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Characterizing the role of extracellular matrix in non-small cell lung carcinoma metastasis using a 3D microenvironment mimetic in-vitro culture system.

Douglas John Owulaku Saforo
University of Louisville

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CHARACTERIZING THE ROLE OF EXTRACELLULAR MATRIX IN NON-
SMALL CELL LUNG CARCINOMA METASTASIS USING A 3D
MICROENVIRONMENT MIMETIC IN-VITRO CULTURE SYSTEM

By

Douglas John Owulaku Saforo
B.S., University of Louisville, 2013

A Dissertation
Submitted to the Faculty of the
School of Medicine
in Partial Fulfillment of the Requirements
for the Degree of

Doctor of Philosophy
in Pharmacology and Toxicology

Department of Pharmacology and Toxicology
University of Louisville School of Medicine
Louisville, KY

August 2019

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A Dissertation approved on
July 23, 2019

By the following Dissertation Committee:

Leah Siskind

Levi Beverly

Paula Bates

Geoffrey Clark

Howard Donninger

Robert Mitchell

DEDICATION

I dedicate this dissertation to my mother, Susan B. Saforo.

She illuminated the beauty of the world and gave me the courage

to fight to make it better for all of us.

ACKNOWLEDGMENTS

I would like to thank my advisors, Leah Siskind and Levi Beverly, for allowing me the chance to work on a project that I was passionate about. During these past four years, I had the opportunity to assist with several projects in the lab and each was intellectually stimulating. I would also like to thank the members of my dissertation committee, Paula Bates, Geoffrey Clark, Howard Donninger, and Robert Mitchell for their input throughout the planning and organization of my work. I have surely benefited from their keen and critical scientific minds.

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ABSTRACT

CHARACTERIZING THE ROLE OF EXTRACELLULAR MATRIX IN NON- SMALL CELL LUNG CARCINOMA METASTASIS USING A 3D MICROENVIRONMENT MIMETIC IN-VITRO CULTURE SYSTEM

Douglas J. Owulaku Saforo

July 23, 2019

Lung cancer is the leading cause of cancer related death in the United States and worldwide. Five-year survival rates for non-small cell lung carcinoma patients have not improved in decades, and a majority of patients succumb to metastasis. Identification of therapeutic targets for metastatic disease is essential for the development of novel therapies. However, current 2-dimensional (2D) *in vitro* models are not reflective of the tumor microenvironment (TME) and are inadequate in identifying therapeutic targets.

We adapted a 3-dimensional (3D) extracellular matrix (ECM) *in vitro* model to a hypoxic incubator to approximate physiological hypoxia of the TME in a microenvironment mimetic culture system. Our overall objective is to determine how ECM interactions with lung adenocarcinoma cells can promote hallmarks of cancer metastasis in a physiologically relevant *in vitro* system. We assessed three interactions within the TME: the interaction of cancer cells with ECM, the

interaction of cancer associated stroma with ECM, and the influence of environment on ECM remodeling.

Using established and primary cell lines to produce cell-derived ECM (CDM), we found that lung carcinoma cells grown on 3D CDM adopt a mesenchymal mode of 3D migration and are significantly more invasive compared to 2D. The invasive phenotype was attenuated by inhibitors downstream of “outside-in” integrin signaling including focal adhesion kinase and Src family kinases. 3D CDM and physiological hypoxia enhanced the isolation and proliferation of primary tumor stroma isolated from cancer resections of early stage patients. Patient derived cell lines maintained a stem-like phenotype and expression of tumor promoting factors in an environment dependent manner. Further, they promoted metastasis in a xenograft model and CDM produced from patient derived stroma altered invasive characteristics in cancer cells compared to normal lung fibroblast CDM.

Using our microenvironment mimetic model, we demonstrate that we can study ECM-to-cell interactions in a way not possible for traditional 2D models. These interactions play a pivotal role in determining the fate of cancer cells and modulate metastatic potential. Understanding how remodeling of the TME is involved in late stage disease will allow development of preventative and curative therapeutic strategies to halt tumor progression and metastasis.

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CHAPTER I: INTRODUCTION

Lung cancer characteristics and mortality

Of the solid malignancies, lung cancer accounts for the greatest number of new cancer diagnoses worldwide with an estimated 2.1 million new cases in 2018 (Siegel, Miller, & Jemal, 2019). It is the most commonly diagnosed cancer in men, and second most commonly diagnosed cancer in women after breast cancer. Unfortunately, this particularly insidious disease is the leading cause of cancer related death in men and often the leading cause of death in women each year, accounting for 1.8 million deaths last year. In the United States, these grim statistics have shown little improvement in the last decade. Kentucky bears the highest lung cancer incidence rate and this outcome is correlated with its top-ranking smoking rate among the population. Lung cancer is a disease of aging and commonly affects people over the age of 60. Currently the five-year survival rate of lung cancer is around 16% overall in men and 21% in women according to the Center for Disease Control and Prevention and the National Cancer Institute, with moderately more favorable outcomes associated with the location of the tumor at the time of diagnosis. Data provided by the National Cancer Institute and the Surveillance, Epidemiology, and End Results program indicate that patients in which their disease is detected early, while the tumor is confined to the primary location and has not disseminated to the adjacent lymph nodes have a five-year survival rate of approximately 57.4% (Duggan, Anderson, Altekruse,

Penberthy, & Sherman, 2016). Once the cancer has spread to adjacent regional lymph nodes, this survival rate drops markedly to 30.8%. Tragically, those diagnosed with late stage and distant metastatic disease have only a 5.2% chance of survival past five years. It is a grim fact that a majority of new patients that will be diagnosed with lung cancer, around 80%, will be informed that they have regional or distant disease. Metastatic disease is the true killer in lung cancer.

Lung cancer can classically be divided into two types: small cell and non-small cell lung cancer. While small cell lung cancer is a particularly aggressive and devastating disease, with even locally diagnosed tumors rapidly progressing and a five-year relative survival rate of just 29%, the biology of small cell lung cancer is markedly different than that of non-small cell and will not be discussed in this dissertation. Non-small cell lung cancer is also subdivided into several histological subtypes including adenocarcinoma, a disease arising from the distal glandular epithelial lining of the bronchi, squamous cell carcinoma, arising from the more proximal squamous epithelium of the bronchus, and large cell carcinoma (Cooper, Lam, O'Toole, & Minna, 2013). Notably, the mean doubling time of lung adenocarcinoma and squamous cell carcinoma is around 160 days (Arai et al., 1994). This means that from the time of oncogenic transformation that results in a tumor cell able to continuously proliferate and evade immune detection to the time of a detectable tumor mass by chest radiograph can be over a decade. For the vast majority of this time, patients do not experience pain from the growing mass and do not experience other related symptoms specific to the

disease. Often the first symptoms experienced by the patient are associated with metastatic dissemination such as pain associated with tumor invasion into the chest wall, liver capsule, or bone structure, or a number of other paraneoplastic syndromes. This delays the critical time to diagnosis for the patient, leading to poor outcomes, but also allows ample time for mutated cells to adapt and modulate a local tumor microenvironment that is conducive to support tumor growth, progression, and metastasis.

The tumor microenvironment of non-small cell lung carcinoma

The tumor microenvironment of non-small cell lung carcinoma is a complex and heterogenous combination of cellular and non-cellular tumor promoting elements that exist and coordinate in a dynamic manner to facilitate the growth of transformed malignant cells. The cellular constituents include the malignant transformed cells themselves and possibly their purported stem-like counterparts, vascular endothelial cells, supporting mesenchymal cells such as cancer associated fibroblasts and mesenchymal stem cells, and immune cells that have been modulated toward a wound healing or immunosuppressive phenotype such as macrophages and helper T cells (Quail & Joyce, 2013). The non-cellular components include the factors and cytokines that these cells produce such as vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF- β), hepatocyte growth factor (HGF), and others (Maman & Witz, 2018). Cancer associated fibroblasts and macrophages are primarily responsible for extracellular matrix deposition and facilitate its remodeling respectively (Alexander & Cukierman, 2016; Kalluri, 2016). These processes are facilitated by

enhanced deposition of extracellular matrix proteins like fibronectin, laminin, collagens, and the matrix metalloproteinases (MMP) responsible for degrading these structures. This dynamic structural regulation and biochemical modulation of the tumor tissue is essential in providing a microenvironment that facilitates the transformation of malignant cells and allow further dissemination and survival in distal body sites normal differentiated cells are ill-adapted to survive. One such purported transformation that may mediate this cellular plasticity and has been extensively studied in the context of metastasis is the epithelial to mesenchymal transition (EMT) (Soltermann et al., 2008).

Epithelial to mesenchymal transition

EMT is a normal process of developing cells within the embryo in which cells undergo a transition from an epithelial state characterized by a maintenance of cell polarity, cell-to-cell junctions, and cuboidal morphology to a more mesenchymal state in which cells lose polarity, gain a more spindle-like morphology and an increased migratory capacity, separating from surrounding tissue (Lamouille, Xu, & Derynck, 2014). Upon reaching the appropriate location or upon stimulus from the environment, the mesenchymal cells are thought to undergo the reverse process of mesenchymal to epithelial transition (MET) and revert to an epithelial phenotype. However, this phenomenon is not restricted to fetal development, as adult somatic cells are implicated to undergo this process in a regulated manner in response to organ injury in order to repair damaged tissue in a variety of tissues (Stone et al., 2016). However, as tumors have been described as “wounds that never heal” (Dvorak, 1986, 2015), this process is

hypothesized to be a critical mediator within cancerous tissue to facilitate metastasis.

Driven by certain mutations in oncogenes, epithelial cells undergo uncontrolled replication and begin to influence their local environment to respond to these wound healing signals, and this response aids in the tumors growth as the dysplastic cells undergo additional transformation and the tumor loses normal organization and differentiation (Negrini, Gorgoulis, & Halazonetis, 2010). Under influence from the microenvironment, these atypical cancerous cells undergo EMT to a more mesenchymal phenotype and express MMP in order to penetrate the basement membrane of tissue and facilitate migration through the interstitial fibers of the ECM to reach nutrients and endothelial lined blood vessels or lymphatic vessels. In a process termed intravasation, these cancerous cells penetrate through the endothelium in order to reach the bloodstream. A property that differentiates epithelial cells from mesenchymal cells is the ability of mesenchymal cells to survive anchorage independent growth. Normally, epithelial cells that detach from the ECM undergo a process of anchorage dependent programmed cell death known as “anoikis” (Paoli, Giannoni, & Chiarugi, 2013). In circulation, the mesenchymal-like phenotype of transformed cells provides resistance to this form of apoptosis and potentiates survival during hematogenous dissemination. Accordingly with Paget’s “seed and soil” hypothesis, many transformed cancer cells enter the circulation but only an exceedingly small percentage survive and reach to distant metastatic sites, where they penetrate the blood vessel by extravasation, and migrate to an area

within the pre-metastatic niche with favorable growth conditions (Langley & Fidler, 2011; Paget, 1889). Once established, these mesenchymal-like cells undergo MET to transition to a more epithelial state, anchor to tissue at a distant organ, and begin to respond to local growth factors and proliferate.

The processes of EMT and MET are also regulated intracellularly and extracellularly. Perhaps the most characteristic protein of the epithelial phenotype is the cell-cell adherence protein epithelial-cadherin (E-cadherin) (Jeanes, Gottardi, & Yap, 2008; Onder et al., 2008). E-cadherin is member of the calcium dependent adhesion molecule family of proteins known as cadherins. Cadherins are transmembrane proteins that link cells together through extracellular domains, while their intracellular domains bind to β -catenin and serve as linkages for several other proteins including α -catenin, p120 catenin, and vinculin to form linkages with the actin cytoskeleton in a complex known as the adherens junction. The stability of this complex has been demonstrated to be under the regulation of kinase activity from receptor tyrosine kinases such as epidermal growth factor receptor (EGFR) as well as non-receptor tyrosine kinases such as the Src family. For example, Roura et al demonstrated that Src phosphorylation of tyrosine 654 of β -catenin reduces the binding affinity for e-cadherin and phosphorylation at this site results in disruption of the adherens complex (Roura, Miravet, Piedra, Garcia de Herreros, & Dunach, 1999). Additionally, e-cadherin expression is tightly regulated and repressed by the Snail family of zinc-finger transcription factors including Snail and Slug, the basic helix-loop-helix protein Twist, and others. Importantly, aberrant e-cadherin expression and localization is

a feature of advanced tumor progression and correlates with increased invasiveness of tumors, whereas overexpression of e-cadherin can suppress invasion in some cancer cell types. The loss of e-cadherin and cell-to-cell contacts is coupled with the expression of neural cadherin (N-cadherin) and other proteins such as vimentin, alpha-smooth muscle actin, and fibronectin which are associated with a mesenchymal phenotype. The stabilization of this mesenchymal state also confers an increased motility to cells to facilitate migration of single cells from the primary tumor (Aiello et al., 2018).

Cell migration and invasion processes in 2D and 3D environments

Cell migration is a critical component of cancer metastasis. The molecular aspects of cell migration have been studied extensively in-vitro predominantly using slow moving fibroblasts or rapidly and uniformly motile keratinocytes. These experiments have led to a proposed multistep mechanism of migration by which cells first establish polarity toward an inducing extracellular stimulus creating frontward protruding adhesions at the leading edge and contracting adhesions at the rear of the cell in order to propel the cell forward. Next, actin is assembled at the leading edge of the cell in various cellular protrusions, each with specialized machinery and distinct actin morphologies. One of these protrusions, the lamellopodia, is composed of dense, branched actin networks under the control of the actin nucleator actin-related protein 2/3 (ARP2/3), Wiskott-Aldrich syndrome protein (WASP), WASP-family verprolin homologous protein (WAVE), cofilin, and other proteins (Lai et al., 2008; Machesky & Insall, 1998) that exert a protruding force on the inner surface of the cell membrane in

order to extend the cytoplasm forward. Filopodia, another classification of cellular protrusion, are composed of parallel aligned actin filaments (F-actin) that extend toward the cell membrane with their positive, ATP-bound barbed ends to probe the extracellular environment while the negative, ADP-bound pointed ends are oriented toward the cell interior (Mattila & Lappalainen, 2008). Adhesive force and tension mediated by myosin machinery result in rearward retraction of this actin filament and aid in propelling the cell forward. In the last step of migration, adhesions at the rear of the cell are disassembled allowing localized detachment of the cell body and contractile forces by myosin motor proteins including myosin II to generate force to retract the rear of the cell and propel the cell body forward.

These adhesions are composed of various cell adhesion molecules that form, mature, and disassemble rapidly and dynamically. Adhesion formation is thought to begin with the formation of the nascent adhesion. These nascent adhesions are composed of kindlin-2, α -actinin, vinculin and other proteins that loosely attach the cells actin cytoskeleton to the extracellular environment via a heterodimer of transmembrane proteins known as integrin receptors (Sun, Lambacher, & Fässler, 2014). Integrins bind to several different protein motifs on extracellular proteins such as collagen (DGEA), fibronectin, laminin, and vitronectin (RGD), and others (Horton et al., 2016). As the focal complex recruits additional force sensitive proteins including talin, kindlin-1, and paxillin, more integrin pairs are recruited to the adhesion in a process known as clustering, creating further binding sites for accessory adhesion proteins such as focal adhesion kinase (FAK) and integrin linked kinase (ILK). Force generated by the

extracellular integrin linkage and dense bundles of F-actin cytoskeleton known as stress fibers assists in the maturation of nascent adhesions to larger signal transducing structures known as focal adhesions (Tai, Chen, & Shen, 2015). Focal adhesions generate signals through focal adhesion kinase and other kinases in order to modulate a wide variety of cell processes including actin remodeling, cellular contraction, proliferation, survival, migration, and invasion (Fig. 1). In some cases, these focal adhesions can mature further to fibrillar adhesions, which lose their force reactive properties and do not bind to actin stress fibers. In other cases, focal adhesions are rapidly disassembled in a process dependent upon FAK (Geiger & Yamada, 2011; Hamadi, 2005). Thus, FAK plays a central role in the dynamic regulation of focal adhesions within cells.

While the mechanisms of cell migration are fairly well characterized under in vitro conditions in which cells are grown on a 2-dimensional (2D) substrate, less is known about the migration of cells in-vivo, where cells maintain connections through adherens junctions as they migrate in a process termed collective cell migration and are surrounded on all sides by extracellular substrates including ECM (3D), and these are active areas of research. Fibroblasts and cancer cells grown on plates coated with ECM substrates such as collagen, fibronectin, or vitronectin exhibit markedly different morphological characteristics than those grown on glass or plastic. On surfaces coated with fibrillar collagen gels, fibroblasts form adhesions along the fibers of the matrix, providing them with an elongated spindle shape with parallel arrangements similar to mesenchymal cells embedded within connective tissue in-vivo such as

cartilage (P. Friedl & Brocker, 2000). This change in cell structure is accompanied by a difference in migratory function as fibroblasts and cancer cells grown on 3D substrates alter their cytoskeleton and matrix adhesions to facilitate travel through a more complex architecture compared to a flat surface. The first of these migratory strategies is termed mesenchymal migration in which cells utilize extended cellular projections classified as pseudopodia to form matrix adhesions including integrins to anchor to the matrix stabilized with F-actin, decrease their velocity, and localize matrix degrading enzymes like MMPs to degrade through migratory barriers. In the second form of 3D migration, termed amoeboid migration, cells decrease their matrix adhesions, allowing the cell body to become more rounded, increase contractile activity to produce membrane blebs, and squeeze through structures in an MMP-independent manner similarly to the behavior of leukocytes to accomplish high cellular velocities. Cancer cells in vitro demonstrate both methodologies of migration and can actively alter their migrations strategy, however whether this behavior is dependent upon a cell intrinsic regulatory mechanism or is solely a factor of the encountered extracellular environment are currently active areas of research.

Aside from migration, invasion through the basement membrane into surrounding structures is a critical parameter for cancer dissemination and metastasis, as invasive tumors are characteristic of late stage disease. Frequently this step in the metastatic cascade is modeled through the inclusion of a basement membrane-like material derived from Englebreth-Holm-Swarm mouse sarcoma cells and marketed under the trade name Matrigel. Matrigel

contains a number of constituents found in the basement membrane including laminins, collagen IV, and entactin, as well as an assortment of growth factors and cytokines including EGF, transforming growth factor beta (TGF- β), and platelet-derived growth factor (PDGF), although it is also available in a growth factor reduced form. Classically, this material is used to coat the upper surface of a porous membrane where the gel is polymerized to a thin matrix coating.

Cancer cells are placed on top of this membrane and the number of cells that invade through the matrix and migrate to the opposite side of the membrane are quantified at endpoint. To accomplish this task, cancer cells adapt to their environment and produce cellular protrusions termed invadopodia. Invadopodia are critical structures in cell invasion found in aggressive tumor cells but not normal cells. Their assembly can be promoted through EGFR signaling, and the cortical actin binding protein cortactin has been found to be necessary for the early assembly of an F-actin core within these protrusive structures. Src, downstream of EGFR, has been shown to be a critical mediator of invadopodia maturation through tyrosine phosphorylation of cortactin and other proteins. The mature invadopodia promotes invasion through targeting MMP, particularly MT1-MMP to the ECM.

Extracellular matrix and microenvironment characteristics that promote metastasis

Apart from its protein composition, the ECM possesses multiple properties that promote transformation and plasticity of a variety of cell types. Alterations of the ECM stiffness, linearity, and density are correlated with worse features in a

variety of fibrotic pathologies including idiopathic pulmonary fibrosis, liver cirrhosis, and chronic kidney disease, as well as cancers including those of the breast, pancreas, and others (Biagini & Ballardini, 1989; Cox & Erler, 2011; Herrera, Henke, & Bitterman, 2018; Jena & Janjanam, 2018; H. O. Lee et al., 2011). Fibroblasts and other mesenchymal cells are thought to be primarily responsible for the deposition and assembly of the complex array of proteins and proteoglycans which compose the ECM, however frequently other cells within the microenvironment including epithelial cells and leukocytes also locally organize and remodel the ECM, cross-linking proteins like fibronectin with collagen or degrading matrix through MMP secretion and activation. The assembly and remodeling of the ECM by diseased or desmoplastic tissue can create structures that facilitate migration of cells by creating tracks or tunnels, create barriers to leukocyte infiltration, modulate stiffness, and provide a reservoir of zymogens or matrikines, ECM components that possess binding motifs which can stimulate growth factor receptors like EGFR, that can be liberated from the ECM by MMP mediated proteolysis.

Aside from these structural elements, other components of the tumor microenvironment play an important role in cancer progression and the regulation of processes such as EMT. For example, cancer associated fibroblasts or mesenchymal cells (CAM) produce extracellular matrix proteins, but they have also been shown to secrete a variety of cytokines and growth factors into the tumor microenvironment to facilitate different elements of tumor growth or play a role in mediating resistance to chemotherapeutic drugs. During the reciprocal

cross talk between cancer cells and their supportive CAM stroma, the CAM cells secrete epidermal growth factor (EGF), hepatocyte growth factor (HGF), and insulin-like growth factors (IGF) and others to support tumor growth and proliferation. CAM have also been demonstrated to promote angiogenesis in tumors through secretion of VEGF and PDGF. Aside from these growth factors, in a study by Navab et al., gene ontology analysis of expression profiles from cancer associated fibroblasts and matched normal fibroblasts from NSCLC patients revealed that enriched genes were associated with associated with extracellular proteins and membrane-bound proteins, mostly involved in signal transduction, cell adhesion, and response to cell stress (Navab et al., 2011). Many of these proteins were also associated with TGF- β regulation, another growth factor that is secreted by activated CAM cells. Through these studies and others, it has recently been shown that CAM cells are major influencers of cancer progression, and understanding the mechanisms that govern the activation and aberrant gene expression of these activated cells will be critical to the development of treatments in the future.

