and 3D NHDF co-culture with MDA-MB-231 and SKBR3 cells.

A and B) Representative flow cytometry plots showing Cav-1 expression in NHDF from MDA-MB-231 (A) or SKBR3 (B) co-culture in 2D and 3D. Cav-1-positive cells were gated using the FMO control shown on the left. C) Graph showing data from four experimental repeats with standard error, with the exception of the 3D SKBR3/NHDF co-culture, which shows data from three experimental repeats.

In summary, fibroblasts in 2D culture with MDA-MB-231 and SKBR3 tumor cells showed higher frequency of expression of all the activation markers, CD29, FAP, PDGFRb, and Thy1.1, but lower frequency in Cav-1 than 3D co-cultures, indicating the fibroblasts in 2D were in a more activated state. Moreover, all activation markers were expressed at higher percentage in fibroblasts from SKBR3 2D co-cultures than MDA-MB-231 co-cultures. Fibroblasts in the 3D co-cultures with SKBR3 showed a distinct population positive for FAP, PDGFRb, and Thy1.1, but all three markers showed very little/no expression in fibroblasts from the MDA-MB-231 3D co-culture, indicating that these fibroblasts remained in a more quiescent state.

Fibroblast Marker Expression on Tumor Cells

To determine if the marker expression shown previously was specific to the fibroblasts from the co-cultures, expression in the tumor cells was also analyzed. Tumor cells were gated using a FITC negative gate to distinguish them from the FITC-positive GFP-tagged fibroblast cells as shown in Figure 17A. Expression of the five fibroblast markers - CD29, FAP, PDGFRb, Cav-1 and Thy1.1 – was analyzed on the tumor cells applying the same gates that were described previously, determined from the FMO controls.

Figure 17B shows expression of each marker in MDA-MB-231 from NHDF 2D co-cultures and Figure 17C shows expression plots of SKBR3 from NHDF 2D co-cultures. CD29, which is known to be more widely expressed on various cells types, was positive on a majority of cells in both tumor types. A moderate population of tumor cells were positive for Cav-1, with frequency of expression being higher in the SKBR3/NHDF co-cultures. FAP, PDGFRb, and Thy1.1 showed no expression on tumor cells from either co-culture as expected. The same trend was seen on both types of tumor cells from the 3D co-cultures (data not shown). Data was reproduced across four experimental repeats.

