"DECELLULARIZED NORMAL AND CANCER SUSCEPTIBLE MICE COLONS TO STUDY THE CONTRIBUTION OF THE EXTRACELLULAR MATRIX TO CELL BEHAVIOR AND COLON CANCER PROGRESSION"

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DEDICATION

I would like to dedicate this body of work to my family. To my loving mum and dad, my sisters Biola and Kenny and the best (and only brother), Tman, for their love, support and prayers. The love, support and advice I got from my family throughout my graduate school career has helped guide me mentally, emotionally and physically through every stage of my PhD program.

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To God be ALL the Glory.

DECELLULARIZED NORMAL AND CANCER SUSCEPTIBLE MICE COLONS TO STUDY THE CONTRIBUTION OF THE EXTRACELLULAR MATRIX TO CELL BEHAVIOR AND COLON CANCER PROGRESSION

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DECELLULARIZED MICE COLONS AS MODELS TO STUDY THE CONTRIBUTION OF THE EXTRACELLULAR MATRIX TO CELL BEHAVIOR AND COLON CANCER PROGRESSION

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ABSTRACT

Current 3D culture models to study colorectal cancer lack architectural support and signaling proteins provided by the tissue extracellular matrix (ECM) which may influence cell behavior and cancer progression. Therefore, the ability to study cancer cells in the context of a matrix that is physiologically more relevant and to understand how the ECM affects cancer progression has been understudied. To address this, I developed an *ex-vivo* 3D system, provided by intact wild type (WT) and colon cancer susceptible decellularized mouse colons (DMC), to support the growth of human cancer cells. DMC are free of viable cells but still contain extracellular matrix proteins including subsets of collagens. Stiffness, an important mechanical property, is also maintained in DMCs. Importantly, I observed that the DMC is permissive for cell proliferation and differentiation of a human colon cancer cell line (HT-29). Notably, the ability of cells in the WT DMC to differentiate was also greater when compared to Matrigel ^{TM}, an extracellular matrix extract from a mouse tumor cell line. Additionally, I observed using invasion assays that DMC obtained from polyps from a colon cancer susceptible mouse model facilitated increased cell migration/invasion of colorectal cancer cells and immortalized non-tumor colonic epithelial cells compared to DMC from WT mice. Finally, using mass spectrometry, I identified extracellular matrix proteins that are more abundant in DMC from a colorectal cancer mouse model compared to age and sex-matched WT mice. I propose that these abundantly expressed proteins in the tumor microenvironment are potentially involved in colorectal cancer progression.

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PRIOR PUBLICATIONS

2019 **Alabi, B. R**., LaRanger, R., & Shay, J. W. (2019). Decellularized mice colons as models to study the contribution of the extracellular matrix to cell behavior and colon cancer progression. *Acta Biomaterialia*, *100*, 213-222.

2018 LaRanger, R., Peters-Hall, J. R., Coquelin, M., **Alabi, B. R**., Chen, C. T., Wright, W. E., & Shay, J. W. (2018). Reconstituting mouse lungs with conditionally reprogrammed human bronchial epithelial cells. *Tissue Engineering Part A*, *24*(7-8), 559-568.

2018 Peters-Hall, J.R., Coquelin, M.L., Torres, K.J., LaRanger, **R., Alabi, B.R**., Sho, S., Calva-Moreno, J.F., Thomas, P.J. and Shay, J.W. Long-term culture and cloning of primary human bronchial basal cells that maintain multipotent differentiation capacity and CFTR channel function. *Am J. Physiol. Lung Cell Mol Physiol.* 315: May 3, 2018 doi:10.1152/ajplung.00355.2017.

2014 London, A. S., Mackay, K., Lihon, M., He, Y., & **Alabi, B. R**. (2014). Gel filtration chromatography as a method for removing bacterial endotoxin from antibody preparations. *Biotechnology progress*, *30*(6), 1497-1501.

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2D	Two Dimensional
3D	Three Dimensional
5X PBSAA	5X Phosphate Buffered Saline containing 5% antibiotics and antimycotics
5-HT	5-Hydroxytryptamine
AHR	Aryl Hydrocarbon Receptor
ALI	air-liquid interface
APC	adenomatous polyposis coli
BSD	Bioreactor sodium deoxycholate
BST	Bioreactor sodium dodecyl sulfate Triton X-100
CCMM	Colon Cancer Mice Model
CDK4	cyclin dependent kinase 4
CFTR	cystic fibrosis transmembrane receptor
DAPI	4’,6-diamidino-2-phenylindole
ddPCR	droplet digital PCR
DMA	dynamic mechanical analysis

DMC	Decellularized mice colon
DT	decellularized tissue
DW	deionized water
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EHS	Engelbreth Holm Swarm sarcoma
FAK	focal adhesion kinase
FDR	false-discovery rate
FGF	fibroblast growth factors
GLP-1	glucagon-like peptide 1
GLP-2	glucagon-like peptide 2
GMP	Good manufacturing practice
HBEC	human bronchial epithelial cell
HCEC	Human Colonic Epithelial Cell
H&E	Hematoxylin and Eosin
HSP90	heat shock protein 90

hTERT	human telomerase reverse transcriptase
INSL5	insulin-like peptide 5
LC-MS/MS	liquid chromatography with tandem mass spectrometry
LGR5	Leucine rich repeat containing G-protein-coupled receptor 5
LTBP	latent transforming growth factor beta-binding proteins
MAb	monoclonal antibody
MMP	matrix metalloproteinase
MSCs	mesenchymal stem cells
Myc	avian myelocytomatosis viral oncogene homolog
NTS	neurotensin
PEG	polyethylene glycol
PSD	Petri-Dish Sodium Deoxycholate
PBS	Phosphate buffered saline
PYY	peptide YY
RB1	retinoblastoma protein
RGD	Arg-Gly-Asp

SD	sodium deoxycholate
SDS	Sodium dodecyl sulfate
TASIN-1	truncated APC-selective inhibitor 1
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioixn
TEER	transepithelial/transendothelial electrical resistance
TNXB	Tenascin-X
UTS	ultimate tensile strength
VEGF-A	vascular endothelial growth factor A
WTM	Wild type mice
XAP2	X-associated protein 2
YAP	Yes associated protein

CHAPTER 1

Introduction

There is compelling evidence that monolayer or two-dimensional (2D) *in vitro* culture system, the most widely used system for studying cell behavior and for drug testing, significantly contribute to the low success rate (10%) of compounds progressing successfully through clinical development. (Edmondson, Broglie, Adcock, & Yang, 2014; Langhans, 2018). This is because cells are being studied in an environment that is not similar to their i*n vivo* conditions (Edmondson, Broglie et al. 2014, Langhans 2018, Kapalczynska, Kolenda et al. 2018). Cells in 2D environments lose their phenotypic diversity and morphology (Kapalczynska et al., 2018). Because the cells are grown in monolayer on plastic culture dishes, they also lose their polarity (Kapalczynska et al., 2018). These losses have downstream consequences and effects. They cause changes in cell secretion, cell signaling, cell function and cellular response to internal and external stimuli (Kapalczynska et al., 2018). 2D culture systems have also been noted to cause changes in gene expression and cell biochemistry (Kapalczynska et al., 2018).

3D culture systems, which allow cells to be grown in environments that are more physiologically relevant, are widely being developed and adapted to study cell behavior. In fact, cell responses to drug treatments *in vivo* have been found to be more similar to those of cells grown in 3D versus cells grown in 2D (Edmondson et al., 2014). The environment that the cells are grown in is specific to an organ and is complex biochemically and mechanically (Urciuolo & De Coppi, 2018). The microenvironment contains a number of extracellular matrix (ECM) proteins, such as

collagen which confer tissue specific tensile properties. Tissue decellularization is a process used to harness the desirable attributes of the ECM for drug screening, cell studies, transplantation or tissue engineering purposes by removing native cells of the tissue, However, all these desirable attributes of decellularized tissues can be affected by the decellularization protocol or decellularizing agents used (Fernandez-Perez & Ahearne, 2019).

To this end, as part of my dissertation research, I have optimized and developed a perfusion bioreactor-based protocol to decellularize colonic tissues from normal and colon-cancer susceptible mouse models. I show by DNA quantitation that the decellularization protocol results in a decellularized tissue that has lost all its viable cells and more than 95% of its endogenous cellular DNA. I also show by immunohistochemistry, immunofluorescence staining and dynamic mechanical analysis testing that important tissue architecture, extracellular matrix proteins and biomechanical properties, seen in native mice colonic tissues, still persist in the decellularized mice colon (DMC). Further, I show that this system supports the growth and differentiation of mouse and human colorectal cancer cells. Additionally, I observed that DMC from a colon cancer mouse model (CCMM) facilitated greater cell invasion of colorectal cancer cells and normal colonic epithelial cells compared to DMC from wild type mice (WTM). Using mass spectrometry, I identified and characterized several extracellular matrix proteins, including ECM protein1, that are more abundant in DMC from a CCMM compared to age and sex matched WTM. Production of biological scaffolds such as the DMC that have the vast majority of endogenous DNA removed (with no viable cells remaining), are conducive to reintroduced cells, have preserved mechanical properties, and provide a biologically relevant environment for studying and understanding cancer cell behavior when reintroduced into a decellularized mouse colon. My studies using the DMC as a tool to identify potential oncologic targets for colon

cancer suggest additional utility of the DMC as a drug discovery tool. Finally, since viable endogenous cells that can cause immunogenic responses are effectively removed, the DMC may also be a promising tool for tissue regenerative practices including implantation.

In this introduction to my already published and unpublished studies, I will discuss 2D and three dimensional (3D) culture systems, their usefulness and limitations. Then, I will focus in on 3D decellularized culture systems, how they are made and their desirable biomechanical and chemical properties for cell studies and drug discovery. I will also discuss the need to identify novel and targeted therapies for colorectal cancer and why microenvironment niche proteins, such as those I identified in my studies, may provide a source for these types of targeted therapies. I will conclude my introduction with the application of decellularized and reconstituted biological scaffolds.

2D and 3D Culture Systems; Utilities and Limitations

Cell culture systems are important and robust tools for understanding cell behavior, tissue morphology, mechanisms of drug action and processes involved in disease (Kapalczynska et al., 2018). They are also utilized for biologics production via the suspension culture of mammalian cells such as the Chinese hamster ovary cells and human embryonic kidney cells (London, Mackay, Lihon, He, & Alabi, 2014). Further, cell systems are now used for development of tissue engineering and for compound screening to identify targeted therapies (Kapalczynska et al., 2018). There are two main types of cell culture systems; 2D and 3D. 2D culture systems are versatile, reasonably less expensive, easy to work with and are generally reproducible. Further, they have provided the scientific community with enormous clinical breakthroughs since they

were initially developed by Harison, in 1907 to study the origin of nerve fibers (Kapalczynska et al., 2018; Langhans, 2018). In this 2D method, cells are obtained from living organisms and established in primary culture from tissues. Once established in cell culture, early passage or established cell lines can be obtained commercially from bioresource centers such as the American Type Culture Collection (Kapalczynska et al., 2018). These cells are typically grown as adherent monolayers on rigid glass or specific prepared tissue culture plastic dishes or as suspension cells (Kapalczynska et al., 2018) (**Figure 1.1**) All cell types from a tissue biopsy are not present when established in cell culture.