One element of the tumor microenvironment important to the regulation of gene expression in tumor and stromal compartments is hypoxia. Generally, a growing tumor requires a vascular supply in order to transport nutrients to and waste from the rapidly dividing cells. The availability of blood supply has been shown to be a critical determinant of tumor growth. As the tumor grows, the center of the tumor becomes cut off from the blood supply resulting in a gradual oxygen deprivation toward the center of the tumor mass resulting in necrosis. To

compensate, cells that are undergoing hypoxic and nutrient stress express genes in order to promote the recruitment, migration, and proliferation of endothelial cells that undergo angiogenesis. Within the tumor microenvironment there is an imbalance of pro- and anti-angiogenic factors, and this may contribute to the aberrant vasculature found in tumors characterized by tortuous, dilated and leaky vessel walls. Many of these genes are under the control of an oxygen sensitive regulator called hypoxia inducible factor 1 alpha (HIF1- α). HIF1- α expression is correlated with worse tumor progression and can activate EMT and invasion in cancer cells. It can enhance expression of several enzymes involved in post-translational modification of collagen and can lead to stiffer, aligned ECM fibers that can drive tumor progression. HIF1- α can also alter the expression of MMP and urokinase plasminogen activator receptor (uPAR), thus hypoxia also plays a central role in ECM remodeling (Petrova, Annicchiarico-Petruzzelli, Melino, & Amelio, 2018).

Extracellular matrix remodeling

ECM remodeling is characterized by a balance of ECM deposition and degradation by MMP and other proteases. One of the major proteolytic systems important in various fibrotic pathologies is mediated by plasminogen and its major regulator plasminogen activator inhibitor-1 (PAI-1). The plasminogen-plasmin system modulates migration and tissue remodeling by interactions with the ECM and by regulating signal transduction (Fig. 2). PAI-1 is a serine protease inhibitor and is the main regulator of the plasminogen activation system. PAI-1 inhibits both tissue-type plasminogen activator (tPA) and urokinase-type

plasminogen activator (uPA), decreasing the cleavage of plasminogen to the active enzyme plasmin. Aside from fibrin, plasmin can directly degrade ECM components such as laminin, proteoglycan, and type IV collagen (Liotta et al., 1981), inducing matrikine peptides capable of signal transduction, and also indirectly degrade ECM via activation of several pro-MMPS to their active form including MMP2, MMP3, MMP9, MMP12, and MMP13 (Jaiswal, Varshney, & Yadava, 2018) (Ramos-DeSimone et al., 1999). PAI-1 has important biological activities that extend beyond its inhibition of ECM degradation. PAI-1 activity and expression have been shown to mediate a number of tumorigenic processes through its interaction with uPA and other elements of the ECM. PAI-1 exists in latent and active forms in plasma. Active PAI-1 is unstable and binds to the ECM protein vitronectin to prevent spontaneous conversion to the latent form (Seiffert, Mimuro, Schleef, & Loskutoff, 1990). The binding of PAI-1 to vitronectin prevents integrins from binding to vitronectin, resulting in inhibition of integrin mediated cell adhesion and migration depending on the cell type (Whitley, Palmieri, Twerdi, & Church, 2004). However, PAI-1 has also been shown to enhance cell adhesion and migration of breast cancer cells (Chazaud et al., 2000; Whitley et al., 2004). Further evidence for the ability of PAI-1 to contribute to tumor progression was shown by adding PAI-1 to cells in culture. Exogenous PAI-1 was sufficient to block spontaneous and induced apoptosis, possibly by upregulating c-Jun/ERK and BCLxl, while downregulating BAX expression (Balsara & Ploplis, 2008). Interestingly, inhibitors of PAI-1 block cellular migration and invasion in murine cancer models, as well as the seeding of lung cancer cells in the lungs of mice,

preventing lung cancer metastasis through inhibition of host interactions and independently of tumor PAI-1 levels (Masuda et al., 2013). In the clinical setting, elevated plasma PAI-1 levels are associated with poor prognosis in cancer patients (Look et al., 2002; Pavey, Hawson, & Marsh, 2001). Importantly, increased PAI-1 expression in lung cancer patients correlates with cancer mortality (Di Bernardo, Matakidou, Eisen, Houlston, & Consortium, 2009; Gemma et al., 2001; Pappot, Pedersen, Brunner, & Christensen, 2006; Pappot, Skov, Pyke, & Grondahl-Hansen, 1997). Through these mechanisms and others, PAI-1 has been shown to be involved in cancer development, growth (Jing et al., 2012), and metastasis through inhibition of apoptosis (Balsara & Ploplis, 2008), increased tumor cell adhesion to endothelium (Prager, Breuss, Steurer, Mihaly, & Binder, 2004), proliferation (Y. P. Zhang et al., 2013), and disruption of tumor cell interaction with the extracellular matrix (Czekay, Aertgeerts, Curriden, & Loskutoff, 2003). These studies highlight the importance of studying the stromal and supporting elements of the tumor microenvironment, and particularly ECM interactions of cancer cells.

In-vitro models of the tumor microenvironment

In order to recapitulate structural elements of the tumor microenvironment, researchers have utilized in-vitro strategies that include preparation of collagen gels on plastic or glass slides. Variations in the fibrillar structure of these matrices can be achieved through changing their preparation parameters including varying concentration, temperature, and chemically induced crosslinking of fibers. Fibroblasts and cancer cells grown on these fibrillar gels exhibit distinct

morphological differences, including extension of long, thin, cellular projections termed pseudopodia that extend outward from the cell to connect to the matrix. Alterations of the stiffness of the collagen matrix are capable of altering the invasive capabilities of cancer cells. MDA-MB 231 breast cancer cells exhibit a biphasic invasive response that depends on the concentration, stiffness, and porosity of the hydrogel, where they exhibit greater invasive behavior on soft and crosslinked matrices and stiff, porous matrices (Lang et al., 2015). While these constructs are useful in studying cell-matrix interactions, they may be insufficient in recapitulating some of the ECM alterations associated with the tumor microenvironment. For example, when collagen gels are combined with Matrigel, H1299 lung cancer cells alter their morphology and migration characteristics (Anguiano et al., 2017). In a collagen hydrogel, the cells adopted a mesenchymal migration phenotype with polarization of the cell body and lamellipodia adhesions at the front and rear of the cell. In the combined collagen and Matrigel matrix, the cells shifted to short, blunted lobopodia protrusions to navigate the matrix, and the maintenance of this mesenchymal phenotype required $\beta 1$ and $\beta 3$ integrin matrix adhesion. In contrast to single or dual ECM component approaches, an alternative method to produce ECM is directly from cell culture of the main ECM producing cells in the body, the fibroblast. Cultured at confluence, and supported with supplements of ascorbic acid to promote collagen deposition and crosslinking over the course of a week or more, fibroblasts and other mesenchymal cells produce an ECM that contains protein constituents found in tissue, and is organized and cross-linked by depositing cells to recapitulate the *in*

vivo microenvironment. These culture plates are then denuded of cells using methods which preserve the structural and biochemical components of the ECM and are then carefully washed and treated with DNA digesting enzymes to remove nuclear debris from the glycosylated matrix. Following cell extraction, the cell derived ECM constructs are used as scaffolds for cells to study cell-matrix interactions and downstream behaviors. In a landmark study, Cukierman et al. demonstrated that 3T3 mouse embryonic fibroblasts (MEF) attach to 2D fibronectin differently than fibroblasts attach to the ECM *in vivo*, and that these cell-matrix adhesions could be recapitulated when fibroblasts were grown on a fibroblast-derived ECM. These matrix adhesions were characterized with triple localization of $\alpha 5$ integrin, paxillin, and fibronectin (Cukierman, Pankov, Stevens, & Yamada, 2001). Other components of these matrix adhesions included α -actinin, F-actin, talin, tensin, and vinculin, however these components were shared by focal and fibrillar adhesions.

This work served as a catalyst for investigators to use this system to study cell-matrix adhesions and ECM dependent cell signaling and behavior in a variety of contexts, including vasculogenesis (Soucy, Hoh, Heinz, Hoh, & Romer, 2015), stem cell differentiation (X. D. Chen, Dusevich, Feng, Manolagas, & Jilka, 2007), and cancer biology (Scherzer et al., 2015). Soucy et al. used immunofluorescence microscopy to characterize a lung fibroblast derived ECM to contain fibronectin, tenascin-C, collagen I, collagen IV, and collagen IV, among others (Soucy & Romer, 2009). While many of these proteins colocalized, fibronectin had a more unique distribution. Human umbilical vein endothelial cells

(HUVEC) grown on these lung fibroblast derived ECM were found to have cell matrix adhesions containing $\beta 1$ integrin, phospho-tyrosine 397 FAK, phospho-tyrosine 410 p130Cas, vinculin, and talin. Additionally, these matrix adhesions localized with MT1-MMP, suggesting an association with invadopodia formation. Remarkably, HUVEC grown on lung fibroblast ECM formed tubular networks after 24 hours, demonstrating the benefits of physiologically relevant cell culture systems on tubulogenesis.

Previously in our lab, Scherzer et al extended this lung fibroblast derived ECM to study the interaction of lung adenocarcinoma cells with the extracellular matrix (Scherzer et al., 2015). Among the findings of that study were marked differences in cell morphology of A549 to an elongated appearance, resistance to serum withdrawal and chemically induced hypoxia, increased migration velocity and directionality, and a decrease in E-cadherin expression. The characterization of this model in the context of lung cancer allows us to now ask questions related to lung cancer cell metastatic processes in a physiologically relevant manner. For example, what signaling mechanisms are induced by cell-matrix interactions with a lung fibroblast deposited extracellular matrix? What are the cellular components of these interactions and what ligands do they interact with extracellularly? What characteristics of cell migration are promoted by extracellular matrix interactions in the tumor microenvironment and are there specific signaling mechanisms we could target to inhibit 3D migration? What role does extracellular matrix remodeling play in the context of NSCLC? Would an in-vitro physiological microenvironment promote alterations in extracellular matrix

deposition, and would these altered matrices differentially affect cancer cells or cancer activated fibroblasts? Does a microenvironment that contains the extracellular matrix and physiological hypoxia preserve characteristics of lung tumors found in-vivo? Are the cancer associated stromal elements able to influence cancer progression through extracellular matrix deposition?

To begin to answer these questions, we formed the hypothesis that a microenvironment mimetic in-vitro culture system consisting of a lung cell-derived extracellular matrix and a physiological oxygen environment would preserve and promote in-vivo tumor characteristics and modulate migration and invasion of lung adenocarcinoma cells through cell-matrix interactions.

Overall goal

Determine how ECM interactions with lung adenocarcinoma cells can promote hallmarks of cancer metastasis in a physiologically relevant *in vitro* model.

Overall hypothesis

A microenvironment mimetic in-vitro culture system consisting of a lung cell-derived extracellular matrix and a physiological oxygen environment would preserve and promote in-vivo tumor characteristics and modulate migration and invasion of lung adenocarcinoma cells through cell-matrix interactions.

SPECIFIC AIMS

- 1. Investigate the mechanism and modality of 3D migration of lung adenocarcinoma cells on extracellular matrix.**
- 2. Determine the effect of physiological hypoxia and extracellular matrix on tumor stroma.**
- 3. Determine the effect of extracellular matrix remodeling on lung cancer metastasis.**

Figure 1. Cell-matrix interactions in cancer cells promote cellular processes involved in metastasis.

Interactions with extracellular matrix through integrins activate multiple signaling cascades independently and through crosstalk with growth factor receptors to affect survival, cell adhesion, cytoskeletal remodeling, proliferation, migration, and adhesion. Matrix metalloproteinases released from the cell mediate pericellular remodeling of the ECM by degrading ECM proteins and activate latent growth factors within the microenvironment. These factors and the matrix peptides further potentiate cell surface receptor signaling. This figure was modified and adapted from (Lu, Weaver, & Werb, 2012).

Figure 2. Plasminogen activator inhibitor 1 (PAI-1) regulates pericellular proteolysis and cell migration.

PAI-1 regulates ECM degradation and fibrinolysis directly by inhibiting the catalytic activation of plasminogen, which is localized to the cell surface by Annexin A2 and S100A10 or embedded within fibrin clots, to the active enzyme plasmin by either tissue-type plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA) and its receptor (uPAR). Plasmin degrades extracellular matrix substrates and activates pro-forms of matrix metalloproteinases (Pro-MMP) to their active state (MMP). The uPA/uPAR complex also associates with integrins to induce intracellular signaling through Jak/STAT, FAK, PI3K, and other pathways. Vitronectin, an ECM protein, has binding sites for PAI-1 and uPAR, however PAI-1 binds with higher affinity and can prevent association with the receptor. PAI-1/uPAR complexes are internalized within the cell, where the PAI-1 is targeted for lysosomal degradation and uPAR is recycled to the cell surface to facilitate attachment and migration of the cell. PAI-1 also interacts with intracellular substrates such as caspase 3 to inhibit apoptosis, and may be involved with other intracellular activity associated with migration.

CHAPTER II: MATERIALS AND METHODS

Human tissue samples

Primary human lung cancer resections (six) were acquired and de-identified from patients undergoing resection of pulmonary tumors without preoperative chemotherapy or radiotherapy at the James Graham Brown Cancer Center at the University of Louisville. Patients with lung adenocarcinoma and squamous cell carcinoma were staged according to the tumor-node-metastasis system established by the American Joint Committee on Cancer. This study was approved by the IRB committee of the University of Louisville (#130188) and informed consent was obtained from all patients. All experiments were performed in accordance with relevant guidelines and regulations.

Hypoxic cell culture

Hypoxic cell culture conditions (5% O₂, 5% CO₂, 90% N₂, 37°C) were achieved with a modified water jacketed cell culture incubator equipped with a ProOx P110 oxygen controller (BioSpherix, Parish, NY). The sensor was calibrated and programmed to maintain a setpoint of 5% O₂ via infusion of Ultra High Purity Nitrogen gas unless otherwise stated.

Patient sample processing

Tumor resections were transported on ice, transferred to a sterile 100 mm cell culture plate in ice-cold PBS, imaged and placed in a sterile laminar flow hood. The tissue was diced to approximately 1mm³ pieces. The diced tissue was transferred to a vial containing collagenase/dispase (Sigma, St. Louis, MO) dissolved (1mg/mL) in serum free Dulbecco's modified Eagle's medium-high glucose (DMEM) for enzymatic digestion. The vial was warmed to 37° C and incubated with rocking for at least 1 h 15 m. The tissue suspension was agitated with vigorous pipetting to confirm tissue dissociation and either returned to 37° C if incomplete or inactivated. Cell suspensions passed through a 70 µM cell strainer. A syringe plunger was used to force remaining tissue through the strainer and the strainer washed with ice cold PBS. The tissue suspension was cooled to 4°C and then placed in a centrifuge for 3 minutes at 400 cfg and 4°C. The supernatant was aspirated and the cell pellet suspended in growth medium (DMEM 2% FBS, Pen/Strep, Cipro). Cell and viability counts were obtained with a TC20 automated cell counter (Bio-rad), and cells were placed onto a plate coated with human cell derived extracellular matrix and maintained in a climate-controlled incubator at 37°C, 5% CO₂, and 5% O₂ (Fig. 14A). Cells were monitored for attachment and healthy morphology at 24 hours post culture and the media was changed 4 or 7 days post culture depending on cell attachment.

Cell culture

Human normal fetal lung fibroblast cell lines WI38 and IMR90 were purchased from American Type Culture Collection (ATCC) and maintained in Minimum Essential Medium Alpha modification (MEM-α) supplemented with L-Gln, ribo-

and deoxyribonucleosides (Fisher), 10% fetal bovine serum (Invitrogen) and 1% antibiotic/antimycotic (Sigma). Human bone marrow mesenchymal stem cells (hMSC) were purchased from Lonza and cultured as the fetal lung fibroblast cell lines. Human embryonic stem cell line H1 (WiCell, Madison, WI) was cultured on human embryonic stem cell-qualified Matrigel-coated plates (BD Biosciences, San Jose, CA) in mTeSR1 media (STEMCELL Technologies, Vancouver, Canada), with media changed daily. All assays involving comparisons between tumor derived stromal cell lines (927, 955, 956, 959, 960, and 1072) and mesenchymal cell lines (WI38, IMR90, and hMSC) were performed with early passage cells ($p < 8$) in the same culturing medium, MEM α , described previously. A549, H2009, and H2030 human adenocarcinoma cells were purchased from ATCC and were cultured in RPMI medium supplemented with 10% FBS, and 1% antibiotic/antimycotic. A549 NucLight Red human adenocarcinoma cells (Essen Bioscience) were cultured similarly, and media was supplemented with 1 $\mu\text{g/mL}$ puromycin. All cell lines were maintained at 37°C and 5% CO $_2$, unless otherwise stated.

Extracellular Matrix Preparation

Extracellular matrix preparation was performed as previously described (Scherzer et al., 2015). Briefly, IMR90 or WI38 fetal lung fibroblasts, or patient derived mesenchymal cells, were plated at confluence in matrix growth medium (MEM- α , 10% FBS, 1% antibiotic). On the second, fourth, and sixth days, medium was supplemented with 50 $\mu\text{g/mL}$ L-Ascorbic acid (Sigma) to promote matrix deposition. On the eighth day, wells were washed with phosphate buffered saline

(PBS) and cells were lysed with a detergent (20mM NH₄OH, 0.5% Triton-X-100) for 1-3 minute incubation at 37° C until complete cell lysis was observed. Resulting cell derived matrices were gently washed three times with PBS, and then incubated with Dnase1 (10 U/mL) for 30 minutes to remove residual DNA. The matrices were then washed twice in PBS, and either used immediately or stored at 4°C in PBS supplemented with 1% antibiotic for up to 4 months.

Microscopy and immunocytochemistry

Phase microscopy images were obtained using a Zeiss AX10 inverted microscope. A549, H1, and patient derived cells were fixed with 2% PFA/PBS (10 minutes, 24°C; Electron Microscopy Sciences, Hatfield, PA), permeabilized when needed with 0.05% Triton X-100/PBS (5 minutes, 24°C; Sigma Aldrich, St. Louis, MO), and blocked with 5% normal goat serum/PBS (Sigma-Aldrich, St. Louis, MO). Cells were probed with mouse monoclonal antibodies recognizing SSEA-4 (1:200, ThermoFisher, Walton, MA) and CD90 (PE) (1:200, BD Biosciences, San Diego, CA) overnight at 4°C. Cells were washed with PBS and incubated with goat anti-mouse 488 (1:1000, ThermoFisher, Walton, MA). DAPI nuclear stain (1:2000; ThermoFisher, Walton, MA) was added (5 minutes, 24°C), washed, mounted, and imaged using Olympus IX81 fluorescence microscope (Center Valley, PA). Live cell time-lapse phase and fluorescence microscopy was performed using a Cytation 5 Cell Imaging Multi-mode reader (BioTek). Immunofluorescent images of co-culture experiments with red nuclear A549 were acquired with a Nikon Eclipse Ti microscope with a 4X objective using NIS-Elements AR acquisition software (Nikon Instruments, Inc., Melville, NY, USA).

Nuclear counts from equal image thresholds were quantified automatically using the NIS-Elements AR software.

Cell Proliferation, CFU-F, and Co-culture

Doubling time experiments were performed by plating approximately 4×10^4 cells in technical duplicates. Mean cell counts were collected at 24 and 96 hours while cells were in log phase growth counting at least 100 cells in a hemocytometer.

Doubling time was calculated using the formula $DT = T \ln 2 / \ln(X_e/X_b)$, where T is the incubation time, X_b is the cell number at 24 hours, and X_e is the cell number at 96 hours. CFU-F assay was performed by seeding 50 cells in a 35-mm well.

Media was refreshed on days 7 and 10. Colonies were fixed in methanol and acetic acid (7:1) on days 14-18 and were stained with crystal violet. Greater than 32 cells were considered colonies. For co-culture studies, stromal cells that had grown in 3DH (Fig 14A.) were seeded at confluence density on 35mm glass bottom tissue culture dishes (MatTek) to produce monolayers. After 4 days growth, 1.5×10^4 Red A549 cells were seeded onto stromal monolayers and maintained in RPMI (10% FBS, 1% antibiotic/antimycotic) and monitored daily.

Migration and Invasion

Lung cancer cells were plated at confluence and serum starved overnight following attachment. A micropipette tip was used to create a scratch in the cell monolayer and pictures were captured at the indicated time points. 3D invasion assays were plated using a WI38 derived matrix that had not been supplemented with ascorbic acid during the growth. Ascorbic acid supplementation or use of

IMR90 fibroblast matrices results in a matrix that was too thick to reliably scratch. Invasion assays utilized a layer of Matrigel containing either vehicle or drugs after the scratch had been made. Images were captured at the indicated timepoints and results were quantified using the area measurement of imageJ, comparing the wound closure at the time of the image to the initial wound. Averages of at least three images were captured for each time point and at least two replicates were used for each individual experiment.

Spheroid Preparation

Lung cancer cells were culture under normal conditions on a Corning ultralow attachment 96 well plate (Sigma) at 1×10^4 cells per well for a period of 6 days to allow small, rounded spheroids. Spheroids were observed under microscopy and transferred to matrix plates with a micropipette for further migration studies.

Generation of Human PAI-1 by LentiCRISPRv2

SERPINE1 (PAI-1) ^{-/-} cell lines were generated using CRISPR-Cas9 mediated genome editing as previously described (Shalem et al., 2014). Briefly, we used selected two guide RNA sequences that targeted the first and second exons of the *SERPINE1* gene. Complimentary oligonucleotides containing the shRNA sequences (Guide 2: 5'-CACCGAACCACGTTGCGGTCCTTGG-3'; guide 3: 5'-CACCGCTGGACGAAGATCGCGTCTG-3') were digested by *BsmBI* and cloned into the lentiCRISPR v2 plasmid (Addgene #52961). Lentivirus was generated using 293T cells transfected with 6 µg lentiCRISPR v2 or pLX304 containing *SERPINE1* cDNA (DNASU HsCD00440510) plasmids, 3 µg psPAX2, and 1.5 µg

pMD2.G in optiMEM with a 3:1 ratio of polyethylenimine (PEI) to total DNA. Lentivirus was collected 48 hours after transfection, twice daily over a two-day period, and filtered in a 0.22 μ m filter. A549, H2030, H2009, and IMR90 cells were infected twice with lentivirus over a 48 hour period and then selected with puromycin or blasticidin (for pLX304; 2-50 μ g/mL) containing media (0.5-1.5 μ g/mL). Single clones were generated by plating low densities of puromycin resistant cells and isolating single colonies with cloning cylinders after 10-14 days of colony growth. Cell lines were maintained in 0.5 μ g/mL puromycin containing media after selection.