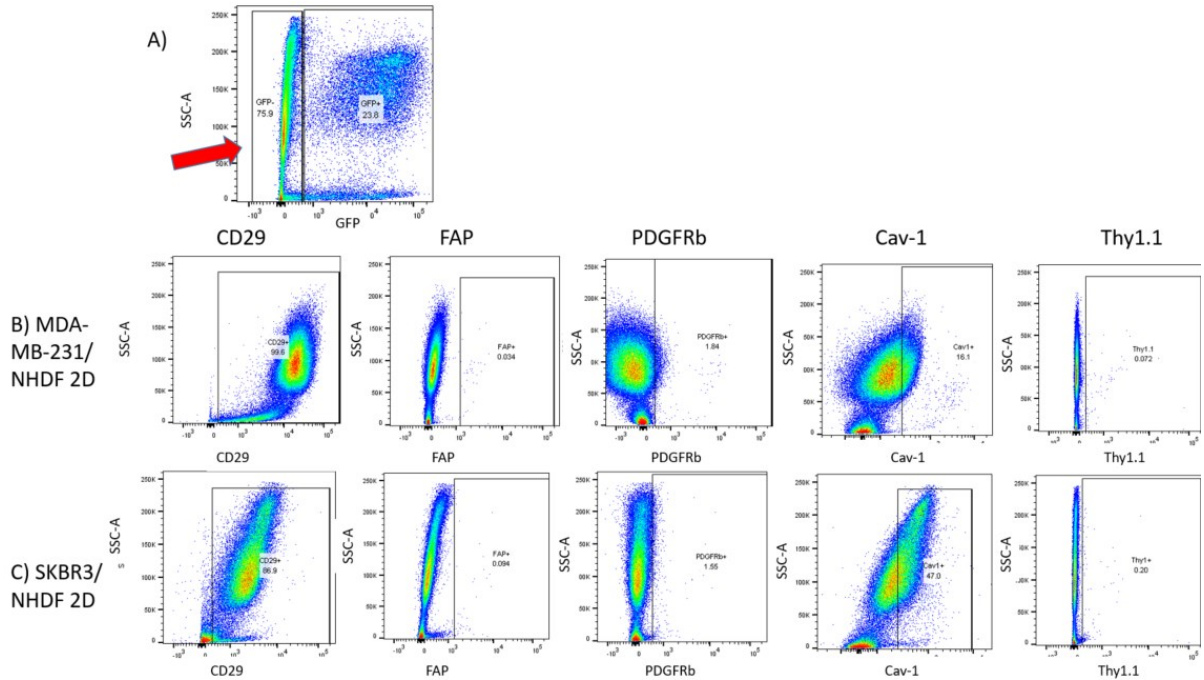


Figure 17. Expression of fibroblast markers on tumor cells in 2D co-culture with NHDF
 A) Tumor cells were gated from co-culture samples using GFP negative gate. B and C) Expression of five fibroblast markers on MDA-MB-231 cells (B) or SKBR3 cells (C) from NHDF co-culture samples.

SKBR3 and MDA-MB-231 cells grown in 2D mono-culture were also analyzed and showed the same expression patterns (Figure 18), with positive populations seen for CD29 and Cav-1 and no expression detected for FAP, PDGFRb, and Thy1.1. Data was reproduced across four experimental repeats.

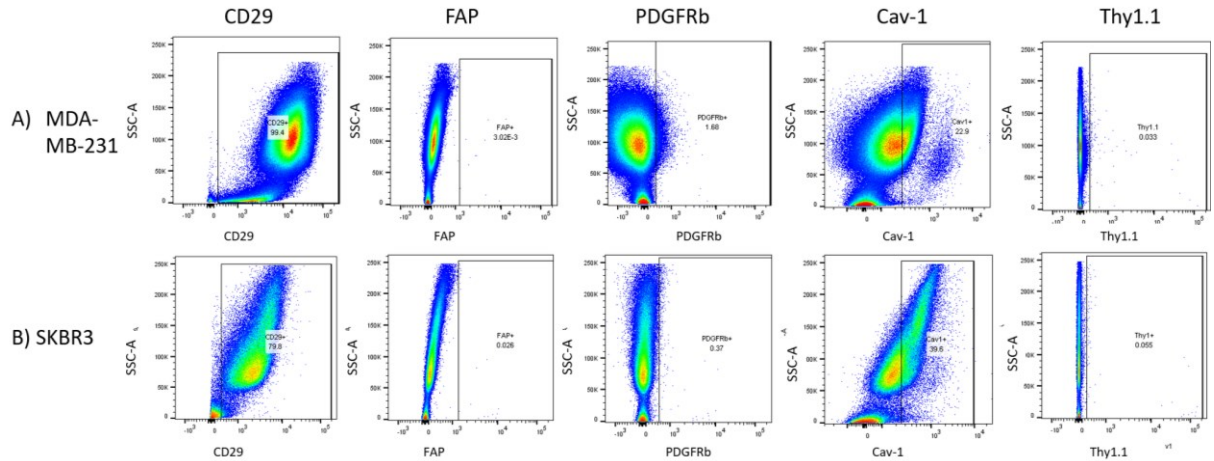


Figure 18. Expression of five fibroblast markers on tumor cells in 2D mono-culture
 A and B) Expression of five fibroblast markers on MDA-MB-231 cells (A) or SKBR3 cells (B) grown alone with no fibroblasts

FAP, PDGFRb, and Cav-1 showed no expression on the tumor cells alone from either mono or 2D and 3D co-cultures but were expressed on fibroblasts. Based on these results, these three markers are shown to be specific to fibroblasts. CD29 was expressed on a majority of both types of tumor cells from all culture conditions, while Cav-1 was expressed in a lower, but still significant percentage. Therefore CD29 and Cav-1 are markers that are not as specific to fibroblasts. The similar results from tumor cells grown in mono- and co-culture indicate that co-culturing with fibroblasts had no effect on expression level of these five markers in either tumor cell type.