However, by definition, the tissues and organs of our body consists of various cell types interacting and influencing each other, for the proper function of that specific tissue and the overall function of the body. For example, in the large intestine or colon there are a number of different cell types with a variety of functions including serving as a barrier between the colon lumen and the internal environment of the body and absorbing salt and water (Colony, 1996; de Santa Barbara, van den Brink, & Roberts, 2003). Specifically, the goblet cells, one of the earliest differentiated cell types of the colon, secrete mucin for lubrication and provide barrier protection from intestinal microbes (Colony, 1996). There are also intestinal stem cells that can differentiate into other cell types of the colon. There are also the absorptive cells that have numerous microvilli which make up the colonic brush border that increases surface area to help absorb water as waste passes along the colon. This is achieved by the establishment of a sodium transmucosal gradient; that allows water to be transported across the mucosa to the serosa permitting electrolytes, such as salt, to be actively transported (Colony, 1996). There are also enteroendocrine cells. These are a number of hormone producing cells located along the gut that bring about the response of the body to food intake (Gribble & Reimann, 2016). Some of the

enteroendocrine cells in the colon and rectum include enterochromaffin cells which secrete 5-hydroxy-tryptamine (5-HT); N-cells which secrete neurotensin (NTS); L-cells which secretes glucagon-like peptide 1, glucagon-like peptide 2, a gradient of peptide YY and insulin-like peptide 5 (GLP-1, GLP2, PYY, INSL5).

To remedy the isolated growth of a single cell type, sometimes cells have been grown in co-culture with other cells such as fibroblasts, which are called the feeder layer (**Figure 1.2)** (Goers, Freemont, & Polizzi, 2014; Kretzschmar & Clevers, 2016; Prasad, Hogaboam, & Jarai, 2014). These co-culture systems have been used to demonstrate the repair ability of fibroblasts in response to epithelial cell injury (Prasad et al., 2014). Some of these fibroblasts also secrete extracellular matrix proteins that can support stem cell growth or promote longevity of primary cells (Kretzschmar & Clevers, 2016; J. R. Peters-Hall et al., 2018). Despite some areas of progress in 2D culture technology, the full *in-vivo* phenotypic make up of cells in organs is not recapitulated. Further, cells are not present on rigid or plastic cell surfaces *in vivo.* Instead, cells in the body lie on top of biologically active extracellular matrices.

The compositions and mechanical properties of the extracellular matrix differ from organ to organ. Regulation of these properties involve the ECM proteins interact with cellular integrins to exert their influence on cell behavior. Cells can exert force on the ECM to change their surrounding environment. The ECM can in turn exert force on cells and modify cell behavior. This allows for an ongoing dynamic relationship between the cells and their environment in the body that is often referred to as dynamic reciprocity (Thorne et al., 2015; R. Xu, Boudreau, & Bissell, 2009).

The drive to mimic these *in-vivo* conditions is what brought about the development and adaptation of the 3D culture models. One of the first 3D cultures was done by Hamburg and

Salmon when they cultured cells in soft agar (Kapalczynska et al., 2018). 3D culture models can be broadly categorized into 2 groups based on the availability or unavailability of scaffolds for cells to attach onto during culture. These groups are termed the scaffold based and the non-scaffold based categories (Edmondson et al., 2014). In some studies, the scaffold based 3D cultures are further broken down into cultures on (natural or synthetic) scaffolds and cultures in gel-like substances (Kapalczynska et al., 2018). In the scaffold free system, cells are grown as suspension or floating cells in shaker flasks or by agitation methods (**Figure 1.3**) (Edmondson et al., 2014). Cells are also grown as spheroids in hanging drop plates and have been used to culture kidney tissues (Foty, 2011; S. Wang et al., 2017).

For the scaffold-based cell type culture systems where cells are grown as spheroids within or on gel-like substances, Matrigel ^{TM} is usually the gel of choice (**Figure 1.4**) (Edmondson et al., 2014). Matrigel is derived from the basement membrane of Engelbreth Holm Swarm sarcoma (EHS). It is rich in laminin, growth factors, nitrogen, type IV collagen and perlecan (Kleinman, 2001). By supplementing cell culture media with niche growth factors such as noggin, scientists have been able to grow spheroids from different cellular or tissue origins into organoids (Kretzschmar & Clevers, 2016). Cell sources for the growth of organoids have come from human and mouse epithelial cells, minced tissues and pluripotent stem cells (Kretzschmar & Clevers, 2016). The tissue origins to establish epithelial organoid cultures have varied from gastrointestinal sources such as the small intestine, large intestine, gall bladder, liver, pancreas and stomach and non-gastrointestinal areas such as prostate, mammary gland, lung and esophagus (Kretzschmar & Clevers, 2016). Understanding of the niche cells and factors has been instrumental to advancing this field. For example, the knowledge that Lgr5 (Leucine rich repeat containing G-protein-coupled receptor 5) as an intestinal stem cell marker drove proponents of

this culture system to isolate these specific cells for organoid culture (Kretzschmar & Clevers, 2016). Further, the understanding of Wnt signaling in stem cell maintenance helped with identifying the appropriate niche factors to drive stem cell renewal, proliferation and differentiation, morphogenesis, and in the case of Lgr5 cultures, self-organize into crypt-villus structures (Gjorevski et al., 2016; Kretzschmar & Clevers, 2016). However, despite the utility and advances that the 3D culture of cells in gel-like matrices has allowed for including modeling tissue environment of stem cells, tissue plasticity and morphogenesis, there are still some limits to this 3D scaffold culture system (Kretzschmar & Clevers, 2016). Firstly, environmental signaling of EHS mice is not well defined and currently there are no techniques that allow for expansion of the organoid cultures in a GMP-compliant manner (Giobbe et al., 2019). Further, expensive growth factors need to be added to the culture media to maintain the intestinal stem cells or to permit them to differentiate (Urbischek et al., 2019). Also, there is usually batch to batch variation in Matrigel which may make it difficult to compare results (Urbischek et al., 2019). Additionally, it is difficult to culture these cells long-term because of the expansion of necrotic cores (Sood et al., 2019). Further, mechanical or biochemical properties of Matrigel are not tunable and may pose risk of immunogen and pathogen transfer thereby stunting the translation of this organotypic model for clinical uses (Gjorevski et al., 2016). All these limitations have made the other scaffold culture types, synthetic and natural scaffolds, desirable.

Synthetic scaffolds can be made by 3D printing or from polymers (**Figure 1.5**) (H. Chen et al., 2019). They are tunable to the biochemical and biomechanical properties specified by the researchers. By modulating synthetic scaffolds, produced by enzymatically crosslinking polyethylene glycol (PEG) polymers, researchers were able to show that the RGD (Arg-Gly-Asp) peptide was the minimal component essential for intestinal stem cell preservation and

growth on this polymer (Gjorevski et al., 2016). By varying stiffness of the PEG gels, it was also shown that on softer matrices cells tend to follow a more differentiated path while cells on stiffer matrices are more proliferative (Gjorevski et al., 2016). They also showed that stiffer matrices allowed for Yes associated protein (YAP) translocation to the nucleus and its activation which in turn allowed for intestinal stem cell activation (Gjorevski et al., 2016). YAP is a transcriptional co-activator that controls cell fate by translocating to the nucleus to activate genes involved in cell proliferation while suppressing genes involved in apoptosis. Softer PEG gels allowed for cell differentiation. However, when the gels became too soft, they became detrimental to the cells resulting in a decrease in cell viability. Despite the utility of synthetic scaffolds, there are still some important limitations. One of those limitations is that it is difficult to define all these properties in a spatio-temporal manner as is observed in ECM obtained from specific organs. Therefore, the appeal of a type of scaffold cell culture systems provided by decellularized native system increases.

3D Decellularized Culture System; Production and Properties

Decellularization is the process of separating the extracellular matrix from the inhabiting cells of an organ. Cell removal prevents immunogenic responses if the decellularized tissue is implanted. There are a number of methods and reagents that can be used to decellularize tissues. These methods and the techniques used to administer them must be carefully optimized to ensure effective removal of cellular components while minimizing alteration and composition of native extracellular matrix (Crapo, Gilbert, & Badylak, 2011; Fernandez-Perez & Ahearne, 2019). Methods or agents used in decellularizing of tissues can be physical, chemical, biological, or a

combination of these methods (Crapo et al., 2011). All these methods have their pros and cons that may allow them to be suitable for one specific tissue versus another.

Physical methods include direct application of force, pressure and freeze-thaw methods to allow force to burst cells or ice to disrupt cell membranes, respectively (Crapo et al., 2011). Unsurprisingly, the force, pressure or ice used to disrupt cells from the tissue may also affect the extracellular matrix architecture (Crapo et al., 2011). Biologic agents include enzymes such as nucleases, trypsin and chelating agents such as EDTA. They work by degrading DNA and RNA either by catalyzing hydrolysis of DNA and RNA or cleaving specific peptides. (Crapo et al., 2011). Some of these biologic agents may not be effective when used alone, may provoke immune responses and can disrupt ECM ultrastructure with prolonged presence in ECM (Crapo et al., 2011). Chemical agents include acids such as acetic and peracetic acids, and bases such as sodium hydroxide. They also include hypotonic and hypertonic solutions which rely on the osmotic differences between the solution and intracellular cell environment to aid with dissociating DNA from proteins or aid with cell lysis (Crapo et al., 2011). Others include ionic detergents such as sodium deoxycholate, non-ionic detergents like triton x-100 and zwitterionic detergents like CHAPS (Crapo et al., 2011). Zwitterionic detergents exhibit properties of both non-ionic and ionic detergents and usually effectively remove cells with mild disruption of ultrastructure in thin tissues like lungs (Crapo et al., 2011). They may, however, not be suited for denser or thicker tissues even when used in combination with stronger detergents (Crapo et al., 2011). The final types of chemical agents that have been used are the solvents such as alcohols and acetones which lyse cells by dehydration. However, they may also precipitate important extracellular matrix proteins such as collagens. (Crapo et al., 2011). Essentially, all these chemical agents have cell lysis abilities, some better than others. However, they can also disrupt

the extracellular matrix proteins, tissue architecture and structure depending on how much they are used or how long the extracellular matrix is exposed to them (Crapo et al., 2011). To apply the decellularization agents to tissue, whole organ perfusion through the tissue vasculature, pressure gradients induced across the tissue and whole immersion and agitation of tissue can be used (Crapo et al., 2011).

The extracellular matrix provided post decellularization, is the non-cellular residual tissue component that originated in cells during tissue development in response to biochemical and biophysical signals between them and the constantly remodeling protein-rich environment. (Frantz, Stewart, & Weaver, 2010). ECM of a tissue is often thought of as an inert scaffold for cell attachment. However, its functions extend beyond this. It is made up of an heterogenous mix of structural proteins, water and polysaccharides (Frantz et al., 2010). The proteins are mainly fibrous and include collagens, elastin, fibronectin and laminins (Frantz et al., 2010). Collagens are the most abundant fibrous proteins and make up 30% of the total protein constitution of a multicellular animal (Frantz et al., 2010). They mainly associate with elastin that provides recoil properties to tissues. Fibronectin, another ECM protein is important for cell attachment. In contrast, the polysaccharides are mainly proteoglycans which are present in the interstitial space in the form of hydrated gels (Frantz et al., 2010). They are useful for conferring tissue specific hydration, binding and force resistant properties (Frantz et al., 2010). All these components are important for cell signaling.