Western Blot

Cells and tissue samples were harvested in CHAPS lysis buffer (1% CHAPS detergent, 150mM NaCl, 50mM Tris pH7, 5mM EDTA) supplemented with cOmplete protease inhibitor and PhosSTOP phosphatase inhibitor cocktail tablets (Sigma-Aldrich, St. Louis, MO). Tissue samples were homogenized in a mini-bead beater. Protein was quantified by using Pierce's BCA Protein Assay Reagent Kit (ThermoFisher, Walton, MA) as per the manufacturer's instructions. Total protein (20 μ g) was heated at 95°C for 5 minutes and separated by 4-12% SDS-polyacrylamide gel. Blots were transferred to PVDF membranes for 120 minutes at a constant voltage of 100 V in 4°C. Membranes were blocked in 5% milk (w/v) in Tris-buffered saline Tween-20 (TBS-T) at 24°C for 1 hour. The membranes were incubated with primary antibody in 5% milk TBS-T overnight at 4°C. A list of antibodies used for analysis is presented in the supplementary material.

<i>Antibody</i>	<i>Host</i>	<i>Catalog</i>	<i>Distributor</i>
HIF-1 α (D2U3T)	Rabbit	#14179	Cell Signaling Technologies
p21 Waf1/Cip1 (12D1)	Rabbit	#2947	Cell Signaling Technologies
α -Smooth Muscle Actin	Rabbit	#14968	Cell Signaling Technologies
Src	Rabbit	#2108	Cell Signaling Technologies
P-Src Family (Tyr416)	Rabbit	#2101	Cell Signaling Technologies
P-Paxillin (Tyr118)	Rabbit	#2541	Cell Signaling Technologies
Vimentin (D21H3)	Rabbit	#5741	Cell Signaling Technologies
N-Cadherin	Rabbit	#4061	Cell Signaling Technologies
Slug (C19G7)	Rabbit	#9585	Cell Signaling Technologies
Snail (C15D3)	Rabbit	#3879	Cell Signaling Technologies
EGF Receptor (D38B1)	Rabbit	#4267	Cell Signaling Technologies
P-EGFR(Tyr1068)	Rabbit	#3777	Cell Signaling Technologies
Akt (pan) (C67E7)	Rabbit	#4691	Cell Signaling Technologies
P-Akt (Ser473) (D9E)	Rabbit	#4060	Cell Signaling Technologies
p38 MAPK (D13E1)	Rabbit	#8690	Cell Signaling Technologies
P-p38 MAPK (Thr180/Tyr182) (D3F9)	Rabbit	#4511	Cell Signaling Technologies
PAI-1	Mouse	#612024	BD Transduction Labs
Zeb1	Rabbit	#05987	Novus biologicals
E-Cadherin (24E10)	Rabbit	#3195	Cell Signaling Technologies
TGF- β	Rabbit	#3711	Cell Signaling Technologies
LC3B (D11)	Rabbit	#3868	Cell Signaling Technologies
Basic Cytokeratin (AE3)	Mouse	#57004	Santa Cruz
p27 Kip1	Mouse	#610242	BD Biosciences

<i>Antibody</i>	<i>Host</i>	<i>Catalog</i>	<i>Distributor</i>
p44/42 MAPK (Erk1/2)	Rabbit	#4695	Cell Signaling Technologies
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	Rabbit	#4370	Cell Signaling Technologies
Annexin II (C-10)	Mouse	#28385	Santa Cruz
p-Annexin II (85.Tyr 24)	Mouse	#135753	Santa Cruz
PAI-2	Mouse	#166539	Santa Cruz
Plasminogen	Mouse	#376324	Santa Cruz
Integrin α V	Rabbit	#4711	Cell Signaling Technologies
Integrin β 3	Rabbit	#4702	Cell Signaling Technologies
P-Stat3 (Tyr705)	Rabbit	#9131	Cell Signaling Technologies
FAK (D2R2E)	Rabbit	#13009	Cell Signaling Technologies
P-FAK (Tyr397) (D20B1)	Rabbit	#8556	Cell Signaling Technologies
P-FAK (Tyr576/577)	Rabbit	#3281	Cell Signaling Technologies
P-FAK (Tyr925)	Rabbit	#3284	Cell Signaling Technologies
RhoA (67B9)	Rabbit	#2117	Cell Signaling Technologies
ILK1 (4G9)	Rabbit	#3856	Cell Signaling Technologies
GAPDH (D16H11)	Rabbit	#5174	Cell Signaling Technologies
β -Actin	Mouse	#A2228	Sigma
Rabbit IgG HRP	Goat	#7074	Cell Signaling Technologies
Mouse IgG HRP	Horse	#7076	Cell Signaling Technologies

Table 1. Antibodies

Flow Cytometry

Cultures of A549, hMSC, and patient sample cell lines were collected with trypsin and washed with PBS. Cells were blocked for 15 min at 4°C with Fc Block (#553142, BD Biosciences, Miami, FL, USA). The samples were then stained with a mix of fluorophore-conjugated anti-human monoclonal antibodies: FITC CD90 (Thy1), PE CD105, and PerCP/Cy5.5 CD73 (Ecto-5'-nucleotidase) from BioLegend (San Diego, CA) for 1 h at 4°C. Stained cells were then examined on a Becton Dickinson FACScan with FlowJo analysis software.

RT-qPCR

Total RNA from cell culture samples was isolated with E.Z.N.A Total RNA Kit (Omega), according to the manufacturer's instructions. Total RNA (350 ng) was then DNase digested with TURBO DNA-free kit (Invitrogen) per the manufacturer's instructions. DNase digested RNA was then reverse transcribed using High Capacity cDNA Reverse Transcription Kit (ThermoFisher, Walton, MA). Gene-specific cDNA was quantified with real-time RT-PCR using self-designed SYBR assays. Primers for qPCR were manually designed with Primer Express 3.0 software (Applied Biosystems, Foster City, CA) per the manufacturer's instructions for SYBR green dye assays. RT-qPCR was performed with iTaq Universal Sybr Green Supermix (Bio-Rad). Expression levels were analyzed using $\Delta\Delta CT$ method and were normalized to $\beta 2$ -microglobulin (B2M) mRNA levels. PCR reactions were analyzed on a BioRad CFX96. Primer sequences are presented in the table below.

<i>Gene</i>	<i>Forward</i>	<i>Reverse</i>
ZFP42 (REX-1)	TTTGAGTCCTTGGAATACCTAAAGAAA	AATTCTCTCCAACCTATCTTTTGTGGAA
TNC (Tenascin C)	GAGAACAAAGAAGAGCATTCTGTCA	GGACTTGAATTCAGGCCTTCA
POSTN (Periostin)	TGGTTATATGAGAATGGAAGGAATGA	GTCAGAATAGCGCTGCGTTGT
NANOG	GAACCTCAGCTACAAACAGGTGAA	TTTCTGCCACCTCTTAGATTTTCATT
POU5F1 (Oct4)	CCAAGCTCCTGAAGCAGAAGA	TTGGCTGAATACCTTCCCAAA
SOX2	GCACAACTCGGAGATCAGCA	TCTCCGTCTCCGACAAAAGTTT
CPSG4 (NG2)	CACTGTCCTGCCTGTCAATGA	CCGTCCGTGCTCCTCAGA
VEGFA	CTTGCTGCTCTACCTCCACCAT	GACATCCATGAACTTCACCACTTC
SERPINE1 (PAI-1)	TGAAGATCGAGGTGAACGAGAGT	GCTGAGACTATGACAGCTGTGGAT
B2M	TGACTTTGTCACAGCCCAAGATA	AATGCGGCATCTTCAAACCT

Table 2. RT-qPCR primers

Tumor Xenograft

NRG (NOD/RAG1/2^(-/-) IL2R γ ^(-/-), #007799) mice were purchased from the Jackson Laboratory and animal procedures were approved by the institutional animal care and use committee under IACUC #18214 and all experiments were performed in accordance with relevant guidelines and regulations. 2.5x10⁶ IMR90, hMSC, 955, and 959 stromal cells were subcutaneously injected alone (N=3) or co-injected with 5x10⁵ Red A549 (N=8) bilaterally into the flanks of NRG mice. Mice were euthanized after 30 days and tumors and lungs were resected from mice. Tissues were fixed in 10% buffered formalin phosphate and paraffin embedded. Tissue sections were stained with H&E.

Statistics

Student's t-tests were used for comparisons between two groups. A one-way ANOVA with Tukey's test for multiple comparison were used to determine statistical significance for multiple groups, unless otherwise stated. Statistical significance was considered for $P\text{-value} < 0.05$.

CHAPTER III: INTEGRIN SIGNALING THROUGH CELL MATRIX ADHESIONS PROMOTE MESENCHYMAL MIGRATION AND COLLECTIVE INVASION OF LUNG CARCINOMA CELLS ON CELL- DERIVED ECM

INTRODUCTION

The extracellular matrix (ECM) is a highly dynamic component of the tumor microenvironment that dictates the structure and organization of cellular and tissue elements through providing structural scaffolding and access to bioactive molecules that mediate tissue growth and differentiation (Burgstaller et al., 2017; Frantz, Stewart, & Weaver, 2010). The ECM and associated structures, termed the matrisome, of the lung are composed of a diverse array of proteins and glycoproteins, such as collagen, elastin, fibronectin, vitronectin, laminin, osteopontin and others, as well as proteoglycans including perlecan and the hyalactins decorin, versican, and lumican (Yue, 2014). These protein components provide much of the tensile and elastic strength of the pulmonary architecture, while hydrophilic proteoglycans bind to growth factors within the microenvironment including transforming growth factor beta 1 (Kolb, Margetts, Sime, & Gauldie, 2001). Both components serve as mediators of cell matrix interactions. Although the ECM is thought to be mainly deposited by stromal elements of the tumor microenvironment including cancer activated fibroblasts

(CAF) or mesenchymal (CAM) cells, cancer cells are also able to interact with and modify the ECM (B. Lee, Konen, Wilkinson, Marcus, & Jiang, 2017; Malandrino, Mak, Kamm, & Moeendarbary, 2018).

Cells interact with the various components of the ECM through a variety of cell surface adhesion molecules including integrins and other glycoprotein families of selectins, syndecans, the hyaluronic acid receptor CD44, as well as the integrin associated molecule CD47. Integrins are transmembrane proteins thought to be the major receptors involved in linking the actin cytoskeleton of the cell to the extracellular matrix. These low affinity receptors exist as noncovalently linked heterodimers of subunits α or β of which 18 α subunits and 8 β subunits combine to form 24 integrins. Each of these integrins contains an N-terminal globular head region that determines its extracellular ligand specificity, a transmembrane region, and a short C-terminal cytoplasmic tail which mediates interactions with intracellular cytoskeletal scaffolding proteins (C. Smith, 2008). These integrins can be grouped according to their ligand specificity with some dimers acting as receptors for laminin, collagen, or the Arginylglycylaspartic acid motif (RGD) found in many ECM proteins including fibronectin, vitronectin, and osteopontin. Upon binding to their extracellular substrates, several scaffolding proteins assemble along the integrin cytoplasmic tail including vinculin, paxillin, the mechanosensing protein talin, the signaling protein focal adhesion kinase (FAK), and dozens of others (Horton et al., 2016). These integrin mediated cell-matrix structures are dynamic and rapidly assembled to form nascent adhesions which mature to focal adhesions and fibrillar adhesions. During this process of

assembly and disassembly, FAK is a major mediator of kinase activity that regulates many signaling pathways that in turn affect multiple aspects of cell behavior including survival, proliferation, migration, and invasion (Appeddu, 1996; Schaller, 2010). Importantly, activation of FAK results promotes the association and stable activation of Src family kinases to promote cell motility, proteolytic activity, and invasion, and this function is activated in the tumor microenvironment (Mitra, Lim, Chi, & Schlaepfer, 2006). Importantly, these cell behaviors are associated with metastasis and epithelial to mesenchymal transition (EMT) in cancer.

Recently, research interests focusing on cell-matrix interactions have utilized an in-vitro model that recapitulates the diverse physiology of the ECM found in different tissues by using three-dimensional (3D) fibroblast cell-derived ECM (CDM) for tissue culture (Cukierman et al., 2001; Yamada & Cukierman, 2007). Fibroblasts and other cells grown on these 3D CDM exhibit different properties compared to traditional culture on plastic or 2D ECM protein coated surfaces, but closely resemble structures and behaviors found in-vivo (Soucy et al., 2015). Interestingly, a common feature of the cell-matrix adhesions on 3D CDM is that they contain integrin, FAK, and paxillin, which colocalized to the ECM protein fibronectin (Cukierman, Pankov, & Yamada, 2002; Soucy & Romer, 2009). Our lab, and others, have shown that cancer cells also alter their phenotype when grown in 3D collagen matrices (P. Friedl & Alexander, 2011) or on 3D CDM (Scherzer et al., 2015). In particular, the characteristics of single cell

migration are vastly altered in 3D and in-vivo environments compared to 2D in-vitro culture (Fraley et al., 2010; Scherzer et al., 2015).

However, the mechanisms of altered cancer cell migration on these 3D CDM environments are not completely understood in lung cancer, and many questions remain. Is collective migration, the behavior of multicellular complexes maintaining cell to cell contact and migrating as a unit, altered on 3D CDM? What role does cell-matrix adhesion signaling play in mediating migration and invasion on 3D CDM and are these pathways similar to 2D signaling? Does culture in a physiological in-vitro microenvironment alter the invasiveness and EMT characteristics of lung cancer, and what role does cell-matrix adhesion signaling play in regulating these phenotypes? To answer these questions, we utilized a microenvironmental culture system that would recapitulate some elements of the tumor microenvironment including a lung fibroblast 3D CDM as well as physiological hypoxia. We hypothesized that within this system, 3D cell-matrix adhesions to the ECM mediate migration, invasion, and EMT of lung cancer cells through signaling downstream of integrin binding to fibronectin within the tumor microenvironment.

RESULTS

Collective migration patterns of lung cancer cells on cell-derived matrix are distinct from 2D and dependent on focal adhesion signaling.

Previously in our lab, Scherzer et al. reported that single cell migration velocity and directionality of lung carcinoma and epithelial cells are increased when interacting with a lung fibroblast derived ECM (Scherzer et al., 2015). However, a characteristic of invasive solid carcinomas *in vivo* is that they maintain cell-cell adhesions and migrate collectively to invade tissue (P. Friedl, Locker, Sahai, & Segall, 2012). To understand the biology of collective cell migration of lung cancer cells, we attempted a variety of models utilizing the cell-derived matrix (CDM).

First, we used ultralow attachment plates in order to prepare spheroids of the lung adenocarcinoma cell line H2030. H2030 originally was derived from a lymph node of a patient with metastatic lung cancer and this cell line is highly motile. We transferred these spheroids to either a collagen coated tissue culture plastic plate (2D), or to a plate coated with CDM from WI38 fetal lung fibroblasts. These plates were then monitored under time lapse microscopy (Fig. 3) for a 48 hour period. Videos obtained from these experiments highlight the dynamic nature with which cells interact with their substrates. Interestingly, we observed that the cells migrating from the spheroid on the 2D surface appeared to spread more rapidly than those on the 3D CDM (Fig. 3A) and covered a greater overall area. As these results are different than we observed from single cell migration studies, this highlights the importance of studying the cell-cell adhesion forces that can

promote migration in a collective manner. We also observed that while cells migrating from the spheroid on 2D travelled relatively linearly from their point of origin away from the spheroid, upon interacting with the matrix some cells appeared to change directing while attached cells followed closely behind. This polarity was much more obvious on the CDM environment, suggesting a microenvironmental control of “leader” cells and “follower” cells characteristic of collective cell migration (Peter Friedl & Gilmour, 2009). Additionally, at later timepoints we observed dozens of cells streaming and breaking away from the migrating mass on 3D but not on 2D, suggesting the ECM also plays a role in dictating the transition from collective to single cell migration.

Further analysis of the tracked individual cells showed that cells interacted with the matrix and oriented their direction to a specific direction on the ECM, revealing an anisotropic velocity (Fig. 3B). Higher magnification of the early timepoints of the videos showed that while cells migrating on 2D possessed broad, flat lamellopodia projections with rapidly assembling and disassembling adhesions at the leading edge of the cell, cells migrating on 3D extended longer, thinner, and more durable protrusions to sense the matrix before migration occurred (Fig. 3C). These structures resemble the extended cellular projections, tipped with lamellopodia, employed by fibroblasts to navigate a 3D environment (Petrie & Yamada, 2012). These structures are known to contain integrins in their focal contacts, so we asked the question of whether inhibition of integrin signaling would inhibit migration on 3D CDM.

During “outside-in” integrin signaling, integrin α and β heterodimers join and attach to an extracellular ligand. This initiates a focal contact between the extracellular matrix and the cell which begins to associate intracellular machinery to strengthen the complex. Major components of this complex are talin, which binds to the cytoplasmic tail of the β integrin subunit as well as actin, and focal adhesion kinase (FAK), a major focal adhesion scaffolding protein that has intrinsic kinase activity and auto-phosphorylates itself upon formation of a focal contact. Phosphorylation of FAK at Tyr 397 induces a conformational shift that allows interaction with the SH2 domains of Src family kinases and phosphatidylinositol 3-kinase (PI3K). Further phosphorylation of FAK by Src at Tyr 925 promotes association the adaptor protein Grb2 (Appeddu, 1996). Ultimately, FAK interacts with dozens of proteins involved in signal activation and transduction including paxillin, Arp2/3, and p190RhoGEF (Schaller, 2010). FAK overexpression is correlated with worse prognosis in breast (Golubovskaya et al., 2009), and head and neck squamous cell carcinoma (Canel, 2006; Miyazaki et al., 2003), and has been found to be overexpressed in many other cancers (Yoon, Dehart, Murphy, & Lim, 2015). Given the relevance of integrin, FAK, and Src signaling in cancer and cell matrix adhesions, we used the pharmacological inhibitors of Src family kinases PP2, and saracatinib, FAK inhibitor Pf-573358 (FAKi), and soluble RGD and RGDS peptides to inhibit integrin on cells grown in 2D and 3D CDM.

After 24 hour exposure to A549 lung adenocarcinoma cells, we found that treatment with the Src inhibitors PP2 and saracatinib yielded similar results in

inhibiting Src phosphorylation at Tyr 416, with saracatinib being slightly more potent and efficacious at inhibiting paxillin phosphorylation and AKT phosphorylation (Fig. 4A). This was coincident with an increase in FAK autophosphorylation upon Src inhibition. Treatment with the FAK inhibitor blocked the autophosphorylation of FAK and decreased Src phosphorylation. The RGD and RGD peptides did not appear to have much effect on integrin signaling and may have increased Src and paxillin activation on 3D. Next, we sought to determine whether these integrin signaling changes were different on 2D fibronectin coated substrate compared to the 3D CDM. We found greater expression of fibronectin, a key component of cell-matrix adhesions and one of the proteins possessing an RGD motif, from cancer cells grown CDM compared to 2D and this expression was not inhibited by any pharmacological inhibitors of integrin or focal adhesion signaling (Fig. 4B). Interestingly, while FAK autophosphorylation was similar on 2D and 3D following treatment with the inhibitors, the soluble RGD peptides seemed to promote a phenotype on 2D which resembled 3D, with phosphorylation of Ser 425 of talin, a site purported to stabilize the talin head region and implicated migration (Huang et al., 2009), and activate Tyr 925 of FAK and downstream targets p-AKT, and p-paxillin. It may be that while the soluble RGD peptides prevent engagement with extracellular ligands, they also activate RGD integrin dependent signaling pathways (G. G. Pinkse, Jiawan-Lalai, Bruijn, & de Heer, 2005; G. G. M. Pinkse et al., 2006). Total FAK protein didn't appear to be affected by treatment with any drugs on 2D or 3D. These findings suggest that RGD binding integrins may be mediating

some of the phenotypes of cancer cells observed on CDM, and that Tyr 925 phosphorylation on FAK mediated by Src may be an important effector.

To determine whether this phenotype was observed in other NSCLC cells, we exposed the lung adenocarcinoma H2009 and A549 to varying concentrations of saracatinib. After 24 hours exposure, we observed drastic changes in cell attachment and morphology to H2009 cells grown on 3D CDM, with cells detaching from the matrix and contracting into distinct cell clusters at concentrations as low as 100 nM, while cells grown on 2D appeared less affected (Fig. 5A). In both cell lines, we observed similar results in which saracatinib appeared to be more potent in inhibiting Src activation on 3D than on 2D (Fig. 5B). In contrast, other pathways downstream of focal adhesion signaling such as p44/42 MAPK (ERK1/2) signaling pathway and AKT appeared less effected on 3D CDM compared to 2D. These findings suggest that the effect of saracatinib on focal adhesion signaling on lung cancer cells grown on 3D CDM may be more specific to Src inhibition than to other pathways, or that other extracellular signals not present in 2D culture compensate for these pathways. Given these results, we asked whether these inhibitors would be capable of inhibiting lung cancer cell migration on a CDM. As we expected, it appeared that collective cell migration on 3D CDM required Src activation and inhibiting FAK kinase was sufficient to significantly stall this migration (Fig. 6A, B).

Figure 3. Cell derived matrix alters collective cell migration morphology and directionality.

Human adenocarcinoma cells H2030 were grown in spheroids for 4 days and transferred to either collagen coated plastic plates (2D) or fetal lung fibroblast IMR90 cell derived extracellular matrix (3D). A. Images of live cell time lapse microscopy depicting cancer cell migration from spheroids at 15 minutes, 6 hours, 12 hours, and 18 hours. Scale bars are 1000 μm . B. Lines denote migration tracks of leading cells from the spheroids on 2D or 3D substrate after 18 hours. C. Image of the leading edge of migrating cells displays cell membrane protrusions of leading edge cells on a 2D substrate compared to a 3D substrate. Arrows denote characteristic flat lamellopodia on the 2D substrate or extended pseudopodia on the 3D substrate.

Figure 4. Pharmacological inhibition of integrin signaling attenuates downstream focal adhesion pathways of lung cancer cells on CDM.

A. A549 lung adenocarcinoma cells were grown on IMR90 cell derived matrix and treated with soluble RGD and RGDS peptides (100 μ M), focal adhesion kinase inhibitor PF-573228 (1 μ M), and Src kinase inhibitors Saracatinib (10 μ M) and PP2 (20 μ M) for 24 hours. Western blot analysis of extracts were probed for total focal adhesion kinase (FAK), its autophosphorylation site (p-FAK^{Y397}), Src and its activated state (p-src^{Y416}), phosphorylated paxillin (Y118) and activated AKT (S473). Actin was used as a loading control. B. Western blot analysis of extracts from A549 grown on either fibronectin coated plastic (2D) or cell derived matrix (3D) and incubated with the indicated inhibitors for 24 hours. These extracts were probed for fibronectin, phosphorylated talin (S425), and the Src phosphorylated site on FAK (Y925).