Marker Expression on Fibroblast in Mono-culture

The data obtained from the fibroblast and tumor cell co-cultures suggested that fibroblasts in 2D have higher frequency of expression of markers associated with an activation phenotype. Moreover, the 3D phenotype appeared to be influenced by the

specific tumor cell line used. Therefore, I wanted to test marker expression on fibroblasts grown in the absence of tumor cells. As shown in table 4, expression of the five markers on fibroblasts from 2D mono-cultures suggested these are in an activated state. However, I noted some variability across six experimental repeats. This may be due to the differences in passage number from thaw of the NHDF at the time of analysis. CD29 was again shown to be consistently expressed in a majority of cells. For the remaining four markers, FAP, PDGFRb, Cav-1 and Thy1.1, the average percentage of positive cells was comparable to what observed for fibroblasts grown in 2D co-culture (Figure 5). Cav-1 had the lowest percentage of expression, compared to the activation markers, also similar to what was observed in the 2D co-cultures. The data obtained from 2D fibroblast cultures indicate that the phenotype of these cells is not significantly affected by the presence of tumor cells and are uniformly activated across the different 2D culture settings.

Table 4. Expression in 2D fibroblast mono-culture

	CD29	FAP	PDGFRb	Cav-1	Thy1.1
Exp 1	90.9	9.86	83.2	41.7	94.5
Exp 2	86.5	57.9	71.9	29.2	67.9
Exp 3	98.1	64.9	44	16.8	84.1
Exp 4	99.8	67.4	73.1	27.8	99
Exp 5	96.2	22.9	10.7	4.8	24.8
Average	94.3	44.6	56.6	24.1	74.1
SD	5.5	26.4	29.5	13.9	30

Table 5. Averages of fibroblast marker expression in 2D mono-culture and co-culture with MDA-MB-231 or SKBR3

	CD29	FAP	PDGFRb	Cav-1	Thy1.1
Fibroblast mono-culture average	94.3	44.6	56.6	24.1	74.1
MDA-MB-231 co-culture average	94.3	38.7	38.5	30.7	44.45
SKBR3 co-culture average	84.6	55.8	64.35	34.7	64.2

I also tried to compare expression of the markers on 3D spheroids formed by fibroblasts alone, but the results were inconclusive as the cells did not form compact spheroids over the course of the experiment and it was not possible to collect enough cells to obtain reliable flow cytometry data. Increasing the amount of fibroblasts seeded per well from 9,000 may help to overcome this issue. In any case, the data obtained from 2D fibroblast cultures indicate that the phenotype of these cells is not significantly affected by the presence of tumor cells in the 2D settings.

Chapter IV.

Discussion

Despite many studies pointing to a key role of fibroblasts in tumor progression, their exact role is not yet fully understood. This is partly due to the lack of appropriate *in vitro* modeling systems that accurately represent the complexities of the *in vivo* tumor microenvironment. Three-dimensional models incorporate an extracellular matrix that allow fibroblasts and tumor cells to self-assemble into spheroid structures. These spheroids much better recapitulate the tissue architecture, cell-to-cell and cell-to-matrix interaction, mechanical properties, and biochemical networks that are seen *in vivo* (Dolznic et al., 2020). This cellular heterogeneity and interaction in the 3D environment is not reproduced by traditional tissue culture techniques on 2D surfaces. The 3D *in vitro* spheroid models more closely mimic features of the *in vivo* situation and thus provides unique possibilities to study the behavior of cancer.