Cells adhere to the ECM using cell surface receptors such as integrins, syndecans and discodin domain receptors (Frantz et al., 2010). The ECM, in turn, interacts with these cell surface receptors and binds growth factors to direct morphological organization and physiological function to regulate cell behavior such as proliferation and differentiation by regulating genes

like YAP/TAZ (Frantz et al., 2010; Gjorevski et al., 2016). When the ECM binds integrins, integrins cluster and initiate the assembly of adapter molecules such as focal adhesion kinase (FAK), tensin and talin to form focal adhesions (FAs) between a cell and the ECM (**Figure 1.6**) (Handorf, Zhou et al. 2015). These focal adhesions act as mechanosensors to direct cells to tissue areas with greater stiffness. (Handorf, Zhou, Halanski, & Li, 2015). Stiffness is the extent to which the tissue resists deformation from an applied force (Handorf et al., 2015). For example, softer tissues like the liver, brain and fat require less force to be stretched or are more easily deformed while the bone needs more force because it a stiffer tissue. (Hinz, 2009). Stiffer matrices tend to have increased FAs and more traction force generated between FAs and ECM (Handorf et al., 2015). The stiff matrix in bone, causes differentiation of mesenchymal stem cells (MSCs) by the activation of ERK1/2 (Handorf, Zhou et al. 2015). The ECM is constantly being remodeled as cells synthesize, breakdown and rearrange its molecular components (Handorf et al., 2015). Also, ECM molecular components can be post translationally modified (Frantz et al., 2010). The Rho/ROCK signaling pathway are another family of mechanosensors and have roles in actin cytoskeleton contraction, cell cycle and myofibroblast differentiation (**Figure 1.7**) (Handorf, Zhou et al. 2015). All of these contribute to the biochemical and biomechanical properties of the tissue including tissue stiffness and elasticity. Stiffness also helps to maintain tissue boundaries and drives cell division and differentiation (Handorf et al., 2015).

Young's modulus is a measure of tissue stiffness. It provides quantitative information of the strain of a material in response to deformation or a force. Materials or tissues, like skeletal muscle and heart muscle, with high Young's modulus need more force to be strained/stretched (Hinz, 2009). The methods used to measure Young's modulus specifically, can be broadly divided into two; dynamic or quasi-static (Farsi et al., 2017). Dynamic methods include

ultrasonic, prism resonance, and impulse excitation while quasi-static methods include micro- and nano-indentations, direct compression and tension tests (Farsi et al., 2017). During dynamic method excitation, the tissue is exposed to cyclic or transient loads and its dynamic response is measured (Farsi et al., 2017). This information is used in conjunction with knowledge of the density and geometry of the tissue. In contrast, in quasi-static methods, continuous or constant loads are applied at a low rate, so inertial effects do not have to be accounted for (Farsi et al., 2017). In contrast, quasi-static method probing require simpler machinery and specimen preparation and are considered more representative of conditions when the specimen is being used and so they are generally preferred (Farsi et al., 2017). Tissue mechanics parameters including Young's modulus can be measured by compression, suction or tension methods using instruments such as the Instron and dynamic mechanical analysis (DMA) (Iivarinen, Korhonen, & Jurvelin, 2014). During DMA testing, stress controlled analyzers or strain controlled analyzers are used to apply an oscillatory force or strain, respectively, to the tissue and the tissue response is recorded (Menard, 2015). The slope of a straight-line portion of the stress-rupture curve of stress versus strain is the Young's modulus.

Targeted therapies for colon cancer

Drugs for treating cancer can be broadly classified into two major groups; the cytotoxic chemotherapies and molecular targeted drugs (Aggarwal, 2010). The cytotoxic chemotherapies are designed to kill rapidly dividing cells which include cancer cells (Gerber, 2008). However, they also kill rapidly dividing cells in normal tissues leading to alopecia, gastrointestinal issues and bone marrow suppression (Gerber, 2008). In contrast, targeted therapies are engineered to

regulate or block production of specific proteins mutated or overexpressed specifically by the cancer cells that are needed for their sustained growth and development (Aggarwal, 2010; Gerber, 2008). There are two main types of targeted therapies including monoclonal antibody (MAb) and small molecule inhibitors (Aggarwal, 2010; Gerber, 2008). MAb are more specific, larger in size (~150,000 Da), can be bioengineered, given intravenously, and bind ligands and receptor binding domains. The molecular targeted inhibitors are smaller (~500Da), chemically manufactured, administered orally or intravenously and can enter cells and block receptor signaling (Gerber, 2008). Some of the advantages of targeted therapies include that they are usually less toxic and better tolerated compared to standard chemotherapy. In many instances they extend overall lifespan of patients with certain cancer types and may be the only type of therapy available for certain categories of patients, such as the elderly, that often cannot be treated with chemotherapies (Gerber, 2008).

Colorectal cancer is one of the top 3 most prevalent types of cancers in male and female population, worldwide (Siegel et al., 2019). There were almost 2 million new cases of the disease in 2018, globally (Siegel et al., 2019). Despite the declining rates of the disease incidence in older adults, new data shows that the diseased rate is increasing among young adults in some high income countries including the United States (Siegel et al., 2019). There are currently no targeted small molecule therapies to treat colon cancer or prevent its progression (W. Wang et al., 2019). Further, the monoclonal antibodies that are available to treat this disease are few and mainly treat the progressed form of the disease or are used in conjunction with other therapies to enhance efficacy (Aggarwal, 2010; Ginsburg & Willard, 2013). One of these therapies include anti-angiogenin antibodies such as the vascular endothelial growth factor A (VEGF-A) targeting Bevacizumab (Aggarwal, 2010; Fakih, 2015). Two other targeted therapy which specifically

work on the receptor called epidermal growth factor receptor (EGFR) are cetuximab and panitumumab (Aggarwal, 2010; Fakih, 2015). However, there are still no targeted therapies for HER2 amplification, activating KRAS mutations or BRAF mutations which frequently occur in colorectal cancer patients (Fakih, 2015). In fact, some of the available targeted therapies may be detrimental to patients with these other genetic mutations (Fakih, 2015). Understanding the genetic alterations of cancer cells and their roles in cancer progression have made it possible to propose and develop targeted therapies to treat colon cancer. A recent example of this comes from work done in the Shay lab. Researchers in the lab used their knowledge of the formation of truncated APC products, a commonly overlooked results of genetic alterations of the APC gene in some human colorectal cancer cells, to screen for and identify a small compound, truncated APC-selective inhibitor 1 (TASIN-1). (Cully, 2016; Zhang et al., 2016). Like the name suggests, TASIN-1, selectively targets colon cancer cells that make truncated products of APC (Zhang et al., 2016). The truncated APC proteins have oncogenic function including migration through Matrigel, growth on soft agar and increase in proliferation (Cully, 2016). TASIN-I is inhibitory to these effects. Further, TASIN-1 reduced the growth of colorectal cancer cells with truncated APC that has been injected into xenograft mice (Zhang et al., 2016). Impressively, it also shrank new and existing tumors in a truncated-APC genetically engineered mice model also with truncated APC (Zhang et al., 2016). As expected, it was not effective in shrinking tumors of genetically engineered mice with wild-type APC (Zhang et al., 2016). This inhibitor could have large implications for colon cancer patients because APC mutations occur in 80% of colorectal cancer patients and 90% of those mutations result in truncated APC products (Cully, 2016). Therefore, close to 3/ 4 of patients with colon cancer may potentially benefit from this small

molecule inhibitor. It has been licensed to Barricade therapeutics for development (W. Wang et al., 2019).

Since the ECM is also remodeled during cancer progression, a more detailed understanding of how the extracellular matrix changes during tumor progression may provide the next generation of targeted therapies against colon cancer (Halim, Markert, & Vazquez, 2018). For example, tumor stromal cells exhibit some of the characteristics found in an unresolved wound and are characteristically stiffer than the surrounding normal tissue. The stiffening of tumors is induced by ECM deposition, remodeling by resident fibroblasts, and by increased contractility of the transformed epithelium (Frantz et al., 2010). The CPC;Apc is an excellent colon cancer mice model to study the changes in ECM and what proteins may be contributing more to cancer progression. This is because these mice form tumors in their colons and rectums that have been found to be morphologically and molecularly similar to human tumors (Kim et al., 2016). In these transgenic mice, tumor suppressor *Apc* is somatically inactivated by Cre recombinase conditionally (Kim et al., 2016). The CDX2 homeobox gene promoter drives expression of this mutated form of *Apc* in epithelial cells of the colon leading to the formation of adenomas and carcinomas. Decellularization and removal of cellular contaminants from CPC;Apc mice colonic tissue can allow direct and specific dissection of the tumor ECM to obtain proteomics information that is specific for the tumor or adjacent normal tissue microenvironment.

Applications of Tissue Engineered Biological Scaffolds

So far there have been a number of practical applications using 3D decellularized tissues. First, decellularized tissue can be processed into hydrogels (Fernandez-Perez & Ahearne, 2019). These

hydrogels have been tested for delivery of human liver and mesenchymal cells made to differentiate into insulin producing cells that are glucose regulated for treating type 1 diabetes (Chaimov et al., 2017; Fernandez-Perez & Ahearne, 2019). This is achieved by transdifferentiating adult human liver cells into insulin -producing cells by ectopically expressing pancreatic transcription factors such as PDX1 and NeuroD1 (Meivar-Levy & Ferber, 2003). Mesenchymal cells, on the other hand, are transduced with the pancreatic transcription factor (Chaimov et al., 2017). They have also been tested for replacing damaged cardiac tissue caused by myocardial infarction as well as treating skin wounds (Chaimov et al., 2017; Fernandez-Perez & Ahearne, 2019).