Figure 5. Saracatinib more potently inhibits Src phosphorylation on 3D CDM.

H2009 and A549 lung adenocarcinoma cells were grown on either 2D or 3D cell derived matrix and treated with varying doses of saracatinib for 24 hours. A. Representative images of H2009 cell morphology 24 hours after treatment with saracatinib. All scale bars are 100 μm . B. Western blot analysis of extracts probed for Src, phospho-Src, phospho-AKT, FAK, phospho-FAK, p44/42, and phosphor-p44/42. Actin was used as a loading control.

Figure 6. Inhibitors of focal adhesion signaling inhibit cancer cell migration on 3D matrix.

A549 lung adenocarcinoma cells were plated on one side of a 3D CDM, and treated with either PF573228 (1 μ M), Saracatinib (10 μ M), or DMSO (V). Images were captured at the collective cell front (0 h) and 48 hours later at the same location. B. Quantification of the average migration distance (μ m) of the cell front from the starting position. Data presented as mean $\pm$ S.E.M. from three independent experiments. All inhibitors were determined significantly different by ANOVA with Dunnett's test for multiple comparison to vehicle, $p < 0.001$.

Environmental hypoxia promotes cell proliferation, migration, and invasion in a cell type specific manner

Tumor hypoxia is a critical parameter in cancer progression as hypoxia inducible factor 1-alpha (hif-1 α) mediates several parameters involved in metastasis (Majmundar, Wong, & Simon, 2010; Masoud & Li, 2015). In an attempt to better replicate the tumor microenvironment, we sought to use hypoxia as a tool to test cancer cells in an environment that more closely resembles their origin. Additionally, we intended to use this system as a tool to test hypoxic regulation of cancer cell processes associated with metastasis, invasion and EMT. In order to determine whether chemical or environmental hypoxia would be more appropriate, we compared an incubator that infused gaseous nitrogen and equipped with an oxygen sensor to treatment with a chemical inducer of hypoxia, cobalt chloride (CoCl₂). A549 cells grown under both hypoxia conditions, as well as 2D and 3D stabilized hif-1 α (Fig. 7A). However, we observed marked activation of a number of signaling cascades under chemical treatment that were not present under environmentally induced hypoxia (2% O₂), with induction of the autophagy related protein LC3B-I, and activation of ERK, p38, and EGFR. Fewer differences were observed between 2D and 3D culture, save for modest reductions in hif-1 α stabilization and AKT activation on CDM. Cancer cells grown on CDM also had a slight activation of EGFR. While we were not directly able to compare the oxygen concentration of media in either of the systems, we determined that environmentally induced hypoxia would allow us to better

observe subtle differences in signaling that may occur on the 3D CDM as well as allow for long term cell culture.

Indeed, we found that A549 grown in physiologically relevant levels of hypoxia (5% O₂) had a slight proliferative benefit after long term culture (Fig. 7B) and were nearly able to restore their doubling time to their traditional normoxic 2D variable (Fig. 7C). To test whether hypoxia could induce invasion, we used a traditional scratch wound assay and overlaid this wound with the ECM compound Matrigel. Hypoxia appeared to only slightly increase invasion into the wound, but biochemical analysis revealed that hypoxia increased expression of the EMT marker vimentin, and the combination of hypoxia and a Matrigel surface induced activation of Src (Fig. 7D, E). This is expected since Src plays a pivotal role in the phosphorylation of invadopodia components cortactin, and N-WASP, and ultimately the recruitment of actin and MT1-MMP to invadopodia tips to promote invasion into ECM substrates (Bailey, Airik, Krook, Pedersen, & Lawlor, 2016; Mader et al., 2011). Next we sought to ask the question of whether this hypoxic environment could promote migration on the CDM.

To answer this question, we created spheroids of the saracatinib sensitive cell line H2009 and transferred them to either a collagen coated 2D surface or a WI38 lung fibroblast CDM. As with the H2030 spheroids, we observed a remarkably different migration pattern on 2D compared to 3D CDM, with cells on the 2D surface migrating in a mostly radial fashion and cells on the 3D CDM migrating from the spheroid in long, connected single cell lines (Fig. 8). However, on both surfaces the cells appeared to migrate farther under hypoxic conditions

compared to atmospheric oxygen culture. Interestingly, we observed different results with the A549 and H2030 spheroids. A549 spheroids were similarly responsive to hypoxia and spread significantly farther from the spheroid (Fig. 9A). This increase in cell spreading was coincident with a decrease in spheroid volume in hypoxia, whereas the central spheroid remained mostly intact in normoxia. H2030 spheroids spread equally in both atmospheric conditions (Fig. 9B). As mentioned earlier, the H2030 cell line was derived from a metastatic lesion and possesses mesenchymal characteristics. It may be that the increased migration observed in the other cell lines is a result of EMT. Alternatively, many cancer cells lines have aberrant expression of hif-1 α and other hypoxia response proteins, and while this level of hypoxia (5% O₂) can induce a response in some cell lines, others may require a more stressful stimulus. As before, spheroid collective cell migration was blunted with saracatinib treatment in normoxia and in hypoxia. Taken together, these data suggest that environmental regulation of hypoxia between 2-5% O₂ can promote EMT, migration, and invasion characteristics in lung adenocarcinoma cell lines in a cell line specific manner. Further, this invasive phenotype is associated with increased Src activation and migration on CDM matrix is effectively inhibited by blocking Src activation. Given that focal adhesion signaling and Src activity were necessary for cell migration of lung cancer cells on CDM, and that Src activity is upregulated during Matrigel invasion, we asked whether interactions between CDM and cancer cells could induce focal adhesion signaling that would also affect invasion in-vitro.

Figure 7. Environmental and chemical induced hypoxia differentially affect cancer cell signaling, proliferation, migration, and invasion.

A. A549 lung adenocarcinoma cells grown on either 2D or 3D CDM in atmospheric O₂, 2% O₂, or treated with 1 mM CoCl₂ for 24 hours. Extracts were probed for Hypoxia-inducible factor 1-alpha (HIF1A), LC3, p44/42 MAPK and phospho-p44/42 MAPK, AKT and phospho-AKT, p38 and phospho-p38, EGFR and phospho-EGFR (EGFR Y1068). Tubulin was used as a loading control. B. Relative cell counts of A549 grown for 20 days in either atmospheric O₂ (normoxia) or 5% O₂ (hypoxia). Data presented as mean ± S.E.M. from three independent experiments. Statistical significance was determined by combining growth data for all timepoints of hypoxia vs normoxia using unpaired student's t test, $p < 0.025$, however individual timepoints showed no significant differences. C. Doubling time of A549 cells following short term growth in different environments (Statistical analysis performed by ANOVA with Dunnett's test for multiple comparison to 2DN, * $p < 0.05$, and **** $p < 0.0001$; data presented as mean ± S.E.M. from three independent experiments. D. A549 cells plated at confluence and a scratch wound was created and overlaid with Matrigel (invasion) prior to placement in normoxia or hypoxia. Quantification of relative wound closure at 32 h, ** $p < 0.01$; Data presented as mean ± S.E.M. E. Western blot analysis of extracts from D, probed for vimentin, total Src, and activated Src (Y416). Actin was used as a loading control.

Figure 8. Physiological hypoxia promotes collective cell migration on 2D and 3D CDM.

H2009 lung adenocarcinoma cells were grown as spheroids for four days and transferred to either a collagen coated dish or a WI38 CDM in atmospheric oxygen (N) or in a O₂ controlled hypoxic environment similar to physiological hypoxia (H). Images were captured an hour after initial plating and at 48 hours. Images are representative of multiple spheroids. Note the distinct migration pattern of migration on CDM where leading cells emerge and sprout in thin migrating stalks.

Figure 9. Hypoxia induced cell migration is cell line specific and inhibited by saracatinib.

A549 and H2030 lung adenocarcinoma cells were grown as spheroids for six days and transferred to either a collagen coated dish or a WI38 CDM in atmospheric oxygen (N) or in a O_2 controlled hypoxic environment similar to physiological hypoxia (H) and treated with DMSO or 5 μ M saracatinib. A. Images were captured an hour after initial plating and at 120 hours (A549) or 72 hours (2030). B. Quantification of total migrated area relative to area of spheroid. Statistical analysis performed by ANOVA with Dunnett's test for multiple comparison to normoxia at each time point, * $p < 0.05$, and **** $p < 0.0001$; data presented as mean $\pm$ S.E.M. from two independent experiments.

Lung cancer cells on 3D CDM promote a mesenchymal mode of migration that potentiates invasion in-vitro

In order to test whether the CDM could affect invasion of lung cancer cells, we opted to combine our two previous assays. First, we created a CDM using the WI38 fibroblast cell line, however we did not supplement the media with ascorbic acid allowing for a less collagen rich matrix that was suitable for scratching without destroying the entire matrix architecture on the plate. Next, we seeded cancer cells at confluence in order to cover the plate and created a scratch wound. After the scratch wound was created, we filled the wells with Matrigel containing either vehicle or drugs involved in inhibition of focal adhesion signaling (Fig. 10A). While previously we had observed a decreased migration of cells from the matrix into the wound area without Matrigel, remarkably we observed rapid wound closure of cells on the 3D CDM into the Matrigel coated wound (Fig. 10B). Significantly increased wound closure occurred at 6, 24, and 48 hours on 3D CDM compared to fibronectin coated 2D surface, with the greatest rates of wound closure occurring in the first 24 hours (Fig. 10C). Interestingly, when the cells were overlaid with Matrigel containing the FAK inhibitor PF-573228, we observed a blunted wound closure response on 3D CDM but not on the 2D substrate, such that the wound closure at 24 and 48 hours was not significantly different between the two substrates. This offers evidence that focal adhesion signaling at least in part is driving this invasive phenotype that is promoted by 3D CDM. Finally, we observed that inhibition of Src activity with saracatinib was

completely able to block wound closure in both 2D and 3D CDM, highlighting the importance of Src activation in supporting cell invasion.

At this point, we had made several observations that would help us characterize the type of migration that is occurring when lung cancer cells were attached to a 3D CDM. Currently, two major forms of migration are thought to occur in a 3D context, amoeboid and mesenchymal. Cancer cells undergo plasticity that allows transition between these two states in order to facilitate migration through soft or dense fibrous matrices. Likely, within a population, both types of migration are occurring but exist on a spectrum of characteristics determined by the balance of signaling which occurs in response to the surface with which cells are interacting. Notably in our experimental context, the morphology of the cells appears to be much more elongated and spindle shaped similarly to fibroblasts as the cells align along the matrix. We also observed long, cellular protrusions from the cells interacting with the ECM. Additionally, the migration and invasion promoted by CDM interaction seem to be blunted by blocking integrin based focal adhesion signaling. Considering these findings, we hypothesized that the CDM was promoting a mesenchymal mode of migration that was also facilitating the invasion into Matrigel.

At the molecular level, the dynamics of the actin cytoskeleton and the contraction of myosin are defined characteristics in determining the modality of migration in 3D (Paňková, Rösel, Novotný, & Brábek, 2010). During amoeboid migration, actomyosin contractility driven by phosphorylated myosin light chain (pMLC) downstream of RhoA and ROCK is high. Conversely, during

mesenchymal migration, Rac1 predominates, inhibiting RhoA signaling and promoting actin assembly and protrusion. To test whether we could recapitulate the invasive phenotype we observed on 3D CDM using a 2D substrate, we overlaid a scratch wound with Matrigel that contained the ROCK inhibitor GSK-269962. We found that treating cells on a 2D substrate with the ROCK inhibitor promoted similar characteristics as cells on a 3D CDM, with long cellular projections and increased wound closure that was significantly different from vehicle treated cells in 2D at 24 and 48 hours, but not significantly different from vehicle treated cells on 3D CDM (Fig. 11A). Western blot analysis of lysates produced from this experiment showed an increase in Src activation with low levels of ROCK inhibition on 2D, but no change in FAK autoactivation (Fig. 11B). Interestingly, further analysis of the microarray of lung cancer cells on 2D and 3D CDM performed previously in our lab showed that expression of myosin light chain kinase (MLCK), another kinase associated with pMLC, is downregulated on CDM. Together, this evidence suggests that CDM interactions may promote a mesenchymal migration phenotype that promotes invasion of Matrigel through activation of Src.

Since we have determined the importance of focal adhesion signaling and Src activation on increased invasion of cancer cells on CDM, we attempted to use western blot analysis to determine some of the molecular changes that were occurring with pharmacological inhibition of different components of this pathway (Fig. 12A). We found that integrin α V, one of the integrin receptor heterodimer components important in binding to fibronectin and other RGD peptides, was

upregulated on CDM compared to 2D, that this expression was not decreased by FAK, Src, or ROCK inhibition, but was decreased by treatment with the cyclic RGD peptide cilengitide. This suggests that the RGD peptides we've used are likely inhibiting their intended targets, however again we see that treatment of cancer cells on a 2D substrate with cilengitide promotes a molecular response that resembles cells grown on a CDM, with similar Src expression and activation, paxillin phosphorylation, annexin II phosphorylation, and RhoA expression. Similarly, EMT markers also appeared to be similar upon cilengitide treatment of cancer cells on a 2D substrate and vehicle treatment on a 3D substrate with decreased e-cadherin expression and increased expression of Zeb1 and vimentin (Fig. 12B). A possible explanation for this result may be due to the internalization of integrin αV following treatment corresponding with a switch to another integrin heterodimer pair at the cell surface. This result was demonstrated to occur by Jim Norman's lab, where ovarian carcinoma cells were treated with cilengitide or osteopontin and as a result recycled integrin $\alpha 5 \beta 1$ to the cell surface to promote cell adhesion to fibronectin and mediate EGFR driven migration into a 3D matrix (Caswell et al., 2008). This suggests the possibility that a similar mechanism is occurring with lung cancer cells adhering to a lung fibroblast CDM as in the original hypothesis, however further characterization of surface integrin expression would be required for verification. On the other hand, treatment with the focal adhesion inhibitors did not appear to affect integrin expression or stat3 activation, however they all seemed to promote increased Src expression and RhoA, perhaps in a compensatory response. Saracatinib

treatment was the only agent that appeared to greatly diminish activation of downstream Src targets paxillin and annexin II. We observed a similar inhibition of the EMT phenotype when cells were treated with focal adhesion inhibitors, with stabilization of e-cadherin of cells on 3D CDM, an increase of zeb1 expression, and a decrease of vimentin. Zeb1, a negative regulator of e-cadherin expression, is promoted by stat3 activation (Xiong et al., 2012), and this pathway appears to play a role in CDM mediated promotion of EMT (Fig. 12B).

Interestingly, blocking focal adhesion signaling restored e-cadherin expression and diminished vimentin expression of cancer cells grown on CDM, even though stat3 was activated and zeb1 expression increased, highlighting the importance of cell-matrix interactions in regulating the EMT phenotype. We investigated other known regulators of EMT, snail and slug, however we were unable to resolve a clear picture of their expression and this may be improved by using a nuclear extract in the future.

The EMT marker plasminogen activator inhibitor-1 (PAI-1) had pronounced expression in cancer cells on CDM upon treatment with saracatinib. PAI-1 regulates pericellular proteolysis by directly inhibiting the activation of plasminogen to plasmin by binding and inactivating plasminogen activators, urokinase and tissue type plasminogen activator. Plasmin has been demonstrated to have a broad spectrum of activity in activating zymogens of a multitude of MMPs in vitro including MMP-1, MMP-2, MMP-3, MMP-9, MMP-13, and MMP-14 (Deryugina & Quigley, 2012). Given the drastically decreased invasion upon saracatinib treatment and the importance of pericellular proteolysis

for invasion, we decided to investigate the role *pai-1* played in lung cancer cells on the CDM. Of the three lung adenocarcinoma cell lines test, H2009, H2030, and A549, only the A549 cell line had high levels of PAI-1 expression, although the other cell lines had variable expression of related serine protease family members PAI-2 and PN-1 (Fig. 13A). To understand the role of PAI-1 in cell migration and invasion, we generated lentivirus containing CRISPR/cas9 constructs containing two different guides targeting PAI-1, a non-targeting guide, and a pLX304 expression vector with the human *SERPINE1* cDNA and stably infected the three different cell lines. Upon expression of PAI-1 in H2009 cells, we observed a slight but significant decrease in cell migration compared to NT, while knock out of PAI-1 did not affect the cells (Fig. 13B).

In order to establish complete knock out of PAI-1 in A549 cells, we isolated and characterized several clones from each of the guides (Fig. 13C) and compared PAI-1 expression to both NT and PAI-1 overexpression clones. Loss of PAI-1 in A549 cells significantly increased migration in clones manipulated with either guide compared to NT, while stable expression of PAI-1 did not affect migration in a scratch wound assay (Fig. 13D). We observed a similar result when we assessed migration on the 3D cell derived matrix, however A549 stably expressing PAI-1 appeared to be less migratory on ECM compared to the NT clone (Fig. 13E). To test the ability of PAI-1 to modulate cell invasion and proteolysis, we employed the 3D CDM Matrigel invasion assay in combination with the pan-MMP inhibitor GM-6001, which targets MMP-1, MMP-2, MMP-3, MMP-8, and MMP-9. Intriguingly, PAI-1 KO clones rapidly invaded into the

wound irrespective of the MMP inhibitor, however 3D invasion was slowed with stable expression of PAI-1 and nearly completely halted with the combination of PAI-1 expression and treatment with the MMP inhibitor (Fig. 13F). It may be that PAI-1 expression of the cells and GM-6001 treatment cover a broad range of potential MMPs involved in Matrigel degradation. Alternatively, unrestricted plasminogen activation may have effects on targets other than MMP as plasmin directly degrades several ECM proteins. Together, these data demonstrate the importance of pericellular proteolysis in invasion into Matrigel, and that interrupting these processes can modulate the migratory and invasive potential of cancer cells in different environments.

C

Figure 10. CDM promotes cancer cell invasion of Matrigel which is inhibited by blocking focal adhesion signaling.

A. Schematic representation of the scratch wound invasion assay. Cancer cells are grown on 2D or 3D CDM at confluence and a scratch wound is created. The well area is then either overlaid with complete media containing Matrigel and treated with focal adhesion inhibitors PF573228 (Faki 1 μ M), saracatinib (10 μ M), or vehicle (DMSO). Images of the scratch wound area are captured at 0, 6, 24, and 48 h. B. Representative images of Matrigel wound invasion by A549 lung adenocarcinoma cells at 0 h and 24 h on 2D surface or CDM. C. Quantification of wound closure over a 48 h period. Each symbol represents the mean of an individual experiment with two technical replicates. Dotted lines indicate means of multiple experiments treated with DMSO, while solid lines indicate means of multiple experiments treated with drug. Statistical analysis performed by ANOVA with Tukey's test for multiple comparison at each time point (DMSO: 2D v 3D; FAKi: 2D vehicle v 3D drug; Saracatinib: vehicle v drug), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$; data presented as mean $\pm$ S.E.M. from at least two independent experiments.

Figure 11. Rho kinase signaling inhibition promotes cancer cell Src expression and Matrigel invasion on a 2D substrate.

A549 lung adenocarcinoma cells were plated at confluence on either 2D (closed symbol) or 3D CDM (open symbol), scratched, and the wound was overlaid with Matrigel containing Rho kinase inhibitor GSK269962 (1 μ M or 5 μ M). A. Quantification of Matrigel wound invasion at 6, 24, and 48 h. Dashed line indicates vehicle treatment from previous figure. Each symbol represents the mean of an individual experiment with two technical replicates. Statistical analysis performed by ANOVA with Tukey's test for multiple comparison at each time point between drug and vehicle on 2DN, ** $p < 0.01$, and *** $p < 0.001$; data presented as mean $\pm$ S.E.M. from at least two independent experiments. B. Western blot analysis of extracts from the 48 hour timepoint of the invasion assay were probed for Src, phospho-Src, phospho-AKT, FAK, and phospho-FAK. Actin and tubulin were used as loading controls.

Figure 12. Analysis of focal adhesion pathway signaling during 3D CDM induced invasion into Matrigel reveals roles for Src and EMT.

Lysates prepared from A549 during the Matrigel invasion assay treated with either DMSO, PF-573228 1 μ M, Saracatinib 10 μ M, GSK269962 5 μ M, or Cilengitide 1 μ M for 48 hours on fibronectin coated 2D or WI38 CDM 3D were probed for members of the focal adhesion signaling pathway and proteins associated with Src activation. A. Western blot analysis of integrins β 3 and α V, one of the heterodimer pairs known to recognize the RGD motif of fibronectin, activated stat3, Src, activated Src, targets of Src: phosphorylated paxillin, annexin II and phosphorylated Tyr 24 of annexin II, and the Rho family GTPase RhoA. B. Western blot analysis of EMT markers E-cadherin, Zeb1, vimentin, slug, snail, and plasminogen activator inhibitor 1 (pai-1). Actin was used as a loading control.

Figure 13. PAI-1 mediates cell migration and invasion of lung adenocarcinoma cell lines on CDM.

A. Western blot analysis of lysates from H2009, H2030, and A549 lung adenocarcinoma cell lines grown in normoxia or physiological hypoxia (5% O₂) for 24 hours were probed for PAI-1, and related serine protease family members plasminogen activator inhibitor-2 (PAI-2) and protease nexin-1 (PN-1). Tubulin was used as a loading control. B. H2009 cells were infected with a lentiviral Crispr/cas9 targeting *pai-1* with two different guides (g2 and g3), a non-targeting guide (NT), and a human *pai-1* expression vector. Relative wound closure of a scratch wound (migration) in modified 2009 cells 18 hours after wounding. Each symbol represents an independent experiment mean of two technical replicates. Statistical analysis performed by ANOVA with Dunnett's test for multiple comparison to NT, ***p* < 0.01; data presented as mean ± S.E.M. from three independent experiments. C. Western blot analysis of single clone cell lines from A549 infected with lentiviral Crispr/cas9 and *pai-1* expression plasmid probed for *pai-1*. D. Relative wound closure of a scratch wound (migration) after 24 hours from selected clones with *pai-1* knock out or overexpression. E. *Pai-1* knock out, NT, and overexpression A549 spheroids were transferred to a IMR90 CDM and images were captured at 0 hours and 96 hours after attachment. F. *Pai-1* knock-out or overexpression A549 cells were plated on WI38 CDM at confluence, scratched, and overlaid with Matrigel with either MMP inhibitor GM-6001 (25 μM) or vehicle. Quantification of relative wound closure (invasion) at 6, 24, and 48 hours. Each symbol indicates an individual experiment with two replicates.