With the work described in this thesis, I set out to develop a 3D model of breast cancer cells and fibroblasts that better captured the physiology of the tumor microenvironment than 2D models, allowing the future testing of therapeutic modalities. To this aim, I utilized a commercially available 3D spheroid assay kit from Trevigen to form spheroids made up of normal human dermal fibroblasts and either MDA-MB-231 or SKBR3 breast cancer cells seeded in co-culture and embedded in a collagen matrix. Fibroblasts with both types of tumor cells were grown in traditional 2D culture simultaneously. After seven total days, the cells were removed from culture and stained

for flow cytometry analysis, specifically looking at CD29, FAP, PDGFRb, and Thy1.1, markers of fibroblast activation, as well as Cav-1 a marker of fibroblast quiescence.

These experiments demonstrated that fibroblast phenotype and expression of activation markers was very different in 2D versus 3D co-culture models. Similar to what had been previously described in fibroblast mono-cultures in 2D and 3D (Smithmyer et al 2019), co-culturing NHDF with breast cancer cells also resulted in higher activation of fibroblasts across the 2D models. FAP, PDGFRb, and Thy1.1, all established markers of fibroblast activation, were shown to be elevated in 2D models compared to their 3D counterparts. The differences were especially clear between the two MDA-MB-231 models, which showed almost no expression in any of these three proteins in the 3D spheroids. On the contrary, expression of Cav-1, which acts as an inhibitor to myofibroblast differentiation, was higher in 3D than in 2D culture. CD29 was highly expressed on fibroblasts irrespective of culture method. CD29 did not appear to be a very specific CAF biomarker, as it was also present on both types of tumor cells, and has been reported to be more ubiquitously expressed (Zeltz et al., 2020). In contrast, FAP, PDGFRb, and Thy1.1 show no expression on the tumor cells, indicating that they are more useful markers to measure fibroblast activation than CD29. Overall, the different culture methods resulted in clear difference in marker expression levels on the fibroblasts. It is especially important to consider that the use of these two methods of culture could produce drastically different results when validating a potential CAF-targeted therapy that could drive key decision making in drug development.

In my experiments, I also looked at the differences between NHDFs cultured with two different types of breast cancer cells. Since MDA-MB-231 cells are more aggressive

and invasive than SKBR3 in an *in vivo* setting (Holliday and Speirs, 2011), I had hypothesized that NHDF in co-culture with the MDA-MB-231 would appear more “CAF-like”. Surprisingly, the fibroblasts in co-culture with SKBR3 cells showed higher expression of FAP, PDGFRb, and Thy1.1 activation markers in both 2D and 3D than those in co-culture with MDA-MB-231. MDA-MB-231 spheroids showed almost no expression of any of these three proteins compared to the SKBR3 spheroid which showed distinct positive populations for all three. Although MDA-MB-231 are known to be a more aggressive tumor type *in vivo* (Holliday and Speirs, 2011), SKBR3 co-culture spheroids show more distinct morphological changes than their respective mono-culture spheroids. SKBR3 alone spheroids maintain a round phenotype, but when co-cultured with the fibroblasts these tumor cells begin “budding” out into the extracellular matrix. Perhaps the increase in fibroblast activation when co-cultured with SKBR3 is driving this invasiveness. It would be interesting to further characterize and co-culture fibroblasts with other tumor cells that have similar three-dimensional budding versus spiky morphology. Use of the three-dimensional spheroid system better reveals the different behaviors and morphology of cancer cell types than the 2D co-cultures, which allows for development of a more highly specific model.

CAFs are often not very homogenous and several subpopulations have been identified (Mhaidy and Mehta., 2020). This heterogeneity might result from numerous causes, including the complex interactions between CAFs and cancer cells or any other components of the tumor microenvironment (TME), in addition to cytokines, chemokines and growth factors that are secreted. Markers such as FAP and PDGFRb are not all regulated similarly or simultaneously in CAFs, thereby highlighting a strong degree of