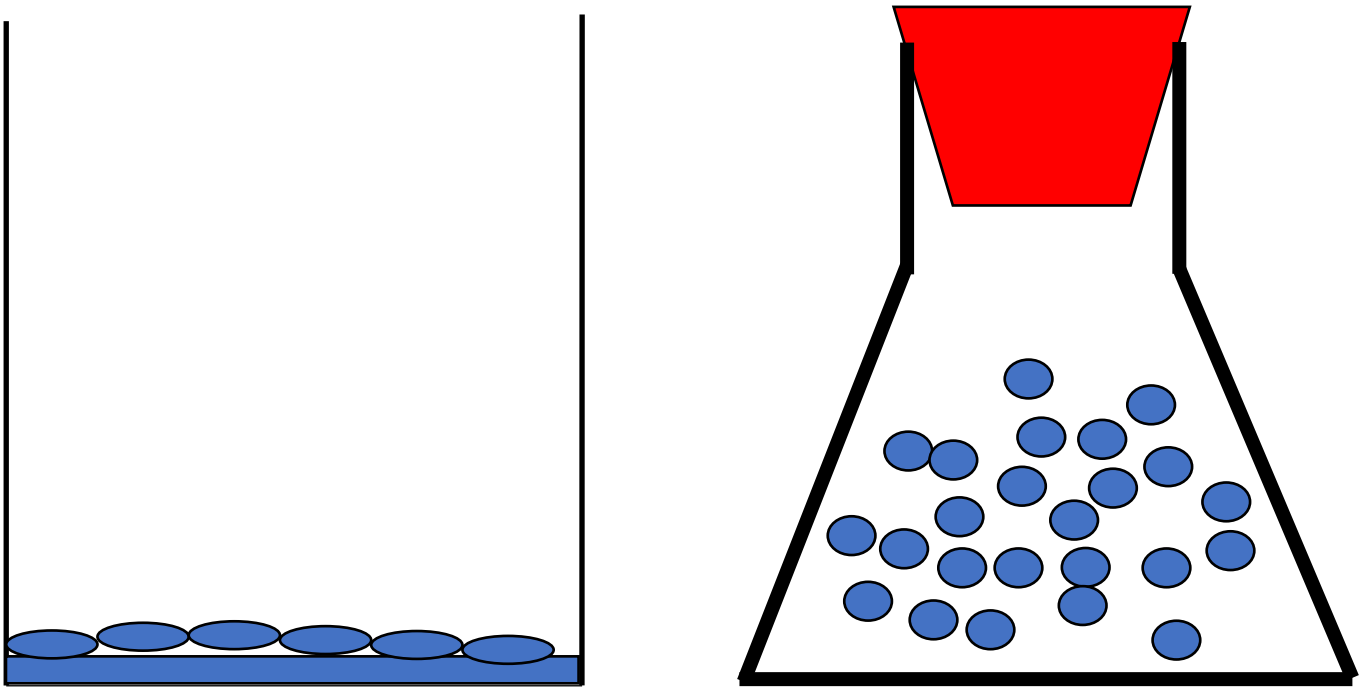
Decellularized human colonic tissues that was manually reconstituted with cell types of the colon were used to identify 6 new genes including ASXL2, CAMTA1, DDX20, FXRI, MITF and PAX7 that modulated invasion of recellularized colonic epithelial cells (H. J. Chen et al., 2016). The involvement of these genes in cancer progression were further validated in *in vitro* studies. In our lab, we have also shown that decellularized mice lungs better support the differentiation ability of repopulated primary human bronchial epithelial cells (LaRanger et al., 2018). The cells differentiated three times faster in the decellularized colon versus cells placed at the air-liquid interface (ALI), a 2D-based differentiation system (LaRanger et al., 2018). Additionally, in ALI, cells differentiate only into upper airway cells, like the MUC5B cells producing cells or alpha-tubulin expressing cells, however, in the decellularized lungs, the cells differentiated into both upper and lower airway cells and maintained a population of basal CK14 cells (**Figure1.5**) (LaRanger et al., 2018).

Whole decellularized tissue have also been implanted and have shown at least some tissue functions. An example is a study done in mice where adipose tissue from humans was implanted

into nude mice for 8 weeks after decellularization and reconstitution with adipose derived stem cells (L. Wang, Johnson, Zhang, & Beahm, 2013). The authors reported that there was no immunogenic responses post implantation and there was vascularization and regeneration of adipose tissue (L. Wang et al., 2013). Currently, there are a number of FDA approved tissue regeneration/replacement products available that were developed from decellularization of tissues. They include Life Cell's AlloDerm Regenerative tissue Matrix which can be used as a human dermal graft and facilitates cell proliferation (Gilpin & Yang, 2017). Another is the Meso Bio Matrix Surgical Mesh by DSM that was derived from pig mesothelium (Gilpin & Yang, 2017). For replacement of the human pulmonary heart valve there is also the CryoLife SynerGraft (Gilpin & Yang, 2017).

The work presented in this thesis describes a decellularization methodology optimized for mice colonic tissue to produce a biological scaffold with preserved native biochemical and biomechanical properties. This thesis also discusses cell behavior in this biological scaffold as well its potential use in the identification of novel and targeted therapies for treating colon cancer.

Figures



2D monolayer **2D suspension cells**

Figure 1. 1: **2D monolayer and suspension cell culture system.**
Adapted from Broglie et al.

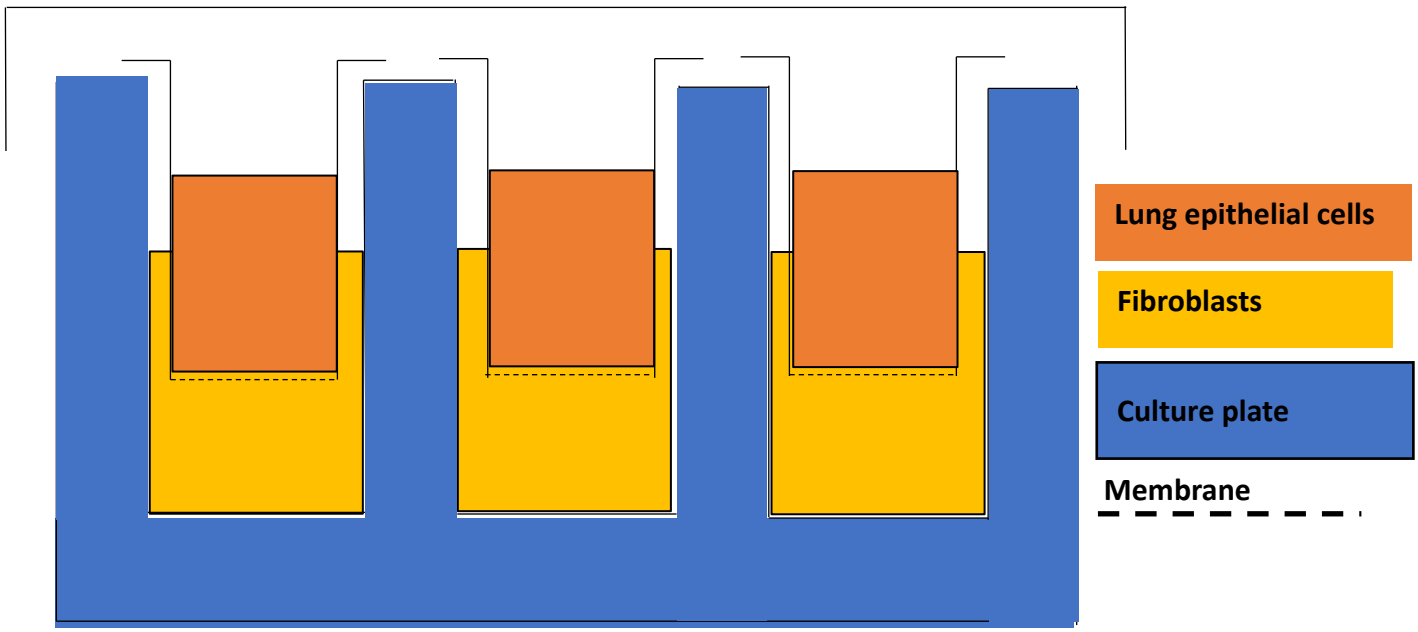


Figure 1. 2: **2D Transwell Co-Culture system**
Adapted from goers, freemont et al. 2014 and prasad, hogaboam et al. 2014.

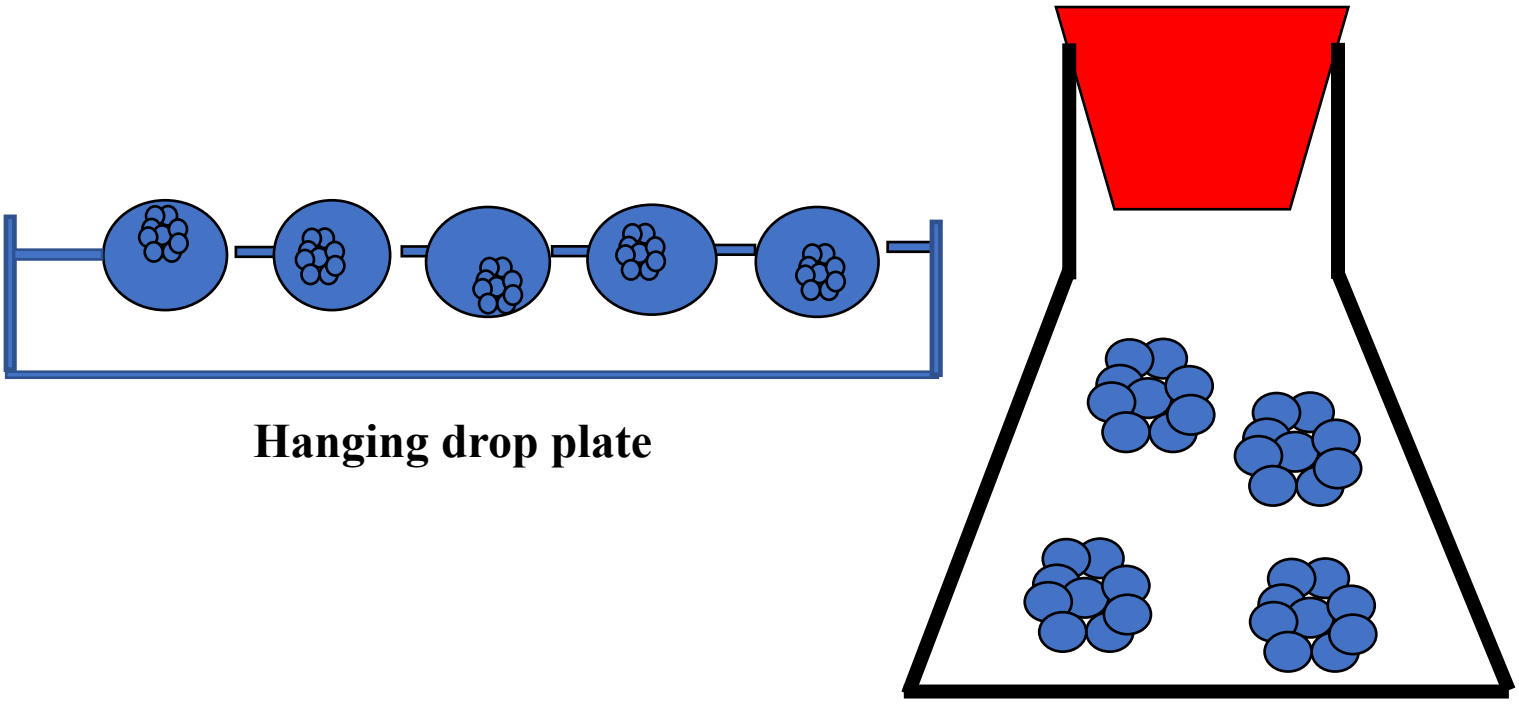


Figure 1. 3: **Scaffold-free 3D culture systems.**
Adapted from Edmondson, Broglie et al. 2014.