DISCUSSION

In the present study, we investigated the influence of integrin signaling in lung cancer cells attached to a fetal lung fibroblast derived extracellular matrix on cellular processes relevant to cancer metastasis including cell migration, invasion, and the EMT. As our lab has previously demonstrated (Scherzer et al., 2015), lung cancer cells on 3D CDM alter their migratory behavior on a CDM compared to a fibronectin or collagen coated 2D surface. In the context of collective cell migration, we found that cells maintain directional migration on a CDM similarly to single cell migration. Surprisingly, we see that during collective cell migration, cells on a 2D surface appear to migrate at an isotropic average velocity that is faster than the anisotropic velocity exhibited by cells on CDM (Fig. 3). Live cell imaging revealed that as cells move collectively on 3D CDM, leader cells extend pseudopodal projections and rapidly assemble and disassemble cell-matrix adhesions to the ECM substrate. While precise identification of cell-matrix adhesions was beyond the scope of this study, we found that signaling cascades downstream of integrin signaling, namely FAK and Src signaling were required for cell migration on 3D CDM as pharmacological inhibitors of FAK and Src slowed migration (Fig. 6).

Inhibition of Src activation appeared to be more potent on cells in the 3D CDM compared to 2D, with diminished perturbations of AKT and ERK signaling pathways (Fig. 5). Considering the frequent association of cell-matrix adhesions with fibronectin, we anticipated that blocking the integrin association with the fibronectin component of the ECM by treatment with soluble RGD peptide may

also block the phenotypic effects observed with cell attachment to the ECM, however upon treatment we found expression and activation of integrin signaling pathways on 2D resembled untreated 3D CDM controls (Fig. 5). As we observed this phenomenon with three RGD peptide based inhibitors (RGD, RGDS, and cilengitide), this warrants further investigation of integrin “outside-in” signaling using other tools of exogenous integrin activation, such as manganese, and inactivation through activation blocking antibodies such as the anti-integrin $\beta 1$ antibody P5D2 or the $\alpha 5\beta 1$ antibody volociximab (Bhaskar et al., 2007; Ye, Kim, & Ginsberg, 2012). As the 3D CDM matrix model is particularly suited to cell microscopy, further evaluation of cell-matrix adhesion components and their localization upon attachment to ECM through fluorescent confocal microscopy would also help in delineating specific integrin interactions.

A critical component of metastasis is cell invasion of dysplastic cells through the basement membrane layer of epithelial tissues and intravasation through endothelial lining. To model this behavior within the tumor microenvironment, we added the element of environmental hypoxia to our cell culture model in order to create a microenvironment mimetic in-vitro system. A benefit of our system is that we are able to control the atmospheric O_2 level to between 2% and 20% that is similar to the O_2 saturation of most tissues within the body. While this is not as hypoxic as some reported levels of hypoxia in the tumor microenvironment (Petrova et al., 2018; Salem et al., 2018), it is sufficient to induce stabilization of HIF-1 α (Fig 7). Additionally, we demonstrate that this level of hypoxia supports and enhances cell proliferation for long term cell culture

and potentiates invasion in A549 cells, which is correlated with induction of EMT and activation of Src. However, hypoxia did not affect all cancer cell lines equally as migration was increased with A549, but not with H2009 or H2030 (Fig. 9). One possibility is the origin of the cell lines, as A549 was originally cultured from a primary lung explant and the H2009 and H2030 were originally derived from metastatic tissue. While they are all KRAS mutant lung adenocarcinoma cell lines, differences may exist in their capacity to undergo and maintain an EMT phenotype (Schliekelman et al., 2015).

Outside of the context of hypoxia, lung cancer cell interaction with 3D CDM promoted Matrigel invasion compared to 2D culture. Treatment with a FAK kinase inhibitor slowed this 3D promoted invasion to 2D levels, whereas Src inhibition decreased invasion in both conditions, albeit to a greater extent on 3D CDM. Analysis of microarray studies performed by Scherzer et al. identified that MLCK was downregulated on two different CDMs in A549 and H358 cell lines compared to 2D fibronectin controls. One of the targets of MLCK is the activation of MLC and blocking this activation through pharmacological inhibition of the Rho/Rock pathway resulted in increasing the invasive capacity of lung cancer cells on 2D (Fig. 10). This also correlated with increased Src activation. Inhibitors of focal adhesion signaling all stabilized e-cadherin and decreased expression of vimentin, suggesting that this signaling pathway is also involved in the regulation of EMT on 3D CDM (Fig. 12). However, PAI-1 expression appeared to be markedly increased by saracatinib treatment on 3D CDM, leading us to ask about the role of ECM remodeling and pericellular proteolysis on cancer invasion.

Of the three cell lines tested, only A549 had appreciable PAI-1 expression (Fig. 13). Restoring PAI-1 in the deficient cell line H2009 decreased migration, whereas KO of PAI-1 in A549 increased migration on 2D and 3D. KO of PAI-1 also increased 3D invasion, but this was not inhibited by MMP inhibitor GM6001, suggesting that loss of PAI-1 in lung cancer cells may increase invasion in a mechanism that does not involve extracellular regulation of MMP but may involve an intracellular target.

Collectively, we see that the interaction of cancer cells with the extracellular matrix alters migration and promotes invasion through focal adhesion signaling. Cancer cell interaction with 3D CDM promotes an elongated cell phenotype, directional migration, EMT, invasion, and is affected by protease regulation. Additionally, cells on 2D can transition to a 3D-like phenotype with inhibition of Rho/ROCK and presumably through altering actomyosin related cortical contractility. These findings suggest that interaction with a lung fibroblast CDM promotes a mesenchymal mode of migration, that this modality of migration is inhibited by blocking focal adhesion signaling, and also promotes local invasion into the basement membrane. While FAK inhibition slows invasion on 3D substrates, Src inhibition blocks invasion in 2D and 3D environments, while not affecting AKT or ERK activation in the cancer cell lines tested. Src inhibition on 3D CDM was associated with increased expression of PAI-1, and modulation of PAI-1 expression in cancer cells altered the migratory and invasive phenotype. Within the tumor microenvironment, cell extrinsic elements such as the interaction with extracellular matrix, and cell intrinsic elements such as the

expression and regulation of proteases and PAI-1 engage in a dynamic interplay to promote cancer cell invasion. Further understanding of essential Src substrates or interactors in 3D environments, particularly related to invadopodia, proteases, and their regulation may lead to improved therapeutic targets in lung cancer metastasis.

CHAPTER IV: TUMOR MICROENVIRONMENT MIMETIC CULTURE AIDS ISOLATION, EXPANSION, AND POTENCY OF TUMOR STROMAL PROGENITORS FROM PRIMARY LUNG CANCER RESECTIONS

INTRODUCTION

The tumor microenvironment consists of a diverse milieu of transformed and non-transformed cells that ultimately coordinate to build and maintain a physical environment that supports tumor growth and potentiates escape and establishment at secondary systemic sites (Quail & Joyce, 2013). These constituents act in concert and dynamically regulate a pathological microenvironment that modulates physical characteristics within the tumor such as tissue stiffness, oxygen tension, and metabolite availability (Erlar & Weaver, 2009; Nelson & Bissell, 2006; Vaupel, 2004). As tumors grow, these elements promote the hallmarks of cancer such as sustaining proliferative signaling, evading immune cell death, inducing angiogenesis, and activating invasion and metastasis (Hanahan & Weinberg, 2011). Recent evidence implicates an activated tumor stroma as enablers of these processes (Bremnes et al., 2011; Pietras & Ostman, 2010).

(Portions of this chapter are published in Scientific Reports. (Saforo et al., 2019))

The constituents of the non-tumor elements within the stroma are multiple and varied, however the cancer associated fibroblasts (CAF) are thought to be a major contributor to the TME stroma (Bremnes et al., 2011). CAF currently lack specific markers but display characteristics similar to activated fibroblasts such as expression of alpha-smooth muscle actin (α SMA) and vimentin (Amatangelo, Bassi, Klein-Szanto, & Cukierman, 2005). They act in paracrine fashion by producing potent proliferative cytokines that elicit mitogenic signaling within tumor cells. Furthermore, these CAF also can directly stimulate angiogenesis via secretion of vascular endothelial growth factor (VEGFA) and activate invasion via production of transforming growth factor beta (TGF- β) (Navab et al., 2011; Y. Zhang et al., 2011). CAF produce a modified extracellular matrix (ECM) which acts as a bioactive mechanical scaffold that can influence tumor cell survival and metastasis, as well as matrix metalloproteinases (MMP) that allow the dynamic regulation of the ECM architecture through degradation of local matrix proteins and activation of latent growth factors within the matrix (Cox & Erler, 2011).

Despite these critical effects on tumor homeostasis and progression, the origin of the CAF remains elusive and may arise from a heterogeneous number of cell types. Evidence has shown that CAF possess many characteristics of mesenchymal stem cells, implicating these cells as a possible origin of the CAF (Kalluri, 2016). Unfortunately, pre-clinical studies of the origin of the tumor stroma are hampered by several challenges. Primary samples from patient tumor resections are limited and this material is valuable for the diagnosis of the patient. Primary cancer cell lines may be more sought after from this material,

and successful transformation of cancer cell lines currently requires repeated xenografts in mice during which time the tumor microenvironment is replaced by mouse stroma (Gaggioli et al., 2007). In-vitro methods to obtain cell lines from primary tissue resection are hindered by time to cell isolation, and these cells can acquire changes in-vitro during the time it takes to passage them in traditional cell culture conditions. During this time progenitor cell types may differentiate, become quiescent, or undergo apoptosis (McKee & Chaudhry, 2017).

Various strategies have been developed to better isolate progenitor cell types. The ECM, which is well known to modulate cell behavior through mechanism of its mechanical stiffness, protein composition, crosslinking, and bioactive components, has also been shown to improve culture of bone marrow mesenchymal stem cells (MSC) (X. D. Chen et al., 2007). Culture dishes are frequently coated with components of this extracellular matrix to promote the adhesion and differentiation of a variety of cell types. Previously, we and others have shown that cell-derived extracellular matrices (CDM) are replicative of the in-vivo environment and influence cancer cell signaling to recapitulate tumorigenic processes in-vitro (Castello-Cros, Khan, Simons, Valianou, & Cukierman, 2009; Scherzer et al., 2015; Yamada & Cukierman, 2007). We have shown that fetal human lung fibroblasts produce a CDM that supports lung adenocarcinoma cells to regulate EMT processes and provide survival signaling to promote survival in conditions of serum starvation and hypoxia (Scherzer et al., 2015).

Hypoxia is a critical factor in the tumor progression of solid cancers (Le et al., 2006). Oxygen tension in physical tissues is around 2-8% and plays a role in regulating metabolic homeostasis. Intratumoral oxygen tension can be much lower (<0.1%) (Carreau, El Hafny-Rahbi, Matejuk, Grillon, & Kieda, 2011). In-vitro systems that control oxygen tension have provided proliferative benefits to a number of stromal cell types compared to traditional culture in atmospheric normoxia (20% O₂) (Mizuno et al., 2009). Culturing at physiological levels of hypoxia has previously been reported to be critical for the cultivation and maintenance of human stem cells (Grayson, Zhao, Bunnell, & Ma, 2007).

We hypothesized that these factors, physiological hypoxia and an in-vivo-like extracellular matrix, are crucial to maintain tumor stroma, and that incorporating these elements in a microenvironment mimetic in-vitro model would improve survival and cultivation of primary cells from small quantities of patient tumor resections. To test this hypothesis, we collected cells from tumor resections of six patients with non-small cell lung carcinoma (NSCLC) and grew them from isolation in different in-vitro environmental conditions. Utilizing a combination of cell derived ECM and physiological hypoxia, we were able to rapidly cultivate and massively expand populations of patient tumor associated stromal progenitors.

RESULTS

Microenvironment mimetic culture system characterization.

Various approaches have been used to attempt to isolate progenitor populations from tumors and bone marrow including serum withdrawal and specific conditioned medium, using specialized culture techniques such as hypoxia and extracellular matrix proteins, and culturing cells using 3-dimensional scaffolds or suspension culture. A commonality of these approaches is that each attempt to simulate certain aspects of the physiological condition to limit the growth of non-progenitor cell types and optimize expansion of rare or quiescent progenitors. In order to test the hypothesis that an in-vitro culturing system resembling the microenvironment of the human lung would facilitate the isolation and expansion of sensitive primary patient tumor cell populations, we developed a microenvironment mimetic culturing system that includes a fibroblast derived extracellular matrix (ECM) and an atmosphere that maintained the oxygen tension at a physiological level (Fig. 14A).

To characterize this system, we assessed each environmental component individually (tissue culture plastic in atmospheric oxygen (2DN), tissue culture plastic in physiological hypoxia (2DH), cell-derived ECM in atmospheric oxygen (3DN), and cell derived ECM in physiological hypoxia (3DH)), and utilized two fetal human lung fibroblast cell lines, IMR90 and WI38, and a human bone marrow mesenchymal stem cell line (hMSC). After 72 hours of growth in each environment, we observed a qualitative increase in WI38 cell number in single (Portions of this chapter are published in Scientific Reports. (Saforo et al., 2019))

conditions of 2DH or 3DN compared to 2DN, as well as an increase in cell number in 3DH (Fig. 14B). Similarly, we observed the greatest increase in cell number for all cell lines in the microenvironment mimetic system that combined physiological hypoxia and cell-derived extracellular matrix (Fig. 15).

Quantification of mean cell doubling time (Fig. 14C) in the 3DH environment showed significant decreases in IMR90 ($P < 0.0099$), WI38 ($P < 0.0039$), and hMSC ($P < 0.0001$), compared to the other conditions. The single condition of physiological hypoxia significantly decreased mean doubling time only for the IMR90 cell line ($P < 0.0387$).

To assess whether the physiological hypoxia environment induced a measurable oxygen response in the cells, we assessed stabilization of hypoxia-inducible factor 1, a global regulator of oxygen homeostasis. Although western blot analysis revealed a stabilization of hypoxia-inducible factor 1 alpha subunit (HIF1A) in the physiological hypoxia environments (2DH and 3DH) after 72 hours, there was a considerable decrease in HIF1A stabilization in the 3DH environment compared to the 2DH environment (Fig. 14D). Previously it was reported that cell-derived matrices generated from stroma of various stages of tumor progression can activate fibroblasts to undergo myofibroblastic and desmoplastic activation (Amatangelo et al., 2005). To ensure that the matrices from human fetal lung fibroblasts in our system were not inducing a desmoplastic response in the cells, we assessed the expression of alpha smooth muscle actin (A-SMA), a marker of activated myofibroblasts and desmoplastic stroma. We found that A-SMA expression decreased in fibroblasts cultured on cell derived

matrix and was variably affected by hypoxia, with a slight decrease in IMR90. Additionally, we investigated regulators of cell cycle progression, and found cell cycle inhibitors p21 Waf1/Cip1 (p21) and p27 Kip1 (p27) were also decreased in the 3DH condition relative to the single 2DH and 3DN conditions. Taken together, our findings suggest that despite long term culture in typical cell culture conditions, commonly used mesenchymal fibroblast and stem cell lines cultured in a microenvironment mimetic system experience a proliferative benefit while not invoking a desmoplastic transformation, properties essential to the establishment of primary cell lines from resected tumor material.

Figure 14. Physiological hypoxia and cell derived matrix enhance proliferation of stromal cell lines and are non-desmoplastic.

A. Schematic of cell derived matrix preparation from IMR90 fetal lung fibroblast cell line and characteristics of microenvironment mimetic conditions. B. Phase contrast images of equal numbers WI38 fetal lung fibroblast cells grown for 72 hours in four environmental conditions (2D Normoxia, 2D Hypoxia, 3D Normoxia, and 3D Hypoxia). Scale bars are 100µm. C. Doubling time of WI38, IMR90, and bone marrow mesenchymal stem cell line hMSC following short term growth in different environments (Statistical analysis performed by ANOVA with Dunnett's test for multiple comparison to 2DN, *p < 0.05, **p < 0.01, and ****p < 0.0001; data presented as mean ± S.E.M.) D. Western blot analysis of hypoxia inducible factor 1 alpha (Hif1a), alpha smooth muscle actin (α-SMA), and cell cycle inhibitors (p21, p27) from WI38 and IMR90 after 72 hours growth in different environments.

Figure 15. Physiological hypoxia and cell derived matrix enhance proliferation of stem and fetal stromal cell lines.

Phase contrast images of equal numbers hMSC and IMR90 fetal lung fibroblast cells grown for 72 hours in four environmental conditions (2D Normoxia, 2D Hypoxia, 3D Normoxia, and 3D Hypoxia). Scale bars are 100 μm .

Enhanced cell isolation and proliferation from patient tumor biopsy.

A challenge to preclinical testing of patient tumors is that resected material is limited and precious for obtaining crucial diagnostic information. Progenitor populations within tumors are thought to be a rare subset of this already limited tissue. To test the hypothesis that a microenvironment mimetic cell culture system would improve the yield of sensitive progenitor cells present within tumors, we obtained six primary tumor resections from treatment naïve patients with non-small cell lung carcinoma (NSCLC), including three squamous cell carcinoma and three adenocarcinoma tumors (Table 3). In these patients, metastasis could not be measured at the time of resection (MX) and samples from one of the patients (1072) was later determined to have cancer present in 2 of 11 lymph nodes (N1). Specimens from the remaining tumor resections did not have metastasis present in the lymph nodes. A portion of these resections were prepared by enzymatic digestion to create single cell suspensions, and equal quantities of cells were seeded onto various cell culture conditions.

The first sample tested, 927, was seeded onto the four environmental conditions detailed above and monitored with microscopy. Seven days post isolation, we observed cell populations of elongated and spindle cell morphology in every environmental condition (Fig. 16A). There appeared to be an increase in the number of attached cells in the 2DH and 3DN environments compared to the traditional 2DN environment, however, remarkably, we observed massive outgrowths of colonies and migrated cells in the 3DH environment. Cells

continued to proliferate in both physiological hypoxia environments but did not passage in the normoxia environments despite presence of extracellular matrix. We found that all tumor samples tested in the 3DH environment showed a marked increase in attached and proliferating cells post isolation compared to traditional cell culture methods (Fig. 16B, D), however we did not observe any increase in primary cell survival from adjacent normal tissue (Fig. 16C). While the 3DH environment greatly enhanced the proliferative capacity of each tumor sample, allowing the first passage after 7 days, in most cases cells grown in traditional methods produced a viable cell line after a moderate delay (Fig. 16D). Similarly, when cells that were isolated in 3DH were transferred to the other environments, we found that the cells were still viable though their proliferation slowed (Fig. 16E). In four biological replicates, we found that the mean doubling time significantly decreased ($P < 0.009$) in the 3DH environment (20.25 ± 0.7147 , $n=4$) compared to transfer to a traditional cell culture environment (42.82 ± 3.643 , $n=4$). These data indicate that a microenvironment mimetic system indeed accelerates the establishment of a heterogeneous cell line from NSCLC resections compared to traditional culture systems.

<i>Specimen Code</i>	<i>Diagnosis</i>	<i>Staging</i>	<i>Histologic Grade</i>	<i>Sex</i>	<i>Race</i>	<i>Age</i>	<i>Cell plating density (cells/cm2)</i>
CDSR00 927	squamous cell	T2aN0MX	III	M	white	56	4300
CDSR00 955	squamous cell	T1bN0MX	II	M	white	68	T:110000; N:12000
CDSR00 956	adenocarcinoma	T1bN0MX	II	F	black	59	150000
CDSR00 959	adenocarcinoma	T3N0MX	II	F	white	75	130000
CDSR00 960	squamous cell	T3N0MX	III	M	white	73	57000
CDSR0 1072	adenocarcinoma	T2N1MX	III	F	white	75	4444

Table 3. Patient Sample Characteristics.

Bolded numbers indicate designations used for cell lines derived from tumor specimens.

Figure 16. Physiological hypoxia and cell derived matrix synergistically enhance proliferation of Patient Tumor Stromal-like cells.

Resected tumors were enzymatically digested with collagenase/dispase and single cell suspensions were grown on four environmental conditions (2DN, 2DH, 3DN, 3DH). A. Representative phase contrast images (from 5 random fields) of patient sample 927 cell growth 7 days post isolation in each condition. Scale bars are 100µm. B. Phase contrast images of patient samples 956 and 959 obtained 7 and 6 days after isolation in 2DN and 3DH. C. Phase contrast images of patient sample 955 tumor and adjacent normal lung cells 9 days post isolation. D. Total cell numbers and days post isolation of 4 patient samples grown in 2D Normoxia and 3D Hypoxia at the time of first and third passaging. E. (Upper) Doubling time of 3DH derived 927 cell line upon transfer to 4 environmental conditions. (Lower) Doubling time of patient derived cell lines maintained in 3DH or transferred to 2DN. Statistical analysis performed by two-tailed T-test, ***p < 0.001, n = 4; data presented as mean ± S.E.M.

Figure 17. Population doubling level of patient sample cell lines.

A. Images of patient tumor resections prior to dissection and dissociation. Scale bars are 1 cm. B. Phase contrast images of dissociated tumor cell growth from sample 956 7 days post isolation showing a heterogenous cell population. Arrowheads indicate clumps of non-fibroblastic colonies. Scale bars are 100mM. C. Population doubling level of cell lines derived from patient tumors and cultivated in 3DH.

Primary tumor cell mesenchymal lineage characterization and immunophenotyping.

While we observed a heterogeneous population of cell morphologies in the first week of cell isolation (Fig. 17), the spindle-shaped cells quickly outpaced the other cell populations. We tested protein expression of epithelial and mesenchymal cell lineages and found that while the 927 tumor resection was highly positive for e-cadherin expression, cell lines derived from the resection were highly vimentin positive with undetectable e-cadherin expression (Fig. 18A). Similarly, cell lines derived from the other tumor resections did not express detectable levels of E-cadherin or Pan-cytokeratin but were also highly vimentin positive. Given the high proliferative capability and strict cell culture conditions utilized in the isolation, we suspected this cell type may be a mesenchymal stem cell. We measured surface expression of well characterized mesenchymal stem cell markers CD90 (Thy1), CD73 (NT5E), and CD105 (Endoglin) (Fig. 18B). The tumor derived cell lines expressed CD73 in the highest quantity, with the majority also staining positive for CD90 as well as CD105.