heterogeneity of these cells in TME (Mhaidy and Mehta., 2020). Fibroblast populations are also more heterogenous in 3D than 2D cultures, where they are highly proliferative (Beacham and Cukieran., 2005). In different types of three-dimensional culture systems, fibroblasts have displayed a diverse array of features - from the activated phenotype like that observed in 2D cultures and by myofibroblasts, to a quiescent state that likely better represents fibroblasts at rest (Woodley et al., 2021). In my experiments, fibroblast showed consistent activation in 2D, irrespective of whether they were grown in mono-culture or co-culture with different tumor cells. The 3D system showed more variability among the different CAF markers, which may better recapitulate the heterogeneity of fibroblasts *in vivo*. Using different types of fibroblasts from different human donor or from a different tissue may produce variable results in marker expression (Kieffer et al., 2020). Other types of normal human fibroblasts primary cell lines commercially available include breast, lung, cardiac, bladder, gingival, lung prostate, and uterine, among others. Fibroblasts can also be isolated from human tissue sources through dissociation methods, and assessed for differences between donors (Beacham and Cukieran., 2005). Fibroblast heterogeneity makes comparisons between studies especially challenging, but could provide opportunities to create highly specific tissue engineered models (Woodley et al., 2021).

Another benefit of the three-dimensional culture system is the ability to adjust, morphological parameters such as diameter, perimeter, area, volume and sphericity, all of which may affect the reproducibility of the results obtained (Zanoni et al., 2016). The spheroids in these experiments were optimized to fall within a predetermined 300 to 500 μm range, as suggested by manufacturer's protocol. Increasing or decreasing the number

of tumor cells from 3,000 per well would impact the spheroid diameter and likely the viability of the cells in the core where there is limited oxygen and nutrient penetration. Addition of fibroblasts to the tumor spheroids was shown to have no effect on the spheroid size but only morphology (in the case of SKBR3 co-cultures) therefore, an optimized 1:3 ratio of tumor to fibroblast cells was used to induce spheroid invasiveness. The expression of fibroblast activation markers was not compared to spheroids of other ratios, which could easily be adjusted. These thesis experiments were cultured for seven days before being analyzed by flow cytometry. Continuing to culture the spheroids for additional time would likely change their size and sphericity, and potentially lead to differences in expression. Zanoni et al (2016) found different spheroid formation protocols results in differently shaped spheroids that grew differently over time. These experiments used a commercially available 3D Spheroid Formation Assay and other kits and protocols were not tested with the same cells and conditions. Adjusting any of these assay parameters could produce different results.

The experiments in this thesis work also solely used the matrix included in the commercially available Trevigen kit and did not explore other types of matrices. There are many types of scaffold platforms for 3D culture are made of synthetic or naturally-derived polymers that provide a support for cell growth and mimic extracellular matrix conditions (Zanoni et al., 2016). The three-dimensional model system is very versatile and matrices of different components or stiffness would likely have an effect on the fibroblasts phenotype. Smithmyer et al. (2019) found that the stiffness of the hydrogel matrix had a significant effect on fibroblast activation. Different concentrations of collagen, hydrogel, etc. can produce different matrix stiffness that effect the phenotypic

properties of fibroblasts (Zanoni et al., 2016). Physical changes in ECM, including rigidity of collagen fibers and modulation of elasticity, govern fibroblast phenotypic modifications (Mhaidy and Mechta., 2020).

This work only focused on quantifying a limited number of membrane proteins as CAF biomarkers all of which can be analyzed by flow cytometry methods. However there are several other membrane proteins that have been used in the identification of CAF populations, including podoplanin, CD49, CD51, and Cadherin-11 (Zeltz et al., 2020). Cytoskeletal proteins can also be useful fibroblast biomarkers, most popularly α -SMA as an indicator of activated collagen-producing stromal cells, as well as fibroblast specific protein-1 (FSP-1). These cytoskeletal proteins could be quantified by embedding the spheroids and performing immunohistochemical staining (Zeltz et al., 2020). A variety of proteins secreted specifically by fibroblasts could be quantified through analysis of the supernatant from the co-cultures. Some of these secreted proteins include tenascins, osteopontin, and periostin (Zeltz et al., 2020). Genes involved in invasion, extracellular matrix remodeling, inflammation, and angiogenesis were reported differentially regulated in a 3D carcinoma model (Dolznic et al., 2011). A broad analysis of gene expression could help understand the interaction of cancer cells with stromal fibroblasts and any differences in cancer expression profiles between culture methods. Incorporating additional biomarker and readouts including but not limited to those discussed will help to obtain a more complete picture of fibroblast phenotype in these models.