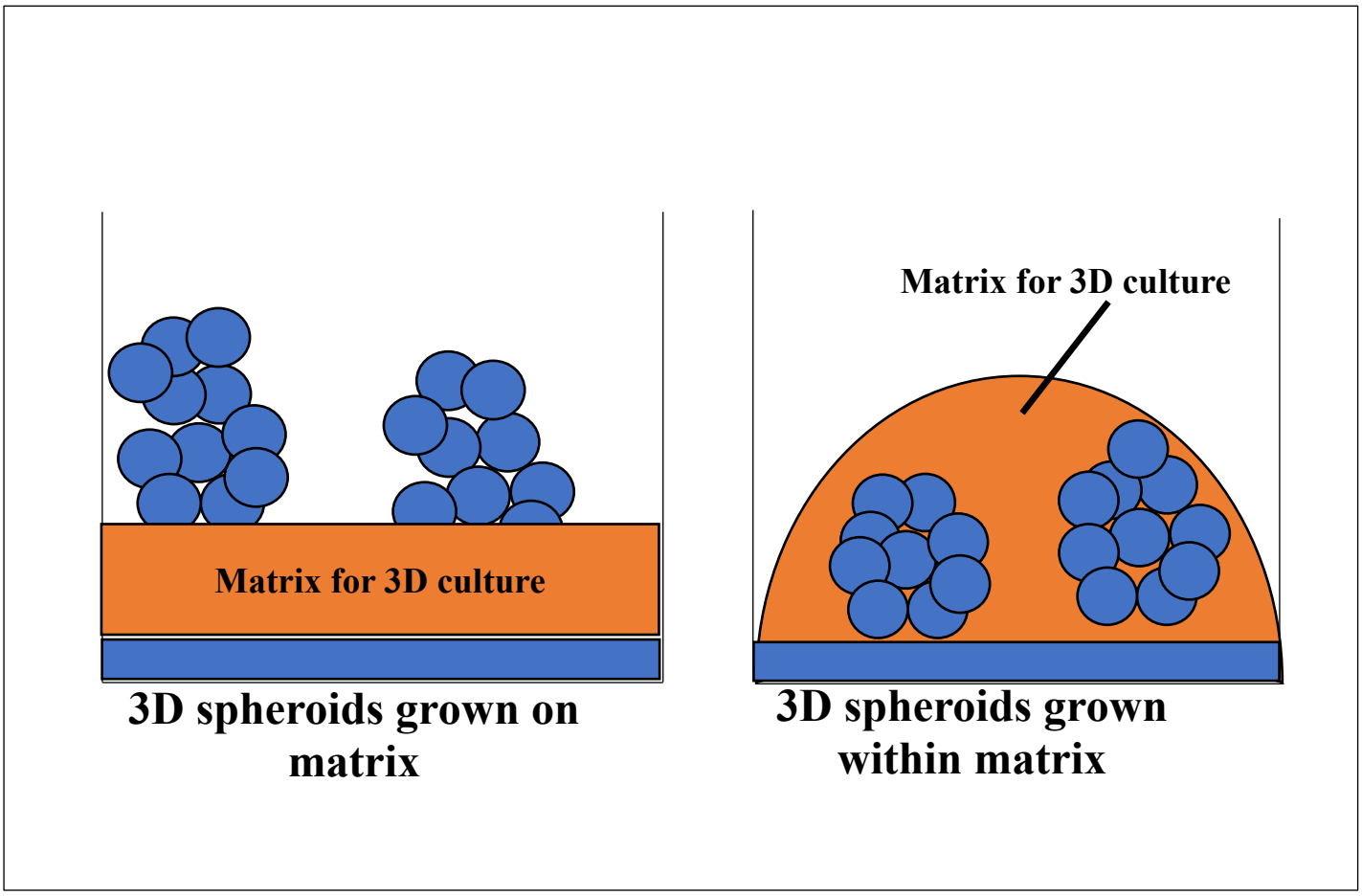
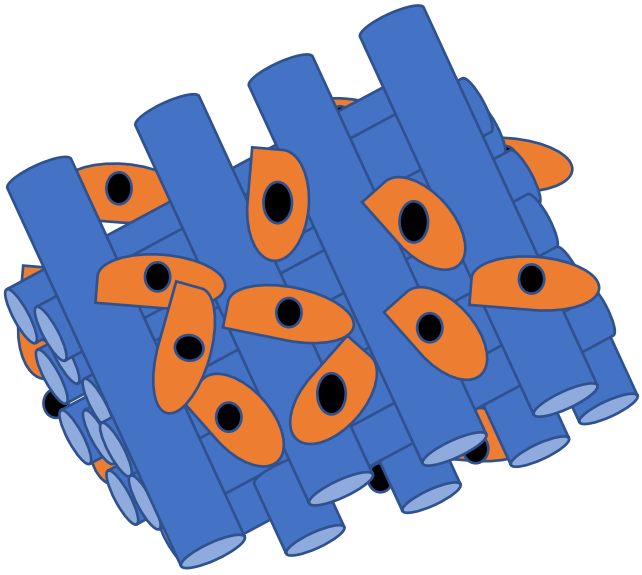


Figure 1. 4: **Gel based 3D scaffold culture systems.**
Adapted from Edmondson, Broglie et al.



3D printed multilayer synthetic scaffold with C2C12 cells

Decellularized mice colon to be used for reconstituting human bronchial epithelial cells.

Figure 1. 5: **Synthetic and native scaffold-based 3D system.**
Adapted from Chen, Zhong et al 2019 and LaRanger R. et al. 2017.

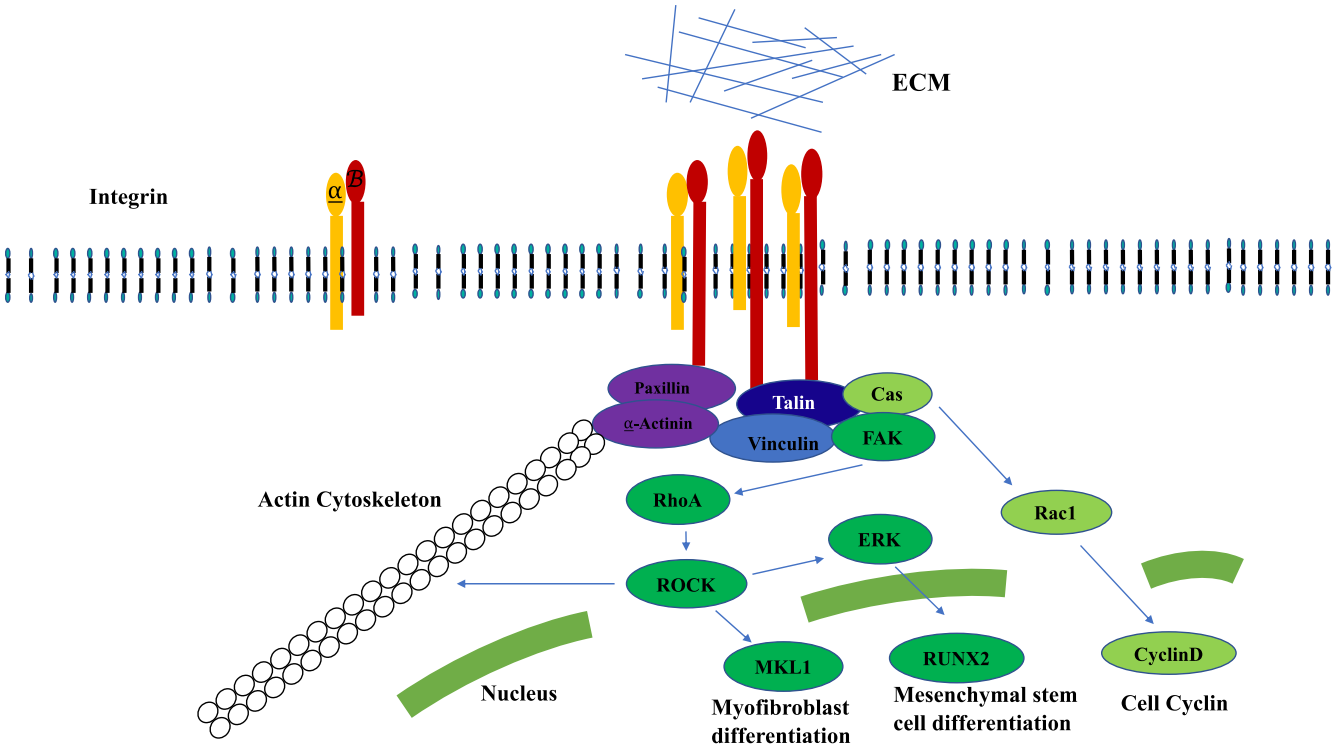


Figure 1. 6: **Regulatory pathways activated by tissue stiffness.** Adapted from Handorf, Zhou et al. 2015).

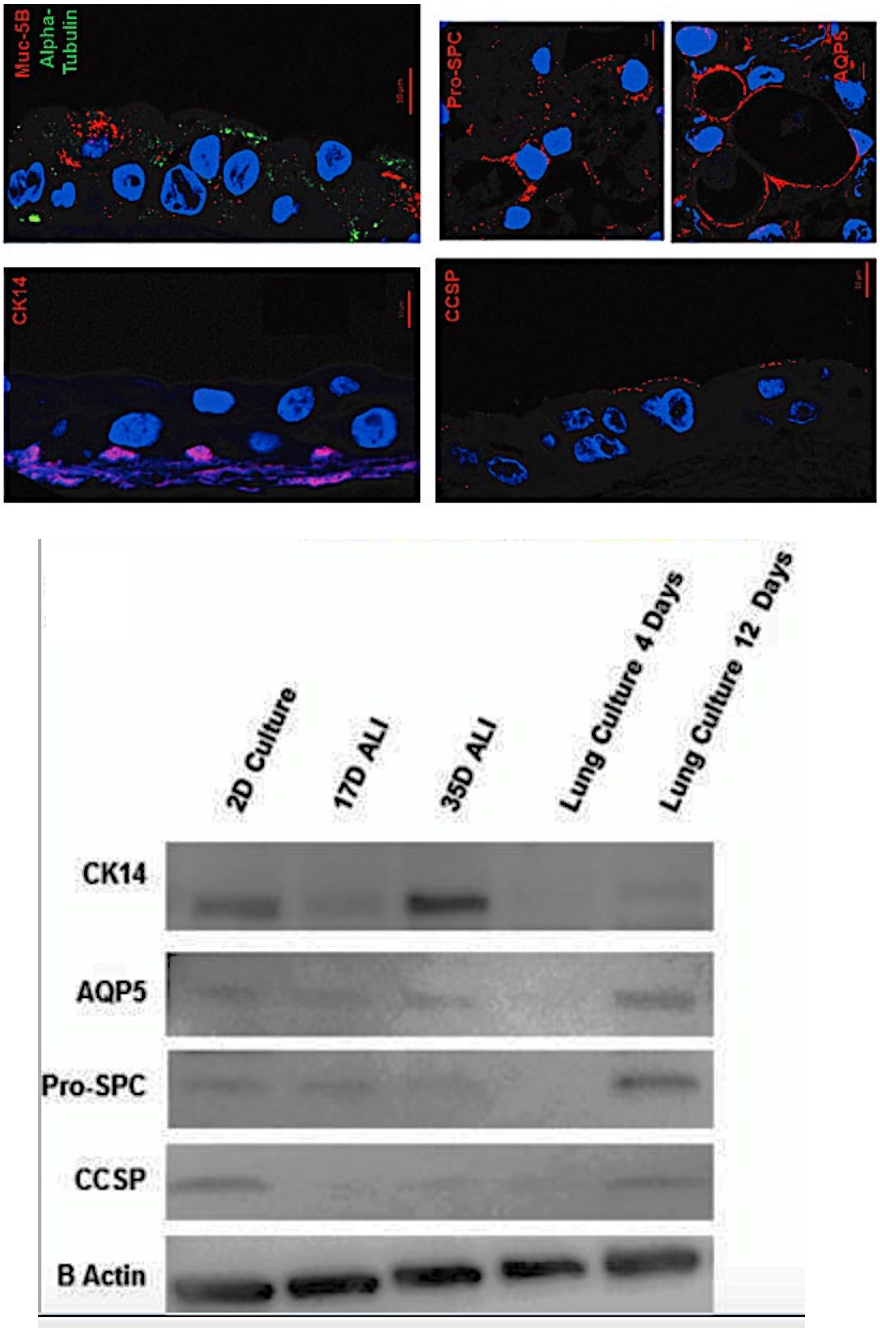


Figure 1. 7: **Immunohistochemistry and western blot shows enhanced differentiation ability is observed in cells used to reconstitute decellularized tissues versus cells at air-liquid interface (ALI).**
Adapted from LaRanger R. et al. 2017.