Mesenchymal stem cells are also defined by their ability to adhere to tissue culture plastic and form colonies from colony-forming unit-fibroblasts (CFU-F). Populations of CFU-F were significantly different between patient sample cell lines, IMR90 fetal fibroblasts, and hMSC ($F(6,18) = 21.32$, $P < 0.0001$) by one-way ANOVA. Upon multiple comparisons, all patient sample cell lines possessed significantly greater CFU-F populations compared to IMR90 fetal

fibroblasts ($P < 0.0314$), however tumor sample 959 derived in 3DH was distinguished with significantly greater CFU-F compared to either hMSC or the other tumor samples ($P < 0.0003$). There was no significant difference in tumor samples 927, 955, or 1072 in colony forming ability compared to hMSC (Fig. 18C). Interestingly, the environment that tumor sample 959 was originally cultivated in had a significant effect ($P < 0.0003$) on maintenance of colony progenitors, where the 3DH environment maintained a greater quantity of progenitors compared to traditional 2DN conditions. Taken together, these findings suggest that culturing cells derived from NSCLC in a lung microenvironment mimetic environment rapidly selects for mesenchymal stromal cells with stem-like characteristics. Given the delay in growth in traditional conditions and that at least one of the samples showed a difference in progenitor characteristics depending on the environment in which it was derived, we asked whether cells cultivated from the same tumor in different environments were phenotypically similar.

Figure 18. 3DH Patient derived stromal cell lines are mesenchymal and possess mesenchymal stem cell-like characteristics.

A. Western blot analysis of tumor resection 927 and its tumor derived cell lines, fetal lung fibroblast cell line IMR90, and lung adenocarcinoma cell line A549 (upper). Western blot analysis of additional tumor resections and their derived cell lines probing for expression of pan-cytokeratin (lower). B. Immunophenotyping of 3DH tumor derived cell lines with commonly identified antigens associated with MSC (Y-axis: CD90, X-axes: CD105 (left) and CD73 (right)). C. Quantification of Colony Forming Unit-Fibroblast (CFU-F) per 25 cells plated in 3DH tumor derived cell lines, a matched 2DN derived cell line (2DN 959), bone-marrow derived mesenchymal stem cell line (hMSC), and IMR90 after 16-18 days of growth. Statistical analysis performed by ANOVA with Tukey's test for multiple comparisons to the indicated groups, *p < 0.05, and ***p < 0.001; data presented are mean ± S.E.M.

Environment dependent MSC multipotency markers.

Mesenchymal stem cell markers and their differentiation capacity into various stromal cell types vary greatly depending on the sites and species from which they are derived, and the methodology used in their isolation (Hass, Kasper, Bohm, & Jacobs, 2011). However, human adult bone marrow mesenchymal stem cells, similarly to pluripotent human embryonic stem cells, were previously demonstrated to express a stage-specific embryonic antigen, SSEA-4 (Gang, Bosnakovski, Figueiredo, Visser, & Perlingeiro, 2007). To test whether primary cells isolated in the microenvironment mimetic environment maintained multipotent characteristics similar to MSC, we assessed SSEA-4 expression on sample 955 via immunofluorescent labeling. We confirmed that the majority of the cell population expressed CD90 (Fig. 19A) and observed that most of the cells maintained SSEA-4 expression (Fig. 19B). We also tested sample 927, which only produced viable progenitor cell lines in hypoxic environmental conditions (2DH and 3DH). We found that only the sample that had proliferated in the microenvironment mimetic 3DH environment maintained strong expression of SSEA-4 (Fig. 19C). The 3DH cells were then passaged in the other environments for 72 hours, and each of the environments maintained SSEA-4 expression. This suggests that certain characteristics induced in the microenvironment culture may be maintained or the rate of expression reduction is slowed upon transfer to traditional cell culture methods.

In order to verify that a physiological microenvironment indeed plays a role in the potency characteristics of derived cell lines, we assessed several markers of stem cell potency in hMSC and the 959 cell line that had been grown in the 3DH environment. Each cell line was split to both environments and allowed to grow for 72 hours before collection. Cells that were maintained in the 3DH environment expressed higher amounts of the markers SOX2 and REX1 (also known as ZFP42) compared to 2DN, but Nanog and Oct4 (also known as POU5F1) were not significantly affected (Fig. 19A). Interestingly, when the hMSC were transferred to the physiological condition, SOX2 and REX1 were undetectable. Therefore, a physiological microenvironment mediates potency characteristics of primary tumor derived stromal progenitor cells but may not be sufficient in enhancing potency of established cell lines.

Figure 19. 3D Physiological hypoxia cell culture selects for SSEA4 expressing tumor derived stromal cells.

A. Phase contrast and immunofluorescence staining of IMR90 lung fibroblasts and patient sample 955 patient tumor stromal cells for stromal marker CD90/Thy1 and nuclear stain DAPI. B. Staining of stromal cells and human pluripotent embryonic stem cell line H1 for stage specific embryonic antigen-4 (SSEA4). C. (left) SSEA4 expression of patient tumor stromal cell line 927 derived in either 2D or 3D hypoxia. (right) SSEA4 expression of patient tumor stromal cell line 927 3DH transferred to other environments. All scale bars are 100 μ m. D. hMSC and 3DH derived 959 were cultured in their native culture environments or transferred for 72 hours prior to collection of RNA and protein for RT-qPCR and western blot. Relative expression levels of pluripotency markers assessed by RT-qPCR of hMSC and 3DH 959 in different environments. Non-detectable products were confirmed on agarose gel (lower).

Tumor stromal cells derived in 3DH preserve tumor promoting characteristics.

Given the possibility that we may have selected for an MSC subtype in our microenvironment mimetic culturing system, we aimed to understand whether our system generally activated MSC to become tumorigenic and whether tumor derived stromal progenitor cells also produced pro-tumorigenic factors. Thus, we assessed stromal genes related to worse prognosis and tumor progression in both cell types. Gene expression of vascular endothelial growth factor (VEGFA) was significantly increased ($P < 0.0368$) in tumor stromal cells maintained in 3DH environment, but not greatly affected upon hMSC transfer to 3DH (Fig. 20A). Similarly, periostin (POSTN), a secreted extracellular matrix protein that plays a role in metastasis and is associated with a worse prognosis in a variety of cancers (Zhu et al., 2010), had significantly higher gene expression when tumor stromal cells were grown in 3DH ($P < 0.0164$) but was slightly decreased when hMSC were transferred to 3DH. Neuron-glia 2 (NG2, also known as CSPG4), an angiogenic pericyte protein associated with the pre-metastatic niche, and tenascin-C, an extracellular matrix protein frequently overexpressed in lung cancer, were not greatly affected by environment transfer in either hMSC or tumor stromal cells. Transforming growth factor beta 1 (TGF- β) was also increased in the 3DH environment, however when 3DH cells were transferred to 2DN they maintained slightly higher expression (Fig. 20B). Our findings suggest that only tumor derived cells cultured in microenvironment mimetic culture alter

their phenotype to produce a variety of pro-tumorigenic factors, and we sought to test whether these factors might alter the phenotype of NSCLC cells themselves.

In summary, mesenchymal stromal stem-like cells derived from early, non-metastatic tumor resections in a microenvironment mimetic environment maintain tumor promoting characteristics in-vitro in an environmentally dependent manner.

A

Figure 20. 3D Physiological hypoxia cell culture maintains expression of tumor promoting characteristics of tumor derived stromal cells.

hMSC and 3DH derived 959 were cultured in their native culture environments or transferred for 72 hours prior to collection of RNA and protein for RT-qPCR and western blot. A. RT-qPCR of stromal derived tumorigenic factors of hMSC and 3DH 959 in different environments. B. Western blot analysis of HIF1-a and TGF-b expressed in 2DN or 3DH derived 959 stroma and transferred to different environments for 72 hours. Statistical analysis performed by ANOVA with Sidak's test for multiple comparisons to the indicated groups, *P < 0.05; data presented are mean $\pm$ S.E.M.

DISCUSSION

Previously, we have shown that lung fibroblast derived extracellular matrices provide various benefits to lung adenocarcinoma cell lines in-vitro such as providing resistance to apoptosis, serum withdrawal and chemically induced hypoxia. Since the environment is a critical determinant of cell fate in NSCLC, we expanded our in vitro system to include a physiological oxygen tension that would resemble the microenvironment of the lung. Initially, we sought to test each individual element of our proposed microenvironment mimetic culturing system (Fig. 14A) and found that the individual components of hypoxia (2DH) or cell derived ECM (3DN) enhanced mesenchymal cell growth after 72 hours (Fig. 14B). Interestingly, the combination of hypoxia and ECM appeared to have the greatest effect on cell growth. Others have demonstrated the mitogenic benefits of low levels of hypoxia (1-5% O₂) to multiple mesenchymal cell types. Grayson et al. showed that hMSC grown in 2% O₂ improved their cell proliferation after extended periods but did not enhance their proliferation rate (Grayson et al., 2007). Instead, they concluded that hypoxia prolonged the proliferative state and the cells were less affected by inhibitory signaling. Our analysis of cell doubling time agreed with their finding as we did not observe significant differences in doubling time for hMSC in hypoxia, and only IMR90 fetal lung fibroblasts had a significantly hastened proliferation rate in hypoxia (Fig. 14C). However, all cell lines tested exhibited a significant reduction in doubling time when cultured in the combination of physiological hypoxia and ECM (3DH), suggesting that there may be an increase in proliferation kinetics and cell cycle progression. This is

supported by the relative decrease in cyclin-dependent kinase inhibitors p21-Cip1 and p27-Kip1 in 3DH grown cells compared to 2DH (Fig 14D). This may yet highlight another benefit of microenvironment mimetic culturing, as these factors, as well as effects mediated by P53 and Akt, play important roles in the induction of cell senescence, a major challenge in the culture and expansion of human mesenchymal progenitor cells and fibroblasts (Estrada et al., 2013; Kim et al., 2017; S. Lee et al., 2011). Indeed, we found that most cell lines tested continued to proliferate rapidly for more than 15 passages (Fig. 17). It is possible that the combined decrease in inhibition of cell cycle progression with the increased 3-dimensional surface area allotted by the ECM act synergistically to allow proliferation at high cell densities, resulting in a sustained proliferative response.

Given this sustained proliferative response, and the reported capability of ECM derived from desmoplastic and tumor conditioned fibroblasts to induce desmoplastic differentiation in stromal cells grown on those matrices (Amatangelo et al., 2005), we also sought to determine if our system was sufficient to promote desmoplastic differentiation of stroma. In accordance with their finding, our fibroblast derived matrix system did not induce expression of a desmoplastic response in normoxia or hypoxia (Fig. 14D). In addition, we found that cells grown in 3DH had less HIF1- α stabilization compared to their 2DH counterpart, indicating a role for the ECM in the regulation of the oxygen sensing response and homeostasis. Taken together, these data provided evidence that stromal cells would receive a proliferative benefit in a microenvironment mimetic

system and that the system may not artificially accelerate the tumorigenic potential of that stroma.

Next, we obtained small tumor resections from five treatment naïve patients diagnosed with NSCLC (Table 3 and Fig. 17). A small portion of these samples was dissected, enzymatically digested, and equal quantities of cells in single cell suspensions were aliquoted into separate environmental conditions. The first of these samples, 927, was tested in each condition (2DN, 2DH, 3DN, 3DH). After 7 days of growth, we observed cell attachment in every condition (Fig 16A), and while there was increased cell number in 2DH and 3DN, the 3DH condition had a substantial increase in cells and numerous large colonies covering the plate. Of the environments tested, only the hypoxia environments were capable of cultivating cell lines. Due to the marked increase in long term cell viability and the limited quantities of our patient samples, we chose to consolidate our testing to traditional cell culture methods (2DN) or microenvironment mimetic methods (3DH). Further sample isolations confirmed our previous results, with every sample tested yielding very rapid expansion of cells into cell lines in 3DH compared to 2DN (Fig. 16B, and D). For one biological specimen, we were also able to acquire adjacent normal tissue, however we did not observe the same growth benefit in 3DH (Fig. 16C). It is possible that the number of progenitor cells present in this tissue was less than that which resides within the tumor microenvironment and was unable to sustain a population.

Alternatively, stromal progenitor cells within the tumor microenvironment may also undergo metabolic reprogramming to allow them to adapt more easily

to a hypoxic cell culture system (Kalluri, 2016). Considering that we were only able to test one specimen, it is difficult to draw conclusions and further studies are required to determine the viability of 3DH culture in isolating primary human tissue resident stromal progenitors. Importantly, while traditional methods produce cell lines after a delay, 3DH isolation increases cell viability of tumor derived stroma, and cells isolated in this environment can be transferred to traditional cell culture (Fig. 16E), although at a slowed proliferation rate, allowing for testing via common pre-clinical research techniques.

It should be noted that initially every sample allowed for the attachment of a heterogeneous population of cell morphologies (Fig. 17), and some of these cell types were present and maintained their in-vivo cell polarity characteristics upon passaging in 3DH. This suggests that our microenvironment mimetic platform can be further manipulated to preserve other populations of cancer stem or progenitor cells, perhaps with supplementation of specific growth factors. Nonetheless, the majority cell type that proliferated was of mesenchymal origin (Fig. 18A) and possessed surface expression of common markers for human mesenchymal stem cells (Fig. 18B). Recently, there has been some discussion in the field of the tumor associated stroma regarding the hypothesis that cancer associated fibroblasts (CAF) may actually be quiescent mesenchymal stem cells that become activated within the tumor (Kalluri, 2016). This is supported by research showing that breast cancer derived CAF are remarkably similar to hMSC in their morphology, surface marker expression, cytokine production, and trilineage differentiation potential (Paunescu et al., 2011). The isolation and

cultivation of CAF and hMSC is also similar and dependent upon adherence to tissue culture plastic either from single cell suspensions or from cells that migrate from dissected tissue fragments. When comparing the CFU efficiency of the isolated cell lines, they were similar to hMSC (Fig. 18C), however the efficiency of at least one cell line appeared to be significantly higher when it was isolated in 3DH compared to traditional methods. It is possible that cells isolated with traditional methods possess MSC-like characteristics but gradually lose them and progenitor capability over time, whereas cells isolated and maintained in 3DH preserve those characteristics.

Multipotency is one of the defining characteristics of hMSC (Dominici et al., 2006). Markers associated with potency are an active area of research (Samsonraj et al., 2015), and these markers vary depending on the species, age, and tissue of origin of the isolated cells (Chamberlain, Fox, Ashton, & Middleton, 2007; W. C. Lee et al., 2014). We found that tumor stromal progenitor cells cultured in 3DH expressed SSEA4 (Fig. 19B, C), similarly to hMSC derived from bone marrow. We did not observe expression of SSEA4 in 2DN derived samples, however SSEA4 was detectable when the 3DH derived cells were transplanted to other environmental conditions. We hypothesize that the 3DH condition selects for and rapidly amplifies the SSEA4 positive population, whereas this population grows at a delay in standard culture conditions before amplifying, similarly to unsorted bone marrow samples examined by Gang et al (Gang et al., 2007). 3DH derived cells also expressed pluripotency markers Oct4, Nanog, Rex1, and Sox2 (Fig. 19D), however only Rex1 and Sox2 appeared to be dependent upon

the environmental condition of the cells. This corresponds with earlier studies of bone marrow MSC and embryonic stem cells grown in hypoxia (Grayson, Zhao, Izadpanah, Bunnell, & Ma, 2006; Westfall et al., 2008). Considering these data and that ECM produced by bone marrow MSC plays a pivotal role in their maintenance of stem-like characteristics, the combination of a native ECM and hypoxia seems to isolate multipotent progenitor cells from the pulmonary tumor microenvironment and that these cells may be MSC.

It is well known that MSCs from the bone marrow have a remarkable capacity to home to tumors of a variety of different organs and affect their growth, as well as their response to treatment. While it is currently not possible to definitively identify whether the progenitor cells in the patient tumors we studied were tissue-resident or bone marrow derived, we asked the question of whether these tumor associated MSC had different tumor promoting effects compared to hMSC. We found that the 3DH environment significantly increased gene expression of angiogenesis promoting VEGF-A (Ferrara, 2004; Imoto et al., 1998) and metastasis promoting periostin (Hong, Wei, Chen, & Shi, 2013; Zhu et al., 2010) in a patient derived cell line, but not in hMSC (Fig. 20A), and also increased TGF- β expression even after the cell line was transferred back to the 2DN condition (Fig. 20B).

In toto, this study demonstrates the benefits of an in-vitro culturing system that mimics two important elements of the tumor microenvironment, physiological oxygen tension and native extracellular matrix structure, in the isolation of stromal progenitor cells from lung tumor biopsies. These progenitor cells express

some commonly used markers of stem cells within our cell culture system, and function similarly to hMSC in supporting an in vitro tumor-like environment. We realize that many cells express these markers and more studies are needed to fully characterize their specific identity, and given our data, especially the potential role they may play in supporting a purported cancer stem cell phenotype. Indeed, our findings highlight the importance of investigation using in vitro models that reflect the in vivo condition as several tumor promoting characteristics were revealed only in physiological hypoxia and on extracellular matrix. Targeting factors that are producible in a physiological model may yet lead to greater drug targeting efficacy for future anti-tumor and anti-tumor associated stroma therapy. Additionally, this methodology may be refined to provide further insight into progenitor cells of other tissues and assist in rapidly expanding stem populations and preserving multipotency.

CHAPTER V: PATIENT DERIVED CANCER ASSOCIATED MESENCHYMAL STROMAL CELLS DERIVED FACTORS AND EXTRACELLULAR MATRIX PROMOTE METASTASIS

INTRODUCTION

The tumor microenvironment is composed of a diverse cellular milieu that acts in concert to promote a pathological environment that promotes hallmarks of tumor progression, including sustaining proliferative signaling, genome instability, inducing angiogenesis, and activating invasion and metastasis (Hanahan & Weinberg, 2011). Among these cellular components are transformed malignant cells, cancer activated mesenchymal cells or fibroblasts (CAM), endothelial cells, and inflammatory cells such as cancer associated macrophages. Importantly, each of these cell types exist in a dynamic and occasionally labile manner as they communicate with direct cell to cell communication or paracrine signaling. Indeed, the cancer associated stroma is a potent source of growth factors and microenvironment modifying enzymes that act both locally in the primary tumor and in distal sites of the pre-metastatic niche (Hoshino et al., 2015; Li et al., 2017; Wortzel, Dror, Kenific, & Lyden, 2019).

Recently, increased focus has delineated fibroblasts and mesenchymal stem cells as important mediators in the tumor microenvironment as they

(Portions of this chapter are published in Scientific Reports. (Saforo et al., 2019))

commonly secrete these growth factors and are primary producers of extracellular matrix. When these cells interact with and are activated by cancer cells, they in turn also modulate the activity of tumor infiltrating immune cells including monocytes, NK cells, and macrophages, which play a role in extracellular matrix remodeling (Kuen, Darowski, Kluge, & Majety, 2017; Yu et al., 2017; R. Zhang et al., 2019). Matrix metalloproteinases (MMPs) and other enzymes released by cells of the tumor microenvironment degrade the ECM, but also activate latent growth factors and cytokines that lay dormant within the matrix architecture (Das, Monteiro, Barai, Kumar, & Sen, 2017; Nguyen et al., 2016; Shay, Lynch, & Fingleton, 2015). The organization of the extracellular matrix as well as the dysplastic deposition of matrix proteins have been shown to affect the metastatic potential of tumor cells. For example, in breast cancer, breast density is an independent risk factor for breast cancer progression and cancer associated modifications to extracellular matrix, including cross-linking, stiffening, and alignment of collagen and other ECM proteins alter proliferation, migration, and invasion of breast cancer cells (Freer, 2015; Lampi & Reinhart-King, 2018; Ondeck et al., 2019). Recently, matrikine and ECM protein profiles have shown promise in early diagnosis of lung cancer and as predictors of therapeutic benefit to adjuvant chemotherapy in early stage tumors (Andriani et al., 2018; Lim, Tan, Lim, & Lim, 2017). These studies demonstrate that alterations within the tumor microenvironment and ECM occur early during the progression of lung cancer, however the effect of the stromal components of early stage lung cancers are not completely understood.

Previously we demonstrated that a microenvironment mimetic cell culture system can amplify the isolation of a stem-like population of mesenchymal cells from primary tumor resections. These cells differed from bone marrow derived mesenchymal stem cells in their ability to respond to environmental factors such as physiological hypoxia and extracellular matrix, and exposure to these environments elicited transcription and expression of tumor promoting proteins including transforming growth factor-beta and the matricellular protein periostin. However, whether these cells were able to also affect the growth and invasion of cancer cells remained an unanswered question. Do these cancer associated cells effect the microenvironment in direct or indirect mechanisms? Are the responses to a “normal” stromal environment and a cancer associated stromal environment different in-vivo? What role does extracellular matrix remodeling play in the progression of cancer?

To answer these questions, we employed physiologically relevant in-vitro and in-vivo models including cell-derived matrices and tumor xenograft to assess the cancer promoting characteristics of patient derived cancer associated mesenchymal cells.

RESULTS

Cancer associated mesenchymal cells promote proliferation, invasion, and metastasis upon direct cancer cell interaction

Our previous findings suggest that, unlike their bone marrow derived counterparts, tumor derived mesenchymal (CAM) cells cultured in microenvironment mimetic culture alter their phenotype to produce a variety of pro-tumorigenic factors (Fig. 20). We sought to test whether these factors might alter the phenotype of NSCLC cells themselves.