In conclusion, this thesis work demonstrates the complexities involved in using different fibroblast and tumor cell co-culture *in vitro* methods to study fibroblast

behavior. Markers of fibroblast activation had varying levels of expression between two co-cultures with breast cancer lines, SKBR3 or MDA-MB-231, that may be reflected in the different morphology of the 3D spheroids. Fibroblasts grown in traditional 2D plastic culturing appeared to be in a more uniformly activated state than 3D spheroid models, suggesting that 3D culture may be a better for understanding differences between tumor models. The development and use of *in vitro* spheroids models provides opportunity to study the different roles played by CAFs in the tumor microenvironment as well as a system for validating potential targets for anti-tumor therapies. Characterizing fibroblast subsets and their different expression patterns of various activation markers may provide targeting opportunities for CAF specific anti-tumor therapies.

References

- Agorku, D., Langhammer, A., Heider, U., Wild, S., Bosio, A., & Hardt, O. (2019). CD49b, CD87, and CD95 Are Markers for Activated Cancer-Associated Fibroblasts Whereas CD39 Marks Quiescent Normal Fibroblasts in Murine Tumor Models. *Frontiers in Oncology*, (9):716.
- Beacham, D. & Cukierman, E. (2005) Stromagenesis: The changing face of fibroblastic microenvironments during tumor progression. *Seminars in Cancer Biology*, 15: 329-341.
- Benton, G., DeGray, G., Kleinman, H., George, J., & Arnautova, I. (2015). *In Vitro* microtumors provide a physiologically predictive tool for breast cancer therapeutic screening. *PLOS ONE*, 10:1371.
- Cummins, K., Bitterman, P., Tshumperlin, D., & Wood, D. (2021). A scalable 3D tissue culture pipeline to enable functional therapeutic screening for pulmonary fibrosis. *APL Bioengineering*, 5(4).
- Dolznic, H., Rupp, C., Puri, C., Haslinger, C., Schweifer, N., Weiser, E., Kerjaschki, D., & Garin-Chesa, P. (2011). Modeling Colon Adenocarcinomas *in Vitro*: A 3D Co-Culture System Induces Cancer-Relevant Pathways upon Tumor Cell and Stromal Fibroblast Interaction. *The American Journal of Pathology*, 179(1): 487-501.
- Haines, P., Hant, F., Lafyatis, R., Trojanowska, M., & Bujor, A. (2012). Elevated expression of cav-1 in a subset of SSc fibroblasts contributes to constitutive Alk1/Smad1 activation. *Journal of Cellular and Molecular Medicine*, 6(9): 2238–2246.
- Han, J., Chang, H., Giricz, O., Lee, G., Baehner, F., Gray, J., Bissel, M., Kenny, P., & Parvin, B. (2010). Molecular Predictors of 3D Morphogenesis by Breast Cancer Cell Lines in 3D Culture. *Computational Biology*, 6(2).
- Hewitt, K., Shamis, Y., Knight, E., Smith, A., Maione, A., Alt-Holland, A., Sheridan, S., Haggarty, S., & Garlic, J. (2012). PDGFR β expression and function in fibroblasts derived from pluripotent cells is linked to DNA demethylation. *Journal of Cell Science*, 125(9): 2276–2287.
- Holliday, D., & Speirs, V. (2011). Choosing the right cell line for breast cancer research. *Breast Cancer Research*, 13(4); 215.