CHAPTER TWO

Abstract

Current 3D culture models to study colorectal cancer lack architectural support and signaling proteins provided by the tissue extracellular matrix (ECM) which may influence cell behavior and cancer progression. Therefore, the ability to study cancer cells in the context of a matrix that is physiologically more relevant and to understand how the ECM affects cancer progression has been understudied. To address this, I developed an *ex-vivo* 3D system, provided by intact wild type (WT) and colon cancer susceptible decellularized mouse colons (DMC), to support the growth of human cancer cells. DMC are free of viable cells but still contain extracellular matrix proteins including fibronectin, extracellular matrix protein 1 and subsets of collagens and laminins. Stiffness, an important mechanical property, is also maintained in DMCs. Importantly, the DMC is permissive for cell proliferation and differentiation of a human colon cancer cell line (HT-29). Notably, the ability of cells in the WT DMC to differentiate was also greater when compared to Matrigel ^{TM}, an extracellular matrix extract from a mouse tumor cell line. Additionally, I observed in invasion assays that DMC obtained from polyps of a colon cancer susceptible mouse model facilitated increased cell migration/invasion of colorectal cancer cells and immortalized non-tumor colonic epithelial cells compared to DMC from WT mice. Finally, using mass spectrometry, I identified extracellular matrix proteins that are more abundant in DMC from a colorectal cancer mouse model compared to age and sex-matched WT mice. In summary, these abundantly expressed proteins in the tumor microenvironment are potentially involved in colorectal cancer progression.

Introduction

The extracellular matrix (ECM) provides structural support for cells in all solid tissues (Filipe, Chitty, & Cox, 2018; Frantz et al., 2010). Structurally, the ECM is composed of water, polysaccharides, and proteins such as fibrous collagens and proteoglycans, that are organized in a tissue-specific manner (Filipe et al., 2018; Frantz et al., 2010; Yue, 2014). The structural arrangement, tensile strength and biochemical properties of the ECM support the homeostatic function of tissues (Frantz et al., 2010; P. Lu, Weaver, & Werb, 2012; Wells, 2008). Other functions of the ECM include tissue morphogenesis, differentiation, transmission of forces and macromolecular filtration (Filipe et al., 2018; Frantz et al., 2010; Mouw, Ou, & Weaver, 2014; Yue, 2014)**.** The ECM is remodeled during the aging process and throughout the progression of diseases such as cancer (Frantz et al., 2010). Further, malignant progression of cells during cancer may be influenced by permissive cues from the surrounding ECM in addition to intrinsic genetic alterations (Crotti et al., 2017; Filipe et al., 2018).

Tissue decellularization uses enzymatic, chemical or/and physical processes to isolate and separate the extracellular matrix (ECM) from the cells of an organ (Crapo et al., 2011; Gilbert, Sellaro, & Badylak, 2006; Gilpin & Yang, 2017). Effective decellularization balances removal of DNA with preservation of tissue-specific mechanical properties and maintenance of ECM structural and functional proteins. Previously, we demonstrated that the extracellular matrix of decellularized mouse lungs are able to support the proliferation and differentiation of human bronchial epithelial cells (HBECs) (LaRanger et al., 2018; J. R. Peters-Hall et al., 2018). Usually, the differentiation potential of HBECs is tested at the air-liquid interface (ALI). In these cell culture conditions the cells differentiate into the upper airway epithelium after 35 days

(LaRanger et al., 2018). However, when these cells were used to reconstitute the decellularized lungs, they were able to form both upper airway bronchial epithelium and lower airway alveolar structures in only 12 days (LaRanger et al., 2018).

The basement membrane is a unique form of ECM. It is a thin dense sheet of specialized and self-assembled ECM in most multicellular animals (Jayadev & Sherwood, 2017). It is comprised of independent heterotrimeric laminin and covalently-linked type IV collagen networks that are linked by other ECM proteins such as nidogen (Jayadev & Sherwood, 2017). However, it also comprises of combinations of other proteins, proteoglycans and glycoproteins including fibulin and type VIII collagen (Jayadev & Sherwood, 2017). It is important for directing cell polarity, signaling the development and maturation of tissues via growth factors, shapes tissues, provides mechanical support for cells and is important for cell differentiation (Jayadev & Sherwood, 2017) In the colon, it separates epithelial cells from adjacent connective tissue. During cancer progression, only a subset of epithelial cells acquire the ability to invade through the basement membrane (Dunn et al., 2012). These cells may secrete matrix-degrading zinc endopeptidase called matrix metalloproteinases (MMPs) (or) and use actin-rich structures called invadosomes to degrade the basement membrane (Jacob & Prekeris, 2015; Jayadev & Sherwood, 2017). Another subset of these invasive cells can intravasate into blood vessels, extravasate and undergo metastasis to form new tumors in distant sites such as the liver, leading to the vast majority of colorectal cancer associated mortalities (Hanahan & Weinberg, 2000). Many of the complex genetic, epigenetic, and biochemical processes responsible for the development of malignant colon cancer cells remain incompletely understood (Hanahan & Weinberg, 2000). By providing an unconventional approach for studying the interaction between the colon ECM, tumor formation, and invasion/metastasis, the biological models described in the current series of

experiments may provide a better understanding of this disease and lead to the development of new therapies that target the process of invasion and metastasis. Discovery and development of novel targeted therapeutic options for colon cancer is important as it is one of the most prevalent types of cancer in both males and females world-wide but there are few available targeted therapeutic options (Fang et al., 2015; Lee & Yun, 2010).

To create *in vivo*-like differentiation in a colonic system that is similar to the lung system that we had produced in the lab, I developed an *ex-vivo* 3D model system using decellularized mouse colons that still maintain structural ECM proteins and the mechanical properties of native tissue. I tested the ability of such decellularized matrices to support the proliferation and differentiation of HT-29, a human colon cancer cell line. This cell line was chosen because it has been reported to be multipotent; the cells can reversibly form brush borders on their apical surfaces, characteristic of absorptive cells, after being cultured for 21days in forskolin treated medium (Cohen, Ophir, & Shaul, 1999; Gout et al., 2004; Huet, Sahuquillo-Merino, Coudrier, & Louvard, 1987). The cells can also produce mucin, which is characteristic of intestinal goblet cells, upon substitution of glucose, in standard medium, with trehalose and are actively used for (Bourgine et al., 2012; Huet et al., 1987; X. Lu, Walker, MacManus, & Seligy, 1992). Also, gene expression profiling data comparing the most widely used colon cancer cells for intestinal pharmacological and toxicological studies showed that HT-29 cells (and T84 colon cancer cells) are the most similar, genetically, to normal human cells (Bourgine et al., 2012). Furthermore, I used the CPC;Apc colorectal cancer mouse model, whose tumors have similar characteristics to human tumors, to identify extracellular matrix proteins that influence colorectal cancer progression via mass spectrometry and western blot analyses (Hinoi, Akyol et al. 2007, Kim,

Bozeman et al. 2016). Finally, i show that the DMC derived from the mice containing polyps influences a cell invasion phenotype of both normal and cancer cell lines.

Results

The decellularization process clears tissue, removes DNA and viable cells but maintains tissue stiffness.

The decellularization process results in an opaque colon becoming translucent (**Figure 2.1A-B**). Greater than 99% of the starting DNA is removed (**Figure 2.1C**). Decellularized tissue was placed in media for 7 days to see if left over DNA contained intact cells capable of proliferating. No cell growth occurred in the tissue after 7 days (**Figure 2.1D**). Young's modulus (*E*) measures the resistance of materials such as the ECM to deformation. The tissue resistance was measured by using dynamic mechanical analysis to apply a force (stress) to the colon and then calculating the resulting change in length (strain) (**Figure 2.2A**). *E* is the slope obtained from the stress versus strain plot. Young's modulus of mice colonic tissue, ~4.2MPa was also maintained post decellularization (**Figure 2.2B**). The nature of the decellularization agents chosen, deionized water (DW), sodium deoxycholate (SD) and DNAse, used in this protocol (BSD) helped to balance cell removal with architectural preservation of the DMC. DW causes hypo-osmotic shock to cells, sodium deoxycholate is an anionic detergent that causes cell lysis by solubilizing cytoplasmic and nuclear membranes and DNAse1 is an endonuclease. These decellularization agents were perfused through the lumen of the colon using a peristaltic pump at a flow rate of 1ml/min. These decellularizing agents have been previously used (Totonelli et al., 2012).Triton X-100 and Sodium dodecyl sulfate (SDS) were also tested as a decellularization pair (BST)

(**Figure 2.3A**). Triton X-100 is often used in conjunction with sodium dodecyl sulfate because it can effectively remove highly cytotoxic sodium dodecyl sulfate from biological scaffolds. However, this pair did not result in a decellularized matrix with the least amount of DNA and so I used the SD and DNAse. Further, I tested tissue agitation using an incubator shaker set to 200 rpm. However, overall, bioreactor based method using detergents SD and DNAse (PSD) pairs, provided decellularized tissue with most amount of DNA removed from mice colonic tissue (**Figure 2.3B**) . Therefore, I continued future decellularization experiments using the BSD protocol.

Extracellular matrix proteins persist post decellularization

To characterize the behavior of cancer cells in their native environment, it was important to maintain specific properties of the tissue such as mechanics and extracellular matrix proteins. Fibrous collagens such as collagen I, are major components of the interstitial matrix of the ECM of the colon. I found that they were maintained post decellularization as shown by trichome staining (**Figure 2.4A-B**). Interestingly, muscle proteins were also maintained post decellularization as evidenced by the persistence of red color in the trichrome staining (**Figure 2. 4B**). However, post decellularization the black nuclei staining was absent in the smooth muscle layer of the colon and along the crypts (**Figure 2.4B**). Therefore, even though muscle and epithelial cells were effectively removed, at least some muscle proteins and extracellular matrix proteins persisted. Also, laminin, fibronectin, and collagen IV, which are all part of the basement membrane, were also preserved post decellularization as shown by immunofluorescence staining (**Figure 2.4C-4F**). Together, these results suggest that this decellularization method produces a

DMC that may effectively model a native colon environment even after removal of its constituent cells.