To determine if direct interaction with patient derived stroma would alter cancer cell growth, we co-cultured fluorescent nuclear labeled A549 lung adenocarcinoma cells on different stromal monolayers. Following seven days of growth, A549 co-cultured with patient stroma rapidly expanded (Fig. 21A) and formed large, densely nucleated colonies compared to smaller, more linearly arranged growth on fetal lung fibroblast monolayers. This colony forming ability was also maintained on the MSC monolayer (Fig. 21B). Fluorescently labeled nuclei were quantitated and one-way ANOVA revealed that different stromal monolayers significantly affected the growth of A549 cells ($F(6,9) = 36.51$, $P < 0.0001$). We found that there was no significant difference of cell growth on either IMR90, WI38, or hMSC monolayers, however all patient samples significantly increased cancer cell growth compared to lung fibroblast monolayers IMR90 and WI38 (955: $P < 0.0088$, $P < 0.0495$; 956: $P < 0.0021$, $P < 0.0126$; 959: $P < 0.0001$, $P < 0.0001$; 960: $P < 0.0009$, $P < 0.0052$). Three of the patient derived stromal monolayers significantly increased growth compared to hMSC (956: $P <$

0.0380; 959: $P < 0.0001$, 960: $P < 0.149$). Of the patient derived cell lines, 959 significantly increased cancer growth compared to the others (955: $P < 0.008$; 956: $P < 0.0023$; 960: $P < 0.0053$).

In order to test the ability of CAM cells to alter the invasion characteristics of lung cancer cells, we employed a spheroid migration assay we used previously to establish migration characteristics of cancer cells on CDM. Briefly, we grew spheroids that contained a mixture of A549 lung adenocarcinoma cells with either IMR90 fetal lung fibroblasts or the CAM cell line 959 (5 parts A549:1 part stroma). Next, we transferred these spheroids to a WI38 CDM and observed migration for several days. Interestingly, we observed several cell migration modalities that we had not previously observed when growing spheroids on CDM with cancer cell lines alone. First, we observed that occasionally, a small cluster of cells would separate completely from the spheroid and the cells would migrate collectively in a small group away from tumor mass (Fig. 22A). Since we utilized the A549 cell line that stably expressed a red fluorescent protein, we were able to confirm that cells within this detached cluster contained a mix of cancer cells and stromal cells (Fig. 22A). Secondly, we also observed the formation and migration of long strands of connected cells that emerged from the central portions of the spheroid mass (Fig. 22B). These strands were present in mixed spheroids of both CAM and IMR90 with A549 but not in A549 spheroids alone, suggesting that this phenomenon is a characteristic of tumor and stromal cell interactions. Again, the strands appeared to have a mixed composition. Notably, both of these invasion modalities are characteristic of tumor invasion in-vivo (Peter Friedl &

Gilmour, 2009), suggesting further applications for our CDM and CAM/Cancer spheroid model in future studies.

Since we observed a significant increase of cancer cell growth upon direct interaction with patient tumor stromal progenitors and observed invasion modalities characteristic of cancer cells in-vivo, we sought to determine whether these cells isolated and grown in 3DH also displayed pro-tumorigenic ability in-vivo. To that end, we subcutaneously injected combinations of A549 lung adenocarcinoma cells and stromal cell lines, separately and co-administered (1:5), into flanks of immunocompromised NRG mice. After 30 days of growth, tumors successfully developed in all mice bearing A549 (Fig. 23A), whereas no tumors developed in any mice injected with only stromal cells (Fig. 23B). One-way ANOVA comparison of mean tumor weights revealed statistically significant differences in tumor growth with stromal co-administered tumors ($F(4,35) = 3.710$, $P = 0.0128$). While generally the addition of stroma resulted in increased tumor growth, statistically significant increases in tumor weight compared to solely A549 injected tumors revealed these changes may primarily be driven by hMSC ($P < 0.0450$), and 955 ($P < 0.0124$) co-injections. Histological sections of the subcutaneous tumors revealed that stromal populations were present and localized toward the core of tumors in co-injections (Fig. 23C), but a prominent stroma was not evident in the solely A549 tumors. Pulmonary histological analysis revealed small metastatic nodules in IMR90 co-injected mice, but prominent metastatic lesions in hMSC, 955, and 959 co-injected cohorts (Fig. 23D). Pulmonary metastasis was not detected in solely A549 injected mice. In

summary, mesenchymal stromal stem-like cells derived from early, non-metastatic tumor resections in a microenvironment mimetic environment maintain tumor promoting characteristics in-vitro and promote a full metastatic program in a normally non-metastatic adenocarcinoma cell line xenograft.

Figure 21. Cell-cell interactions between 3DH Patient derived stromal cell lines and lung adenocarcinoma promote cancer cell proliferation and colony formation.

Stromal cells were grown to a confluent monolayer for four days, and then equal amounts of red nuclear labeled A549 cells were plated atop monolayers. A. (Upper) Phase contrast and fluorescent images of A549 grown on stromal monolayers of IMR90 and patient stroma 959. Images obtained 1 and 7 days after cancer cell seeding. (Lower) Comparison of A549 colony formation on IMR90 or 955 monolayers on day 7. B. Comparison of A549 colony formation on hMSC, 956, and 960 monolayers. C. Quantification of red nuclei on day 7 (mean nuclear counts were obtained from multiple images of technical duplicate plates from 2 different experiments.) Statistical analysis performed by ANOVA with Tukey's test for multiple comparisons to the indicated groups (a: vs. IMR90, $P < 0.01$; b: vs. WI38, $P < 0.05$; c: vs. hMSC, $P < 0.05$; d: vs. 959, $P < 0.01$); data presented are mean $\pm$ S.E.M. All scale bars are 100 μ m.

Figure 22. Co-culture of stromal cells and cancer cells promote distinct migration patterns on CDM.

Spheroids containing a mixture of red nuclear labeled A549 lung adenocarcinoma cells and either IMR90 or patient derived CAM (5 A549:1 Stromal cell) were grown for 4 days and then transferred to a WI38 CDM. Images of cell migration were captured and 4 days post transfer. A. Images of cells migrated from a spheroid containing A549 and IMR90 (left) and images of a small detached cluster that migrated from the spheroid (right). Note that areas of the detached cluster contain cells without red nuclei (white arrowheads). B. Comparative images of mixed spheroids containing CAM cell line 959 or IMR90 with A549 or A549 alone. Note the distinct multicellular invasion strands only present emanating from the mixed spheroids (right).

B A549/Stroma coinjection

C Xenograft Tumors

Figure 23. Patient derived cancer stromal cells promote metastasis in-vivo.

955 and 959 stromal cell lines were subcutaneously injected into alone or mixed with A549 cancer cells (5:1) and subcutaneously injected into flanks of NRG mice. IMR90 and hMSC co-injections served as controls. A. Representative images of tumors excised after 30 days of growth. B. Quantification of excised tumor weights (g) (statistical analysis performed with ANOVA and Tukey's test for multiple comparisons vs. A549 injection alone, $*p < 0.05$; data represented as mean $\pm$ S.E.M.) C. Histology of subcutaneous xenograft tumors. Arrows indicate areas of stromal cells; scale bars are 1 mm. D. Pulmonary histology of lungs from mice receiving xenograft tumors (left; scale bars are 1 mm). 955/A549 and 959/A549 co-injection tumor bearing mice with pulmonary metastasis (inset; scale bars are 500 μ m). Arrows indicate metastatic lesions.

CAM derived extracellular matrix promotes different migration characteristics and molecular patterns associated with invasion

Considering that mesenchymal cells are thought to be the major producers of ECM in tissue, and that we showed CAMs alter the expression of extracellular matrix proteins such as periostin in response to environment specific characteristics, we asked the question of whether CDM produced by the CAM cells would alter the behavior of cancers cells differently than a fetal lung fibroblast derived ECM we utilized for previous studies. To accomplish this task, we adapted our CDM protocol for use with primary CAM cells and produced ECM coated substrates. Visually, these matrices appeared to be more complex than IMR90 CDM, with fewer thick distinct bundles of matrix proteins and appeared less oriented, however these characteristics remain to be accurately quantified. We solubilized and separated the ECM produced by IMR90, as well as samples 955, 956, 959, and 960 using SDS-PAGE and stained the total protein using Coomassie blue (Fig. 24A). We noted several distinct bands that were present in the patient samples but were not present in the IMR90 CDM, as well as an altered relative abundance of high molecular weight proteins. A proteomic analysis of the precise identification of these proteins, their abundance, localization, and gross linearity would likely inform future studies, however from our current data we can determine that each of these samples are different. In order to determine whether these changes in CDM could alter the migration of cancer cells, we transferred A549 and H2030 spheroids onto the different ECM and observed their migration (Fig. 24B, C). Surprisingly, with both cell lines we

observed decreased migration on the patient derived CDM compared to the IMR90 CDM. Again, we observed a directionality of migration on the patient derived CDM (Fig. 24C) and what appeared to be distinct “leader” and “follower” cell phenotypes. Qualitatively, it appeared as though the number of cells transitioning from collective to single cell migration was increased on the CAM CDM, and that the velocity of cells that separated from the mass appeared greater than that of cells connected to the spheroid, however we were unable to find a method of analyzing these parameters in an unbiased manner with potentially thousands of cells migrating independently.

In an attempt to assess single cell migration behavior, we plated the nuclear labeled A549 cells onto the CAM CDM, IMR90 CDM, or collagen coated plastic (2D) and used time lapse microscopy to observe the cells in real time. Again, note that cancer cells grown on IMR90 CDM possess morphologies that resemble mesenchymal migration, with clearly polarized cell bodies that align themselves along the matrix and cellular projections that navigate along the matrix strands in an ordered fashion (Fig. 25A). Immediately, we were able to recognize distinct difference in cancer cells grown on the CAM CDM, where cells made multiple and multipronged pseudopodia adhesions to the matrix that appeared to be maintained for much of the observed period. Concurrently, many of these cells were not motile in this condition and the fluorescent nuclei appeared to be squeezed into oblong shapes as the cells sensed and adjusted to the extracellular matrix. This altered cell adhesion also appeared to affect cell division, as some cells on the CAM CDM would appear to enter prolonged cell

division and then either complete cytokinesis to emerge with multinucleated cells (Fig. 25B) or exit mitosis without completing cytokinesis and becoming multinucleated (Fig. 25C). Recently, increasing emphasis is placed on nuclear membrane integrity in cancer as tension and stress induced by extracellular forces promote nuclear membrane instability and deformability, resulting in genomic instability, MMP mediated invasion and associated with aggressive malignant disease (Denais et al., 2016; Dornier & Norman, 2018; Infante et al., 2018). Considering that cell-matrix adhesions on all CAM CDMs appeared to be stable compared to the IMR90 matrix, we hypothesized that cancer cells on these matrices may also have sustained focal adhesion signaling. Indeed, western blot analysis of FAK activation showed increased phosphorylation at Tyr 397, Tyr 925, and Tyr 576, as well as associated phosphorylation of paxillin and elevated expression of integrin linked kinase of cancer cells grown on the patient CAM CDM compared to IMR90 CDM (Fig. 26). Additionally, cancer cells increased activation of Src on all three patient CAM CDM, although showed little change in AKT from 2D conditions. Strikingly, interaction with the CAM CDM also greatly decreased PAI-1 expression in the cancer cells. Collectively, we have previously shown that this molecular pattern in A549 cells is associated with increased invasion into Matrigel, and here we can see that such a pattern can be promoted solely by interaction with extracellular matrix derived from cells isolated from patient tumors.

Figure 24. Patient derived CAM CDM has distinct protein composition and alters cancer cell migration.

IMR90 or patient CAM cells were grown at confluence for 8 days with media supplemented with ascorbic acid every other day to produce CDM. Matrices were denuded of cells and then solubilized and separated using SDS-PAGE. A. Total proteins were visualized with Coomassie blue to show distinct expression patterns. A549 and H2030 lung adenocarcinoma spheroids were grown for six days and transferred to either IMR90 CDM, patient CAM CDM, or 2D collagen. B. A549 spheroids on ECM exhibited less migration on patient CAM CDM compared to IMR90 CDM after 120 hours. C. H2030 spheroids migrated over less total area on patient CAM CDM than IMR90 CDM, with cells frequently transitioning to single cell motility on CAM CDM.

Figure 25. Patient CAM CDM stabilizes cell-matrix adhesions and alters cell division in cancer cells.

Red nuclear labeled A549 were grown on either IMR90 CDM or patient derived CAM CDM and cell behavior was observed via time-lapse microscopy with images captured every 15 minutes for 24 hours. A. Cell morphology of A549 cells on either IMR90 CDM, with clear cell polarity and mesenchymal appearance, and patient derived CAM 959 CDM, with cells exhibiting multiple projections to the matrix and constricted nuclei. B. A cluster of A549 cells on CAM 956 CDM demonstrates aberrant cell cycle progression with a binucleated cell (left; white arrow) dividing into a cell with three nuclei (right; white arrows). C. A binucleated A549 cell attempts and aborts cell division, resulting in a cell with four nuclei.

Figure 26. Patient CAM CDM promotes activation of focal adhesion signaling pathways and an invasive molecular phenotype in cancer cells.

A549 cells were grown on either a 2D surface, IMR90 CDM, or patient CAM CDMS (956, 959, and 960) CDM and lysates were collected and probed for proteins involved in focal adhesion signaling and 3D invasion, including focal adhesion kinase (FAK), and its phosphorylated sites Tyr 397, Tyr 925, and Tyr 576, integrin linked kinase (ILK), Src, activated Src (Tyr 416), AKT, activated AKT (Ser 473), and plasminogen activator inhibitor-1. Note that the three patient derived CDM similarly increase FAK and SRC activation, as well as down regulate PAI-1 expression, characteristics associated with invasion in cancer cells.

Revisiting the PAI-1 paradox using an in-vitro model of the tumor microenvironment

For decades, the plasminogen-plasmin activation system has been intensely researched due to many elements of the system regulating tissue processes aside from fibrinolysis and the coagulation cascade, including cell senescence, proliferation, angiogenesis, and migration. Indeed, intracellular signaling downstream of the urokinase plasminogen activator receptor (uPAR) has been found to be related to activation of many integrin linked proteins found to be associated with tumor invasion used in this study including FAK, Src, paxillin, AKT, and EGFR (Mauro et al., 2017; H. W. Smith & Marshall, 2010). However, in a well-known paradox, PAI-1, the main inhibitor of uPAR mediated plasminogen activation is also associated with poor prognosis in several solid cancers, including breast and lung (Foekens et al., 1994; Lin et al., 2017; Look et al., 2002). Further, PAI-1 is expressed by adult and fetal mesenchymal stromal cells in the human lung (Rolandsson Enes et al., 2016). In order to understand the PAI-1 paradox in the context of our co-culture system of cancer cells and patient derived CAM cells, we first set out to verify that the stromal cells were also expressing PAI-1.

We found that while lysates prepared from resected tumor material from patients initially had little or low PAI-1 expression, the stromal cell lines and the IMR90 fetal lung fibroblast cell line had relatively high expression (Fig. 27A). Additionally, to understand the contribution of cancer and inflammation to PAI-1 expression in the lung, we used a repeated cisplatin dosing model in FVB mice

bearing lung tumors to recapitulate chemotherapeutic treatment of patients in the clinic (Sharp et al., 2016). Interestingly, while tumor bearing mice did not have different differences in PAI-1 expression in the lung, animals treated with cisplatin had a moderate increase in PAI-1 expression and the cohort of tumor bearing mice that received chemotherapy had the highest measured expression (Fig. 27B). This lends further evidence that the stroma, and particularly the tumor reactive stroma, is a major contributor of PAI-1 in the tumor microenvironment.

Next, we sought to understand the consequence of loss of PAI-1 expression in cancer cells during co-culture of PAI-1 expressing stromal cells. Interestingly, we found that in the early days of co-culture, cancer cells segregated into small cell clusters surrounded by thick layers of stromal cells in an organized pattern, regardless of PAI-1 KO or overexpression in the A549 cells (Fig. 27C). While this delineation was maintained after a week of co-culture in PAI-1 expressing A549, PAI-1 KO A549 proceeded to invade and grow into the stromal sections of the culture plate. These data indicate that loss of PAI-1 in cancer cells results in a phenotype that cannot be rescued by extracellular PAI-1 secretion. Next, we assessed whether migration of the PAI-1 manipulated cells would be affected by the CAM CDM, as previously we observed a downregulation of PAI-1 in cells plated on the patient derived substrates. Previously, we found that overexpression of PAI-1 in A549 resulted in less spheroid migration compared to NT or PAI-1 KO on an IMR90 matrix (Fig. 13E), however on the CAM derived matrix we observed little difference in migration between the NT and PAI-1 Overexpression spheroids (Fig. 28A). As before,

saracatinib completely blocked migration and loss of PAI-1 expression in A549 greatly increased spheroid migration. To model the effect of PAI-1 secretion from the stromal cells, we also added recombinant PAI-1 to the media of migrating spheroids. Interestingly, the addition of PAI-1 had the effect of enhancing cell migration in all the cell lines tested, with the greatest migration observed in the A549 PAI-1 KO cells treated with recombinant PAI-1 (Fig. 28 B, C). According to our previous findings, this condition of high extracellular stromal PAI-1 and low intracellular cancer PAI-1 expression would most accurately resemble the conditions of cancer cells responding to a tumor reactive extracellular matrix within the tumor microenvironment. Further consideration of these parameters in a microenvironment mimetic system may add to our understanding of the PAI-1 paradox, particularly in cell migration and cell-matrix interactions.

Figure 27. PAI-1 is expressed by cells of the tumor stroma, and loss of PAI-1 in cancer cells promotes invasion into tumor stroma.

To assess the expression of PAI-1 in stromal cells, we used the CRISPR/cas9 lentivirus containing guides for PAI-1 to stably modify the fetal lung fibroblast cell line IMR90, and probed for PAI-1 via western blot analysis (left). In addition, we compared the total PAI-1 expression in lysates derived from the primary tumor resections from patients and compared them to the CAM cell lines derived from those tumors (right). Gapdh was used as a loading control. B. PAI-1 mRNA expression was measured in the lung of FVB mice 8-weeks of age (Y) or middle-age (6-months old) (M) with or without non-small cell lung carcinoma and treated with 4-doses of vehicle or cisplatin, 1-wk apart and sacrificed 72 hr after the last injection, demonstrating increased PAI-1 expression in a physiologically relevant cancer model in-vivo. C. Images of co-culture of CRISPR KO or overexpressed PAI-1 A549 cells and WT IMR90 fibroblasts (10:1) 2 and 7 days post culture. Note delineated tumor margins in NT and PAI-1 expressing A549 while PAI-1 KO A549 invade into the stromal margin.

Figure 28. Exogenous PAI-1 promotes spheroid migration on CAM CDM.

CRISPR/cas9 PAI-1 KO, NT, and PAI-1 overexpressing A549 were grown in spheroids and then transferred onto CDM produced by either patient CAM cell lines 959 or 960. A. Quantification of cell migration from PAI-1 manipulated spheroids treated with either vehicle or saracatinib (5 µM) 72 hours after transfer to CDM. To assess the role of non-tumor derived PAI-1 on cell migration, NT, PAI-1 KO (B) and PAI-1 overexpressing spheroids were transferred to a WI38 CDM and treated with complete media, saracatinib (5 µM), or recombinant human PAI-1 (1.33 µg/mL) and images were captured at 72 hours. C. Quantification of spheroid migration. Each symbol represents one spheroid. Data presented are means $\pm$ SEM from one experiment.

DISCUSSION

In the present study, we assessed the contributions of tumor derived stroma in affecting the metastatic characteristics of lung adenocarcinoma cells. To accomplish this goal, we used cell lines that were derived from tumor resections from patients with treatment naïve early stage lung cancer (Table 3). Previously we established that several genes associated with tumor progression and stemness were upregulated in these cells in an environment dependent manner, where a microenvironment mimetic culture system stimulated many of these factors (Fig. 20). We employed direct co-culture of A549 cells with primary tumor stroma or fetal lung fibroblast cell lines to test the effects of the tumor derived cells on cell proliferation. Patient stroma also significantly increased A549 lung adenocarcinoma cell growth in this direct co-culture model in-vitro compared to hMSC and fetal lung fibroblasts (Fig. 21C). Interestingly, A549 clustered into dense nests of cells when cultured on hMSC or patient stromal monolayers, but not on the fibroblast monolayers (Fig. 21A, B). These cell nests, as well as nests observed in early culture of NSCLC derived cell lines (Fig. 17), resembled sphere forming cancer stem cells (CSC) induced in a CSC/CAF co-culture system (W. J. Chen et al., 2014). It is possible that the microenvironment mimetic culture method also plays a role in establishment or maintenance of CSC populations, especially in the context of additional paracrine signaling with tumor stroma. Since CAF have been demonstrated to produce exosomes that regulate tumorigenic properties of other cancers, it would be interesting to know whether direct interaction of cancer cells with tumor stroma is required to incite

these properties or treatment of conditioned media from the stroma is sufficient to alter cell proliferation and stem-like characteristics (Richards et al., 2017).

Remarkably, while co-culture of cancer cells and stromal cells didn't drastically alter spheroid morphology, transfer of these spheroids to extracellular matrix revealed some intriguing differences in collective cell migration. While we had observed cell streaming from spheroids in a collective manner previously, upon co-culture we witnessed collective migration phenotypes normally observed in-vivo including the migration of cells in small, separate clusters or in long, thin strands that remained connected to the spheroid mass (Fig. 22). Fluorescent imaging showed that these multicellular structures contained a majority of cancer cells with a small quantity of stromal cells dispersed in between. Future in-vitro studies may focus on elucidating the molecular mechanisms underlying this association of cells as these structures are proposed to play a role in the survival of circulating tumor cells to promote metastasis (Clark & Vignjevic, 2015; Fabisiwicz & Grzybowska, 2017; Giuliano et al., 2018).

As we established that stromal cells potentiate tumor migration patterns in-vitro that are normally observed in-vivo, we asked the question of whether these tumor-stromal cell interactions would drive tumor growth and metastasis in-vivo. To understand these interactions in vivo, xenograft studies demonstrated that subcutaneous co-injection of A549 with stroma significantly increased tumor size compared to A549 alone (Fig. 23A, B), and that this stroma remained present and localized toward the core of the tumor (Fig. 23C). Further, both cohorts of mice bearing patient stromal tumors developed metastasis to the lung,

similarly to hMSC injected mice while not in A549 alone (Fig. 23D). This suggests that patient stroma can promote a normally non-metastatic cancer cell line to undergo the full metastatic process.