- Hu, G., Huang, L., Zhong, K., Meng, L., Xu, F., Wang, S., and Zhang, T. (2021) PDGFR- β ⁺ fibroblasts deteriorate survival in human solid tumors: a meta-analysis. *Open Access Impact Journal on Aging*, 13(10): 13693–13707.
- Huyhn, L., Hipolito, C., & Dijke, P. (2019) A Perspective on the Development of TGF- β Inhibitors for Cancer Treatment. *Biomolecules*. 9(11): 743
- Kieffer, Y., Hocine, R., Gentric, G., Pelon, F., Bernard, C., Bourachott, B., Lamerias, S., Albergante, L., Guyard, A., Tarte, K., Zinovyev, R., Baulande, S., Zalchman, G., Vincent-Salomon, A., & Mechta, F. (2020). Single-Cell Analysis Reveals Fibroblast Clusters Linked to Immunotherapy Resistance in Cancer. *Cancer Discover*, 10(9).
- Koumas, L., Smith, T., Feldon, S., Blumberg, N., & Phipps, R. (2003). Thy-1 Expression in Human Fibroblast Subsets Defines Myofibroblastic or Lipofibroblastic Phenotypes. *The American Journal of Pathology*, 163(4): 1291–1300.
- Kozlova, N., Grossman, J., Iwanicki, M., & Muranen, T. (2020). The Interplay of the Extracellular Matrix and Stromal Cells as a Drug Target in Stroma-Rich Cancers. *Trends in Pharmacological Sciences*, 41(3).
- Lindner, T., Loktev., Giesel, F., Kratochwil, C., Altmann, A., & Haberkorn, U. (2019). Targeting of activated fibroblasts for imaging and therapy. *Radiopharmacy and Chemistry*,
- MacDonald, A., Priess, M., Curran, J., Guess, J., Farutin, V., Oosterom, I., Chu, C., Schultes, B., Cochran, E., Getchell, K., & Krause, S. (2019). Necuparanib, a multi-targeting heparin sulfate mimetic, targets tumor and stromal compartments in pancreatic cancer. *Molecular Cancer Therapeutics*, 18(2): 245-256.
- Mahaidly, R. & Mechta-Grigoriou, F. (2020). Fibroblast heterogeneity in tumor micro-environment: Role in immunosuppression and new therapies. *Seminars in Immunology*, 48.
- Malik, R., Lelkes, P., & Cukierman, E. (2015). Biomechanical and biochemical remodeling of stromal extracellular matrix in cancer. *Trends in Biotechnology*, 33(4).
- Pelon, F., Bourachot, B., Kieffer, Y., Mgagna, I., Mermet, F, Bonnet, I., Costa, A., Givel, A., Attieh, Y., Barbazan, J., Bonneau, C., Fuhrmann, L., Descroix, S., Vignjevic, G., Silberzan, P., Parinni, M., Salomon, A., & Mechta, F. (2020). Cancer-associated fibroblast heterogeneity in axillary lymph nodes drives metastases in breast cancer through complementary mechanisms. *Nature Communications*, 11: 404.
- Ravi, M., Paramesh, V., Kaviya, S., Anuradha, E., & Solomon, F. (2014) 3D Cell culture systems: advantages and applications. *Journal of Cellular Physiology*. 230(1): 16-26.