Greater presence of differentiated HT-29 human colon cancer cells in decellularized matrices compared to Matrigel™

The decellularized matrix was repopulated with ~10 million HT-29 cells with the aid of a peristaltic pump producing a flow rate of 0.05 ml/min for 2 hrs. Static culture was done afterwards. Cells were only perfused once though the colon lumen. **Figure 2.5A** shows a schematic of the recellularization process. For comparison of this approach to a more standard 3D culturing method for colon cancer cells, six hundred HT-29 cells were also seeded in 50ul of Matrigel and placed on cover slips in a 24 well plate. After 5 days, the cells from both Matrigel and recellularized colonic tissue were stained with KI-67, a proliferation marker, and MUC-2, a mucous or goblet cell differentiation marker (**Figure 2.5B-C**). I observed a significantly higher presence of differentiated cells in DMC **(Figure 2.5D**) suggesting that the immediate environment contributes to the physiological state of the cells.

In other experiments, the cells reseeded in Matrigel or used to reconstitute the colon were isolated after 5 days and droplet digital PCR (ddPCR) was used to quantify expression of MUC2 and villin, a colonic absorptive epithelial differentiation marker. As illustrated in **Figure 2.5E-F** there is >10-fold increase in MUC2 transcripts, as well as >3-fold increase in villin transcripts after 5 days in DMC. Additionally, there are very few villin or MUC2 transcripts in Matrigel after 5 days. This experiment further confirms the greater presence of differentiated cells in DMC versus Matrigel.

Invasion assays and extracellular matrix proteins in polyps versus adjacent normal areas

I next tested how the extracellular matrix influences colon cancer cell invasion, a hallmark of advanced cancers. 24 well tissue culture inserts containing 8 um holes were coated with Matrigel and extracellular matrix from either wild type (WT) mice or mice from a colorectal cancer (CC) mouse model (**Figure 2.6A-B**). The 8um holes allow invasive cells escape through after breaking down the Matrigel ^{TM}. As shown in **Figure 2.6C**, the DMC from a CC mouse facilitated greater cell invasion of HCT116 human colon cancer cells and HCECs (immortalized but not tumorigenic human colonic epithelial cells) compared to DMC from wild type (WT) mice. As expected, HCT116 cancer cells were significantly more invasive when compared to non-transformed HCECs through DMC from the CC mouse model.

To identify and quantify abundance of proteins involved in this process I performed liquid chromatography with tandem mass spectrometry (LC-MS/MS) of polyp versus adjacent non-cancerous areas of colonic tissues in mice with progressed cancer polyps. Mass spectrometry was performed by the proteomics core at the University of Texas Southwestern Medical Center. (Colons from 2 female sibling mice (261 days old) were decellularized. At this age the majority of the CC mice have advanced polyps (adenomas) while their counterparts do not have polyps (**Figure 2. 7Ai-Aii**) . Whole sections of the decellularized colon were cut from regions with polyps. that could still be identified even without cells, and from adjacent non-polyp (apparently normal tissue) areas . 35 ECM proteins were identified via mass spectrometry post decellularization. As shown in **Figure 2.7B**, 5 out of the 35 identified ECM proteins were at least

two times more abundant in polyp ECM versus adjacent non tumor bearing ECM regions. Only two of the identified ECM proteins were more abundant in the adjacent non tumor regions. Laminin subunit alpha 1 was the most abundant ECM protein in the polyp regions while collagen type (IV) alpha 6 was the most abundant ECM protein in non-tumor adjacent regions compared to polyp regions. Tenascin-X, which has not been previously implicated with colorectal cancer, was the only other ECM protein that was at least two times greater in normal tissue versus polyps. Western blotting (**Figure 2.7 Ci and 7 Cii**) confirmed the greater abundance of candidate proteins, ECM protein 1 and vitronectin, in polyp (pol) regions.

Discussion

I developed a decellularization method that successfully removes DNA while still maintaining tissue architecture, mechanical properties and ECM proteins. This is important because these preserved properties are essential for tissue function and also modulate some key aspects of cell behavior. Out of the two decellularization chemical agent pairs that I tested, the sodium deoxycholate and DNAse pair removed the greatest amount of DNA from mice colonic tissue. Further, using a bioreactor system which includes a peristaltic pump, resulted in further decrease in residual cellular materials.

Tissue stiffness, which in this study was measured by Young's Modulus, has been shown to work in concert with genes such as YAP/TAZ and Septin9 to regulate cell behavior including proliferation and differentiation (Gjorevski et al., 2016; Wrighton, 2011; Yeh et al., 2012). Therefore, by maintaining tissue stiffness, I was able to study these cells in an environment that mechanically better phenocopies *in vivo* conditions.

The differences in the behavior of the HT-29 colon adenocarcinoma cell line in Matrigel versus decellularized mouse colon (DMC) conditions, with DMC favoring differentiated cell states, demonstrates how varying cell culture conditions can impact corresponding cell behavior. In standard 2D culture conditions, HT-29 cells show high glucose consumption, grow in multiple unpolarized layers and display an undifferentiated phenotype (Cohen et al., 1999; Verhoeckx, 2015). However, in specific culture conditions, such as when glucose in the medium is replaced with galactose or when chemical inducers such as butyrate are added to the medium, HT-29 cells can differentiate into multiple cell types of the intestinal epithelium (Cohen et al., 1999; X. Lu et al., 1992; Verhoeckx, 2015). This differentiation ability has allowed HT-29 cells to be used, sometimes in conjunction with other colon adenocarcinoma cell lines such as CaCo-2, to study colonic cell differentiation, and drug/nutrient permeability across the intestinal mucosa (X. Lu et al., 1992; Strugari, Stan, Gharbia, Hermenean, & Dinischiotu, 2018). Importantly, not only is this the first time, as far as I could determine, that a human colon cancer cell line has been reported to differentiate in a decellularized tissue system. In addition, the cells differentiated faster in the DMC (5 days) compared to 21 days at the air liquid interface. Additionally, detection of increased villin (a marker for absorptive cells of the colon) transcripts via ddPCR further supports the higher presence of differentiated cell types in DMC versus Matrigel. These results highlight how the cell culture environment affects physiological state.

Furthermore, when HT-29 cells are cultured in colons containing polyps (**Supp. Figure 2.1**), there is about a 20% increase in proliferative cells (Ki-67 stain) compared to WT (or polyp free) areas. This observation indicates that the tumor micro-environment favors proliferation of cells rather than differentiation. Possible explanations to attribute these environment/ECM-specific

differences in cell behavior include preserved tissue architecture, mechanical properties and extracellular matrix proteins. This possibility is consistent with other reports showing that extracellular matrix proteins and the mechanical properties of tissue help to regulate several cellular processes including cell development, proliferation, differentiation and even disease progression (Handorf et al., 2015; Kenny et al., 2018; Wells, 2008; J. Xu et al., 2017).

My observations of increased invasiveness of both normal and colorectal cancer cells in matrix derived from DMC from mice containing large polyps shows that the matrix from the tumor environment does facilitate normal cell migration and cancer progression. Additionally, this increased cell invasion phenotype was only statistically significant in the case of the HCT116 colon cancer cells, but not normal HCECs. This suggests that for cancer progression to occur, there is the need for both genetic ('endogenous signaling') and extracellular matrix signaling ('exogenous signaling'). In the case of the HCECs, the exogenous signaling from the ECM was able to drive or support the cells towards a migration phenotype but was not sufficient for inducing invasion. In contrast, HCT116 are inherently invasive and with additional signaling from the ECM were able to significantly invade through the matrix.

I also identified and quantified ECM proteins that were more abundant in the tumor microenvironment that may facilitate this increased invasive cell behavior. To achieve this, I took areas of decellularized colonic polyps and adjacent non tumor tissue from CPC;Apc mice, a genetically engineered mice model of colonic adenoma and adenocarcinoma. In these mice, the CDX2 promoter is used to drive expression of mutated Apc (adenomatous polyposis coli), a tumor suppressor gene that has been implicated in the occurrence of colorectal cancer, specifically in colonic epithelium using the CRE/Lox transgenic system. The tumors developed

by these mice have been shown to be morphologically and molecularly similar to human adenocarcinomas which makes this a translational model for studying colorectal cancer (Hinoi et al., 2007; X. Lu et al., 1992). To prevent or reduce the occurrence of false negative reads in the mass spectrometry data, I was careful to take portions of non-tumor tissue from various areas of the same colon and not just next to the polyp. The mass spectrometry data showed that ECM proteins including collagen xviii, fibronectin and laminin alpha1were more abundant in the polyp regions of colons versus adjacent normal tissue. It is plausible that these abundantly expressed proteins in the tumor microenvironment are involved in colorectal cancer progression such as the observed increase in invasiveness of the HCT116 cells. In fact, it has been shown that the subcutaneous injection of colonic cancer cells transfected with laminin alpha-1chain, the most abundant ECM protein we observed in mice polyps, into nude mice, resulted in mice with significant tumor growth compared to mice injected with control cells (De Arcangelis et al., 2001). Similarly, mice injected with cells that had been transfected with fibronectin had larger tumors compared to their controls via the p53/NFKB pathway (Gouveia-Fernandes et al., 2016). Translationally, researchers have shown that the serum level of fibronectin and laminin in patients correlates with colorectal cancer disease stage (Saito & Kameoka, 2005; Saito, Nishimura, & Kameoka, 2008). Further, vitronectin and collagen xviii alpha1 have been shown to be highly expressed in stromal tissues of patient adenocarcinoma tissue and in the liver metastasis sites (Guenther et al., 2001). Additional ECM proteins identified as being more abundant in the tumor microenvironment including proteoglycan 3, ECM protein 1 and laminin subunit beta 1 have not previously been characterized as contributing to colorectal cancer progression and would be excellent candidates for untested environmental regulators of this disease.

In addition, other ECM proteins, including collagen IV alpha 6 chain and tenascin-X, were underrepresented in polyps compared to adjacent non tumor-associated DMC. While collagen IV alpha 6 chain has been previously implicated in colorectal cancer, this is the first time that tenascin-X has been associated with colorectal cancer. Normally, the type 6 chain of collagen IV is found in the basement membrane of specific tissues such as the mammary glands, smooth muscle cells and the epithelium of the alimentary tract (Ikeda et al., 2006). However, during the early stages of colorectal cancer invasion a hypermethylation event at the tenascin- X promoter region causes the loss of this chain (X. Lu et al., 1992). Tenascin X is largely known for its role in Ehlers-Danlos syndrome. Deficiency of tenascin-X results in this inheritable connective tissue disorder (Burch et al., 1997; Schalkwijk et al., 2001). However, in various pathological conditions tenascin-X expression is frequently inversely correlated to that of tenascin-C which is known for its role in the progression of various cancers (Murakami et al., 2017; Valcourt, Alcaraz, Exposito, Lethias, & Bartholin, 2015). These results can be interpreted to propose that identification of direct and indirect binding partners of tenascin-X, specifically in epithelial cells of the colon, could provide new insights into therapeutic targets for colorectal cancer prevention.