Whether this effect is due to the increased expression of stromal TGF- β , VEGF-A, periostin, or another stromal derived factor remains to be elucidated. However, we did find that we were able to use our CDM model to create extracellular matrices from the patient derived stromal cell lines. Interaction with the extracellular matrix alone altered the normal 3D migratory behavior and promoted an invasive phenotype in the cancer cells. Specifically, we observed sustained cell-matrix adhesions and focal adhesion signaling (Fig. 25). This led to reduced migration and errors in cell proliferation as cells divided and became multinucleated. Considering that the ECM produced by fetal lung fibroblasts and primary tumor stromal cells is different (Fig. 26), an understanding of the specific ECM proteins interacting with cancer cells, as well as other physical characteristics of the CAM CDM are warranted. Targeting these adhesion mechanisms or the downstream signaling cascades following their interaction may provide therapeutic benefits to early stage cancer patients to help limit progression. Additionally, we have not yet tested the ability of these CAM CDM to promote tumorigenic characteristics in non-transformed lung epithelial cells. Are these invasive responses due to sustained signaling downstream of oncogenes such as KRAS or are they independent of driver mutations and a general feature of cell interaction with a cancer associated ECM? Do cancer cells also contribute to the remodeling of the ECM produced by a tumor reactive stroma?

A hint toward the latter question was revealed when we found that PAI-1 is downregulated in cancer cells that interact with a CAM CDM (Fig. 26). PAI-1 prevents pericellular proteolysis and ECM degradation mainly through inhibiting the action of plasmin, and downregulation of PAI-1 may lead to an imbalance of the plasminogen-plasmin system within the tumor and may promote local remodeling. However, we also see that stromal cells within the lung tumor produce PAI-1 in the tumor microenvironment, and when we knocked out PAI-1 in the tumor cells, we still observed an invasive phenotype into the stroma (Fig. 27). Addition of recombinant PAI-1 also promoted migration on ECM, suggesting that extracellular PAI-1 may enhance cancer cell motility regardless of the expression of the cancer cells (Fig. 28). The cell intrinsic and extrinsic effects of PAI-1 may potentiate each other to promote an invasive phenotype. Takashi Yahata et al. found that intracellular, but not extracellular, PAI-1 inhibited the cleavage and activation of the pro-peptide of MT1-MMP by the proprotein convertase furin in murine hematopoietic stem cells (Yahata et al., 2017). A similar mechanism may be occurring in cancer cells. Further molecular characterization of invasion associated genes, as well as understanding of post-transcriptional regulation of PAI-1 on cancer CDM, would provide valuable insight to the PAI-1 paradox in lung cancer. Development of therapeutics to inhibit the extracellular effects of PAI-1 may therefore be worthwhile to investigate. Alternatively, restoring PAI-1 expression in cancer cells may also prevent the invasive phenotype observed. To test this hypothesis would require a transient

CRISPR/Cas9 editing system that would enable stable genetic knockout and plasmid mediated exogenous expression.

This work further enables the clinically relevant study of patient specific tumor features that are sufficient to promote tumor growth and metastasis. Interactions with primary stromal cells from lung tumors, even from patients that clinically do not present with metastasis, are capable of promoting metastasis in a xenograft model. We see that the extracellular matrix produced by these cells is different, and that this matrix can modulate cell behavior and protein expression. On the other hand, an equally important question that remains to be addressed is what factor in the tumor microenvironment of these early stage patients is sufficient to impede the tumor promoting characteristics we see from the cancer associated ECM? How are these factors lost in the course of cancer progression, and could their restoration prevent metastatic dissemination and disease in late stage patients? Targeting factors that are producible in a physiological model may yet lead to greater drug targeting efficacy for future anti-tumor and anti-tumor associated stroma therapy.

CHAPTER VI: SUMMARY AND CONCLUSIONS

The overall goal of this dissertation was to determine how ECM interactions with lung adenocarcinoma cells can promote hallmarks of cancer metastasis in a physiologically relevant *in vitro* model that included a lung fibroblast cell-derived extracellular matrix (CDM) and physiological levels of hypoxia that approximate the amount of oxygen present in the lung microenvironment. This work continued from a study previously performed by Scherzer et al. that demonstrated that lung adenocarcinoma cells cultured on a CDM altered their morphology, proliferation, migration, and survival to serum withdrawal (Scherzer et al., 2015). Additionally, he performed a microarray study which compared the gene expression of two lung adenocarcinoma cell lines, A549 and H358, on two different CDM and a 2D fibronectin coated surface. These data allowed for us to answer more specific questions regarding the regulation of molecular interactions that occur when cells encounter extracellular matrix.

We were able to identify integrin signaling as a key component of extracellular matrix interaction in lung adenocarcinoma cells in promoting migration and EMT. Activation of Src family kinases was required for 3D enabled migration and invasion. Interaction with 3D CDM in a process that appeared to be downstream of integrin binding to fibronectin through an RGD motif switched

the modality of migration and potentiated invasion into Matrigel. This process was also associated with protease activity under the regulation of PAI-1. Inhibition of Rho/ROCK inhibits the activation of MLC and promoted similar invasion of 2D cells as cells on 3D CDM. Together, these data suggest that lung cancer cells on a 3D CDM utilize a mesenchymal mode of migration to facilitate invasion (Fig. 29).

We also utilized a microenvironment mimetic culture system that included the combination of 3D CDM and physiological hypoxia to greatly amplify the isolation and proliferation of cells from enzymatically digested primary human tumor resections. We extensively characterized the cell populations that derived under this condition as a cancer associated mesenchymal stem-like cell, and we found that these cells produced tumorigenic factors in an environment dependent manner unlike their bone marrow derived counterparts. This environment was also critical in maintenance of stem potency gene expression and may be used in the future to isolate rare progenitor cell populations from other tissues.

Finally, we assessed the ability of these primary tumor resected stromal cells to affect lung adenocarcinoma growth and metastasis. These cells stimulated cancer cell proliferation in-vitro and could promote a full metastatic cascade in a xenograft mouse model using a normally non-metastatic human lung adenocarcinoma cell line. We established 3D CDM from these cells and identified that their ECM differs from fetal lung fibroblast CDM in composition and in function of altering the phenotype of cancer cells. Lung adenocarcinoma cells on patient stromal CDM stabilized focal adhesions, displayed slowed migration

and nuclear instability, and demonstrated a highly invasive molecular profile with increased Src activation and decreased intracellular PAI-1. Interestingly, these stromal cells also produce and secrete PAI-1 into the extracellular environment. We found that migration of cancer cells genetically deficient in PAI-1 was increased on 3D CDM following addition of recombinant PAI-1 protein to the media. These findings suggest a novel possible explanation for the PAI-1 paradox, in which cancer associated stroma produces extracellular matrix that results in the downregulation of PAI-1 within the cancer cell, and supports cancer cell migration by secreting PAI-1 into the tumor microenvironment.

In conclusion, we identified molecular mechanisms that promote cancer metastasis in-vitro and in-vivo through extracellular matrix interactions in the tumor microenvironment. Further understanding of these interactions and specific microenvironment related targets may lead to improved therapeutics for lung cancer patients suffering from or at risk of developing metastatic disease.

Strengths

We utilized a physiologically relevant in-vitro model of lung cancer in order to explore molecular targets with possible in-vivo significance. This microenvironment mimetic model provided us with new information about the modality of migration and invasion in lung cancer cells that is triggered upon interaction with a physiological extracellular matrix substrate. In a direct test of this system, we were able to improve the isolation and proliferation of primary cells derived from patients with early stage lung cancer and demonstrated that these cells promote metastasis in an in-vivo model. The techniques utilized in

this dissertation can be widely applied to cancers of other tissues and are highly reproducible.

Limitations

One of the benefits of the 3D CDM system is that it is highly amenable to light and fluorescent microscopy. Data produced from this dissertation suggested that collectively migrating cells are highly diverse and exhibit a “leader and follower” phenotype. However, a majority of the assays used sampled the entire population of cells rather than those at the leading edge of cell migration that may be biologically significant. Further exploration of the localization and phosphorylation of important proteins in integrin signaling and their interactors utilizing techniques such as Fluorescence resonance energy transfer (FRET) microscopy and fluorescence recovery after photobleaching (FRAP) may provide illuminating information in the search for precise molecular targets. Additionally, all lung adenocarcinoma cell lines used in this study harbored KRAS mutations, which only accounts for around 30% of lung adenocarcinoma. Study of cell lines possessing different driver mutations may provide information on whether the increased invasion observed with contact with 3D CDM is a RAS specific effect or general for NSCLC.

A

Figure 29. Model of cell-matrix interactions and signaling pathways that promote migration and invasion on CDM.

Cancer cells grown on 2D substrates differ in their ability to promote migration and invasion compared to cells interacting with 3D CDM. A. Focal adhesion signaling downstream of RGD binding integrin through FAK and Src family kinases is required for 3D cell migration and EMT. Inhibition (i) of Src results in stabilization of E-cadherin, upregulation of protease inhibitors, and decreased invasion, but ERK and AKT activation may be supported by alternative mechanisms. B. Lung cancer cells on 3D CDM downregulate MLCK. Inhibition of RhoA/ROCK signaling phenocopies the invasive phenotype to cancer cells grown on 2D. This suggests contact with 3D CDM may alter the balance of cytoskeletal remodeling to favor actin polymerization over myosin contractility. Collectively these characteristics support the definition of 3D mesenchymal migration.

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CURRICULUM VITAE

NAME: Douglas John Owulaku Saforo

ADDRESS: 2165 Sherwood Ave Apt 2
Louisville, KY 40205
Djsafo01@louisville.edu

EDUCATION: **B.S, Biology with Cellular/Physiology concentration**
(2013), *Summa cum laude*, Biology honors, University of
Louisville
M.D.-Ph.D. Candidate (2013-2021 projected), University of
Louisville School of Medicine

RESEARCH: **Ph.D. Candidate** (2015-present), Department of
Pharmacology and Toxicology, University of Louisville
School of Medicine.

Mentors: Levi J. Beverly, Ph.D. and Leah J. Siskind, Ph.D.

- Significance: Lung cancer is the leading cause of cancer related death in the United States and worldwide. The tumor microenvironment is essential to tumor initiation and development and is altered during aging and in response to injury. However, the role and mechanism by which the extracellular matrix alters cancer development and metastasis is unknown. This proposal seeks to understand the mechanisms by which alterations in the extracellular matrix promote lung cancer development and metastasis using an in-vitro 3D cell culture system and in-vivo animal models for the purpose of identifying novel therapeutic targets.

NIDDK T35 Training Program for Medical Students
(2014), University of Louisville School of Medicine

Mentor: Leah Siskind, Ph.D.

- Significance: This research was focused on studying the effects of currently employed chemotherapy. The major dose limiting toxicity for cisplatin, a common chemotherapy used in a variety of cancers, is kidney injury. We investigated the potential of targeting the metabolism of sphingolipids to protect mouse kidney cells from chemotherapeutic-induced injury. This project employed several techniques concerning lipid metabolism and isolation, molecular biology techniques like overexpression and knockdown of genes using lentiviral delivery of shRNA, qPCR, and multiple proliferation and migration assays. We found that knockdown of neutral ceramidase, an enzyme involved in metabolism of ceramide to sphingosine and upregulated in renal tubule cells, conferred enhanced proliferative and migratory properties to mouse embryonic fibroblasts, but did not enhance migration in baby mouse kidney cells.

NCI R25 Cancer Education Program (2013), University of Louisville School of Medicine

Mentor: J. Christopher States, Ph.D.

- Significance: Upon successful defense of my Honors thesis and completion of my BS, I began to work on the next step of the Anaphase Promoting Complex/Cyclosome (APC/C) project (below) before and during my first year of medical school. This involved cloning cDNA of APC/C subunits into multiple expression vectors to transform bacteria and generate self-cleaving fusion proteins for purification. During this time, in addition to learning these new genetic techniques, I also assisted in teaching a medical student that was rotating in the lab many of the basic cell culture techniques I had learned as an undergraduate.

Independent Research (2013), University of Louisville

Mentor: J. Christopher States, Ph.D.

- Significance: The major goals of this independent research experience were to demonstrate on-target binding of experimental compounds with our molecular target, the APC/C, and to prepare and defend my Undergraduate Honors Thesis.

Undergraduate Research (2012), University of Louisville

Mentor: J. Christopher States, Ph.D.

- Significance: The focus of this independent research was to elucidate the mechanism behind the differential toxicity of our experimental compounds purported to bind to and inhibit the APC/C. The project was expanded into additional cancer cell lines including melanoma and paclitaxel resistant cell lines, and we performed expanded cell cycle analysis through flow cytometry.

NCI R25 Cancer Education Program (2012), University of Louisville

Mentor: J. Christopher States, Ph.D.

- Significance: As an undergraduate, I was selected to participate in the R25 Undergraduate Cancer Education Program and began my training under Dr. J. Christopher States. Based on my fascination with mitosis and the knowledge that mitotic inhibitors were a powerful tool in anti-cancer therapy, I elected to work on a project with Dr. States that investigated a novel class of non-tubulin based mitotic inhibitors. My research focused on targeting a protein complex, the Anaphase Promoting Complex/Cyclosome (APC/C), to disrupt the cell cycle and cause death in ovarian cancer cells while sparing normal tissue. Previously, researchers in Dr. States' lab had performed an in-silico screen of small compounds purported to bind to and inhibit formation of the holoenzyme and my work focused on evaluating several lead candidates from this screen.

At the end of the program, I had evaluated APC/C small molecule inhibitors in multiple ovarian carcinoma and transformed diploid human fibroblast cell lines and produced a poster for my first presentation at a research conference that received first prize in my competition category.

HONORS AND AWARDS:

- AAP/ASCI/APSA Joint Meeting Travel Award (2019).
- Doctoral Basic Science Graduate Student 2nd place award, Research!Louisville, University of Louisville (2018).
- Carl Storm Underrepresented Minority (CSURM) Fellowship, Gordon Research Conference (2017).
- Outstanding Graduate/Professional Student Award (2015-2016), University of Louisville
- Candidacy Fee Award (2016), University of Louisville.

- American Physician Scientists Association Annual Meeting Underrepresented Trainee Travel Award (2016), Chicago, IL.
- Keystone Symposia “Future of Science Fund” Scholarship (2016), Keystone Symposia Scholarship, Breckenridge, CO.
- Woodcock Society Membership (2013), University of Louisville.
- J. Blaine Hudson Scholar (2013), University of Louisville.
- Honors Scholar (2013), University of Louisville.
- *Summa cum laude* with B.S. in Biology with Biology Honors (2013), University of Louisville.
- Best Undergraduate Thesis award finalist for the Natural Sciences Division (2013), University of Louisville.
- Pfizer Undergraduate Student Travel award for Society of Toxicology annual meeting (2013), San Antonio, TX.
One of Five selected recipients in the nation.
- Minority Undergraduate Education Program Travel award for Society of Toxicology annual meeting (2013).
- Research!Louisville Undergraduate First Prize Winner (2012), University of Louisville.
- Dean’s Scholar (Spring 2013, and Spring 2012), University of Louisville.
- Dean’s List (Fall 2012, 2011, and Fall 2010), University of Louisville.
- Dean’s List (Spring 2010, and Fall 2009), College of Charleston.

VOLUNTEER AND ELECTED POSITIONS:

- Cancer Survivor Celebration, Survivor escort, UofL James Graham Brown Cancer Center (2019).
- Meyzeek Middle School Science Fair, Judge in Engineering Category (2019).
- Louisville Regional Science & Engineering Fair, Judge in Biomedical Sciences Junior Category (2019).
- Vice President of the BIG Leadership Team, Big Brothers Big Sisters of Kentuckiana (2019).

- ULSOM Medical Council Graduate Student Representative (2019).
- Louisville Regional Science & Engineering Fair, Judge in Biomedical Sciences Junior Category (2018).
- ULSOM Medical Council Graduate Student Representative (2018).
- BIG Leadership Team, Big Brothers and Sisters of Kentuckiana, Board Member (2017).
- ULSOM Medical Council Medical Student Representative (2017).
- Family Scholar House Job Shadowing Mentor (2017).
- Louisville Regional Science & Engineering Fair, Judge in Biomedical Sciences Category (2017).
- High School Science Fair Student Mentor, Science Policy and Outreach Group, University of Louisville (2016).
- American Physician Scientist Association Undergraduate Mentorship Program, Mentor (2016).
- Preparing the Next Generation of Young Research Scholars: Lincoln Foundation's Whitney Young Scholars Program, Volunteer (2016).
- Cancer Survivor Celebration, Volunteer (2016).
- American Medical Association Doctors Back to School program, Volunteer (2016).

This program sends minority physicians and medical students into communities to introduce children to professional role models.

- Big Brothers and Sisters of Kentuckiana, Youth leader (2016).
- Louisville Regional Science & Engineering Fair Judge (2016).
- Medals4Mettle participant (2014, 2015, 2016, and 2017).

Volunteers represent a cancer patient and race in the Kentucky Derby Marathon or mini marathon. Medals are then donated to the patient in support of their battle with cancer.

- Minority Pre-Med & Pre-Dent Symposium (2016), MD-PhD Student Panelist, University of Louisville School of Medicine.

- High School Science Fair Student Mentor (2015), Science Policy and Outreach Group, University of Louisville.
- Medical School Pre-Orientation Student Panelist (2015), University of Louisville School of Medicine.
- Multicultural Organization of Pre-Med Students (2015), MD-PhD Student Panelist, University of Louisville.
- Convocation of Thanks: Honoring body donors and families for anatomy education (2014), Musical Performer.
- Medical School Advisory College Advocate (2014 – present), University of Louisville
- Medical School Spotlight (2013 and 2014), MD-PhD Student Panelist, University of Louisville.
- Glean Together, Volunteer (2012 – 2016), Kentucky Interfaith Power and Light, Louisville, KY.

This organization collects unsold produce at farmer's markets and donates to local homeless shelters.

- Undergraduate volunteer (2012), Pharmacy Satellite, Baptist Hospital East, Louisville, KY.
- Volunteer Corps (2009), College of Charleston, Charleston, SC.

PRESENTATIONS:

POSTERS:

1. **Douglas J. Saforo**, Brian C. Sils, B. Frazier Taylor, John O. Trent, and J. Christopher States. Candidate Drugs Target the APC/C to Induce Mitotic Arrest in Ovarian Cancer. 2012 Research Louisville, Louisville, KY.
 - *This poster, presented by the candidate, won 1st place in the Undergraduate student category.*
 - *This poster was also presented at the James Graham Brown Cancer Center Annual Retreat in Louisville, KY, and at the Posters-at-the-Capitol meeting in Frankfort, KY.*
2. **Douglas J. Saforo**, B.C. Sils, C.A. France, B.F. Taylor, J. O. Trent, J. C. States. Compounds Targeting Anaphase Promoting Complex Disrupt Mitosis and Induce Apoptosis in Cancer Cells. 2013 Society

- of Toxicology Annual Meeting, San Antonio, TX.
- *This abstract was selected for the SOT Pfizer Undergraduate Travel Award and the Minority Undergraduate Education Program Travel Award.*
 - *This poster was also presented at the Midwest Regional American Physician Scientist Association Meeting at Ohio State University in Columbus, OH.*
3. **Douglas Saforo**, Kumaran Sundaram, Cameron Conway, Gauri Patwardhan, and Leah J. Siskind. Sphingolipids as potential targets for the treatment of acute kidney injury. 2015 Southeastern Medical Scientist Symposium, Vanderbilt University, Nashville, TN.
 - *This poster was also presented at Research!Louisville (2014), in Louisville, KY.*
 4. **Douglas J. Saforo**, Michael Scherzer, Levi J. Beverly, and Leah J. Siskind. In-vitro 3-Dimensional modeling of the tumor microenvironment in non-small cell lung carcinoma. 2015 Research Louisville, Louisville, KY.
 - *This abstract was selected for a scholarship to the Keystone Symposia Conference on Cancer Pathophysiology: Integrating the Host and Tumor Microenvironment in Breckenridge, CO, and an Underrepresented Trainee Travel Award to the American Physician Scientists Association Annual Meeting in Chicago, IL.*
 5. **Douglas J Saforo**, Parag P. Shah, Levi J. Beverly, and Leah J. Siskind. In Vitro 3-Dimensional Modeling of Tumor Microenvironment Reveals Host/Tumor Influence On Progression In Non-Small Cell Lung Carcinoma. 2017 Gordon Research Conference on Fibronectin, Integrins, and related molecules. Ventura, CA.
 - *This abstract was selected for a travel award to the 2017 Gordon Research Conference and Seminar.*
 6. **Saforo D**, Shah P, Siskind L, and Beverly L. (2017) In Vitro 3-Dimensional Modeling of Tumor Microenvironment In Non-Small Cell Lung carcinoma: Hypoxia and Cell derived Matrices. Salk Mechanisms and Models of Cancer Meeting. August 1-4. La Jolla, CA
 7. **Douglas J Saforo**, Levi J. Beverly, and Leah J. Siskind. Tumor microenvironment mimetic culture aids isolation, expansion, and potency of tumor stromal progenitors from primary lung cancer resections. Abstract for poster presentation: 2018 Research Louisville, Louisville, KY.

- *This abstract was awarded 2nd place in the Doctoral Basic Science Graduate Student category.*

PUBLICATIONS:

1. Dupre TV, Doll MA, Shah PP, Sharp CN, Kiefer A, Scherzer MT, Saurabh K, **Saforo D**, Siow D, Casson L, Arteel GE, Jenson AB, Megyesi J, Schnellmann RG, Beverly LJ, Siskind LJ. Suramin protects from cisplatin-induced acute kidney injury. Am J Physiol Renal Physiol. (2016).
2. **Douglas Saforo**, Linda Omer, Andrei Smolenkov, Aditya Barve, Lavona Casson, Noland Boyd, Geoffrey Clark, Leah J. Siskind, and Levi J. Beverly. Primary lung cancer samples cultured under microenvironment-mimetic conditions enrich for mesenchymal stem-like cells that promote metastasis. Scientific Reports. (2019).
3. Shanice V. Hudson, Hunter Miller, Grace Mahlbacher, **Douglas Saforo**, Levi J. Beverly, Gavin E. Arteel, Hermann B. Frieboes. Computational/experimental evaluation of liver metastasis post hepatic injury: interactions with macrophages and transitional ECM. Under revision.
4. **Douglas Saforo**, Parag Shah, Austin Krueger, Geoffrey Clark, Leah Siskind, and Levi Beverly. Integrin signaling through cell matrix adhesions promote mesenchymal migration and collective invasion of lung carcinoma cells on cell-derived matrix. "In preparation."