- Rhee, S. (2009). Fibroblasts in three dimensional matrices: cell migration and matrix remodeling. *Experimental and Molecular Medicine*, (41): 858–865.
- Rothan H., Djordjevic, I., Bahrani, H., Paydar, M., Ibrahim, F., Rahmanh, N., Yusof, R. (2014). Three-Dimensional Culture Environment Increases the Efficacy of Platelet Rich Plasma Releasate in Prompting Skin Fibroblast Differentiation and Extracellular Matrix Formation. *International Journal of Med Sciences*, 11(10):1029-1038.
- Sadlonova, A., Bowe, D., Novak, Z., Mukherjee, S., Duncan, V., Page, G., and Frost, A. (2009). Identification of Molecular Distinctions Between Normal Breast-Associated Fibroblasts and Breast Cancer-Associated Fibroblasts. *Cancer Microenvironment*, 2(1): 9–21.
- Shen, X., Zhang, H., Tang, G., Wang, X., Zheng, R., Wang, Y., Zhu, Y., Xue, X., & Bi, J. (2015). Caveolin-1 is a Modulator of Fibroblast Activation and a Potential Biomarker for Gastric Cancer. *International Journal of Biological Sciences*, 11(4): 370–379.
- Shliekelman, M., Creighton, C., Baird, B., Chen, Y., Banerjee, P., Ahn, Y., Roybal, D., Chen, F., Zhang, Y., Mishra, D., Kim, M., Liu, X., Mino, B., Villalabos, P., Rodriguez, J., Behrens, C., Wistuba, I., Hanahs, S., & Kurie, J. (2017) Thy-1⁺ Cancer-associated Fibroblasts Adversely Impact Lung Cancer Prognosis. *Nature Research*, 7: 6478.
- Smithmeyer, M., Cassel, S., & Kloxin, A. (2019). Bridging 2D and 3D culture: probing impact of extracellular environment on fibroblast activation in layered hydrogels. *The American Institute of Chemical Engineers Journal*, 65(12): e16837
- Stylianou, A., Gkretsi, V., & Stylianopoulos, T. (2018). Transforming growth factor- β modulates pancreatic cancer associated fibroblasts cell shape, stiffness and invasion. *Biochimica et Biophysica Acta*, 1862(7):1537-1546.
- Sung, K., Su, X., Berthier, E., Pehlke, C., Friedl, A., & Beebe, D. (2013). Understanding the impact of 2D and 3D fibroblast cultures on *in vitro* breast cancer models. *Plos ONE*.
- Thannickal, V., Lee, D., White, E., Cui, Z., Larios, J., Chacon, R., Horowitz, J., Day, R., & Thomas, P. (2003) Myofibroblast differentiation by transforming growth factor-beta1 is dependent on cell adhesion and integrin signaling via focal adhesion kinase. *The Journal of Biological Chemistry*, 278(14), 12384-9.
- Tripathi, M., Billet, S., & Bhowmick, N. (2012). Understanding the role of stromal fibroblasts in cancer progression. *Cell Adhesion and Migration*. 6(3): 231–235.
- Vinci, M., Box, C., & Eccles, A. (2015). Three dimensional (3D) tumor spheroid assay. *Journal of Visualized Experiments*, (99): 52686.

- Welsh, J. (2013). Animal Models for Studying Prevention and Treatment of Breast Cancer. *Animal Models for the Study of Human Disease*, 40; 997-1018.
- Wessels, D., Pradhan, N., Park, N., Kleptisch, M., Lusche, D., Daniels, K., Conway, K., Voss, R., Hegde, S., Conway, T., & Soll, D. (2019). Reciprocal signaling and direct physical interactions between fibroblasts and breast cancer cells in a 3D environment. *PLOS One*, 14(6): e0218854.
- Woodley, J., Lambert, D., & Asencio, I. (2021) Understanding Fibroblast Behavior in 3D Biomaterials. *Journal of Tissue Engineering*.
- Yakavets, I., Francois, A., Benoit, A., Merlin, J., Bezdetyanya, L., & Vogin, G. (2020). Advanced co-culture 3D breast cancer model for investigation of fibrosis induced by external stimuli: optimization study. *Scientific Reports*, 10:21273.
- Yoshida, G., Azuma, A., Miura, Y., & Orimo, A. (2019). Activated fibroblast orchestrated tumor initiation and progression; molecular mechanisms and the associated therapeutic strategies. *International Journal of Molecular Sciences*, 20(9): 2256.
- Yoshida, G. (2020). Regulation of heterogeneous cancer-associated fibroblasts: the molecular pathology of activated signaling pathways. *Journal of Clinical and Experimental Cancer Research*, 39(112).
- Zeltz, C., Primac, I., Erusappan, P., Alam, J., Noel, A., & Gullberg, D. (2020). Cancer-associated fibroblasts in desmoplastic tumors: emerging role of integrins.