Aside from the above mentioned ECM proteins I also identified a total of 8 growth factors and growth factor binding proteins that were present in adjacent normal colonic tissue post-decellularization (**Supp. Figure 2.2).** These growth factors include 2 fibroblast growth factors (FGF), 2 latent transforming growth factor beta-binding proteins (LTBPs), 2 transforming growth factor beta-1 induced transcript protein, and 2 epidermal growth-factor like proteins. There are FGF 23 members and are key regulators of tissue homeostasis and organogenesis by

modulating events including proliferation, migration, differentiation, and metabolic activity (Maddaluno, Urwyler, & Werner, 2017; Yun et al., 2010). Therefore, they have been used to repair damaged tissues of various parts of the body including skin, tooth, adipose, muscle and tendon (Yun et al., 2010). They often work in concert with other pathways, such as the Wnt signaling pathway and the RAS/MAP pathway to perform their functions (Maddaluno et al., 2017; Yun et al., 2010). The LTBPs are a family of four isomeric proteins-LTBP1-4 (Robertson et al., 2015). They associate directly with the extracellular matrix proteins and are important for the assembly of elastic fibers and the organization of microfibrils (Robertson et al., 2015). They also regulate activation and target TGF-β to the ECM where it can be released and activated (Vehvilainen et al., 2011). Interestingly, I also identified one growth factor binding protein that was only present in the polyps of the 2 colonic tissues analyzed. It is the latent-transforming growth factor beta-binding protein 3 (LTBP 3). It was found to be increasingly expressed in highly metastatic breast cancers injected in the fat pad of immunodeficient mice. (Naba, Clauser, Lamar, Carr, & Hynes, 2014). Further, knockdown of LTBP3 in the breast cancer cells reduced their invasive and early metastatic abilities (Naba et al., 2014). However, other have reported that expression of LTBP 3 is downregulated in tissue biopsies of malignant mesothelioma (Vehvilainen et al., 2011).

In summary, the decellularization protocol described in this report resulted in the production of DMC with endogenous DNA removed, but tissue architecture and stiffness remained. This model system has implications, not only in understanding cell responses to culture conditions, but also in understanding the role of altered ECM in colorectal cancer progression. Because many ECM proteins are highly conserved across species, a future direction is to reconstitute the

decellularized mice colons with additional cell types that are found in human normal and tumor micro-environment. These cells could include mixtures of tumor cells with normal human colonic epithelial cells, vascular endothelial cells and fibroblasts. Reconstitution with these other cell types will allow the use of the improved decellularized system (from WT or CPC;Apc mice) to probe into how fibroblasts can influence cancer progression or how the ECM can modulate angiogenesis, another hallmark of cancer that is important for tumor survival and progression. These angiogenesis studies have the potential to shed further insights into how ECM proteins, such as collagens and laminins, or ECM associated growth factors and cytokines differentially regulate the angiogenesis process as discussed by others (Mongiat, Andreuzzi, Tarticchio, & Paulitti, 2016; Neve, Cantatore, Maruotti, Corrado, & Ribatti, 2014; Sottile, 2004) These studies may also allow for the identification of new ECM proteins that are critical for angiogenesis and others that are anti-angiogenic in the colon tumor micro-environment.

Finally, it could be envisioned that such a system could be developed for repair of damaged colons through engineering colon tissue from these decellularized matrices. This will allow the creation of more robust clinical models that are physiologically relevant and can be used for *ex-vivo* translational research including drug studies or for regenerative or transplantation purposes.

Materials and Methods

Decellularization

Colonic tissue was harvested from BL6 mice under UTSW IACUC approved protocols. The essential components of the colonic decellularization system include; a peristaltic pump (Cole

Parmer: Master flex EW-07522-30) to perfuse decellularization agents, an autoclavable glass jar and a rubber stop-cork that houses silicone tubings (Cole Parmer: Masterflex EW-96410-14) and covers the glass jar. The tubings are connected to the peristaltic pump and serve as conduits for aseptically transporting decellularization agents perfused in a circular motion by the bioreactor from the glass jar, through the colon and back into the glass jar. After colon was isolated from mice, a male luer lock connector was inserted into one end of the colon. Next, the male luer lock was tied to the colon with black silk suture spools (Surgical Specialties, PA). The colon was then flushed with 1X PBS + 5% antibiotics and antimycotics (5XPBSAA) (Gemini Bio Products, CA) using a 30 ml syringe (BD: 309650) to remove bacteria and fecal matter up to three times. A male luer lock was also inserted and tied to the other end of the colon. Colon was flushed again with 5XPBSAA. Then, an autoclaved glass jar was filled with deionized water (DW). One of the male luer locks was next carefully connected in a clockwise fashion to the female luer lock of a decellularization tubing in the bioreactor jar (**Figure 2.1**) to connect the colon to the peristaltic pump and also set it in place, in the glass jar filled with DW, Bubbles were removed from the colon and tubings using clean 30 ml syringes and the colon was filled with DW water, from the jar, with the same 30ml syringes. Next, DW was perfused through the lumen of the colon, with the peristaltic pump, overnight at 4ºC. The next day, 4% sodium deoxycholate (SD) was perfused through the colon for 2hrs at RT. This is followed by PBSAA (1% antibiotic and antimycotic solution) for 30mins at RT. Next, 2000 Kunitz of DNAse, diluted in 1M NaCl, was perfused though the colon for 1.5hrs. Flow was always generated by the peristaltic pump at a flow rate of 1ml/min for. Colons were stored overnight at 4ºC in the bioreactor jar containing PBSAA without perfusion and then used for downstream experiments. Other decellularization agents tested but not used for further experiments include 1% sodium dodecyl sulfate and 1%

triton X-100 (**Figure 2**). The 2 modes of decellularization tested were perfusion system using a peristaltic pump and agitation using an incubator shaker.(Innova: 4400).

DNA Quantitation

Colons from age-matched male mice (~193 days old) were weighed and split into halves of ~300mg each. Half of each of the colonic tissues were flash frozen immediately while the remaining half were flash frozen after decellularization. DNA extraction is carried out using Roche's DNA isolation Kit for Cells and Tissues based on the manufacturer's instructions. The Quant-It PicoGreen dsDNA Assay was used to quantitate amounts of DNA using fluorescence intensity according to manufacturer's instruction (Fisher Scientific, MA.).

Trichrome and Hematoxylin and Eosin (H&E) Staining

Tissues were fixed in formalin for at least 24 hours and sent to the Simmons Comprehensive Cancer Center histology core at UTSW and processed into paraffin blocks. Processed tissue was sectioned at 5µm thickness, heat fixed, rehydrated and stained following manufacturer's instructions for trichrome staining (American Master Tech Scientific Lab Supplies, CA). H&E staining was done following well established protocols.

Immunofluorescence

Tissue sections were de-paraffinized in xylene, placed in 100%, then 95% ethanol, and finally rehydrated in deionized water. The tissue sections were then unmasked in a microwave in 10mM sodium citrate buffer pH 6 at power 5 for 20 minutes then allowed to cool for 30 minutes. Tissue was then blocked with 1X PBS+5% normal serum + 0.3% Triton X-100 at room temperature.

Tissue was then incubated with primary antibodies overnight. The next day the slides were washed in 1X PBS 3 times and then incubated with a secondary antibody for 1hr in the dark. Finally, the slides were washed with PBS for 5 mins 3 times then mounting media H-1500 (Vector Laboratories, CA) were added with DAPI and covered with coverslips. For immunofluorescence (IF) staining the following primary antibodies, Fibronectin: ab2413 (Abcam) (1:200), Collagen IV: ab6586 (Abcam) (1:200), Laminin: ab30320 (1:200) are used. The secondary antibodies used were Alexa Fluor 488 goat anti-rabbit IgG A21206 (ThermoFisher) (1:400) and Alexa Fluor 586 goat anti-mouse IgG A11004 (ThermoFisher) (1:400).

Tensile Testing

Native and decellularized colons (n =3) were cut open longitudinally and sectioned into rectangular strips of about 20mm length and 3mm width. The thickness of the tissue was measured with a digital carbon fiber caliper (Fisher Scientific, MA). Thickness varied between 0.13mm and 0.15mm thickness. Sandpaper squares were secured to the ends of colonic strips with adhesive gel to help alleviate stress concentrations focused around the grips and to prevent slippage during testing. The colon was mounted on a dynamic analysis testing instrument and allowed to equilibrate in 1X PBS. The colon was preconditioned with 5 loading and unloading cycles at the rate of 0.5mm/min to reach 75kPa. Mechanical testing was performed using an RSA-G2 solids analyzer (TA Instruments, New Castle, DE). Triose software v4.4.0.41651 (TA Instruments, New castle, DE) was utilized to direct the experimental procedure and to collect raw data. Samples were secured between two stainless steel tension clamps within an immersion chamber with an initial grip to grip length of 5 mm. The immersion chamber was then filled

with PBS. Prior to testing, samples were preconditioned using 5 cycles to 2 percent strain. Immediately following the final preconditioning cycle, the samples were extended from the initial grip length at a cross head speed of 0.1 mm/s until failure (sample rupture). Data was collected at a frequency of 10 Hz and used to plot Cauchy stress vs strain plots and then used to identify the ultimate tensile strength (UTS), maximum force, and elongation at failure. The elastic modulus (Young's modulus) was calculated as the slope of the linear curve fit to the initial linear region of the stress-strain curve.

Cell Culture

Cancer cells, HT29 and HCT116 obtained from American Type Culture Collection were cultured in basal medium (4 parts Dulbecco modified essential medium and 1part M199; HyClone) supplemented with 10% cosmic calf serum. Human Colonic Epithelial Cells (HCEC) were cultured on Primaria dishes (Corning, NY) with HCEC medium which is made up of basal medium plus 5nM sodium selenite, 2ug/ml apo-transferrin, 20ng/ml epidermal growth factor, 10ug/ml insulin, 1ug/ml hydrocortisone, 2% cosmic calf serum and 50ug/ml gentamicin). All cell lines were expanded at low passages and preserved in liquid nitrogen. Mycoplasma contamination was tested for using an e-Myco PLUS mycoplasma PCR detection kit (Fisher Scientific, MA). All cells were grown in a 37^{o}C and 5% CO_2 incubator.

Recellularization of mice colon

Decellularized colon was conditioned in media for 1hr in a 37^{o}C and 5% CO_2 incubator. Decellularized colon was coated with fibronectin for 1hr by closing off both ends of the colon using male and female luer locks and manually injecting 1mg/ml human fibronectin into the

lumen of the decellularized colon from one end of it (Thermofisher Scientific, MA, 33016015).Then the colon was placed in a petri dish and inserted into a 37ºC and 5% CO_2 incubator. Afterwards, the bioreactor jar was filled with ~150mls of medium. The colons were connected to the perfusion pump by connecting the female luer lock of the tubing, inside the bioreactor jar, to the male luer lock that was inserted and tied to the colon. Next, air was removed, and the colons were filled with media by using a 30 ml syringe to remove air and draw media from the jar into the colon . 1.2 ml of media with single cell suspensions were manually injected into the upper 3-way stopcock towards the direction of the colon using a 30 ml syringe (**Figure 2.1**). The bioreactor jar and pump were then placed in an incubator (37^0 C, 5% CO_2). The tubings of the bioreactor jar were connected to the Masterflex pump and the cell suspension was perfused through the colon lumen at a flow rate 0.40 ml/min for 2hr then static flow (no perfusion) overnight. Colons were re-filled with media every other day till the culture was completed by using a 30ml syringe to draw media from the jar into the colon.

ddPCR

Recellularized colons was placed in an RNA stabilization agent (Thermofisher Scientific, MA) in 4ºC until use. Tissues were homogenized on ice and the QIAGEN RNeasy Plus Kit was used to extract RNA from cells in recellularized colonic tissues. Cells in Matrigel were extracted using cell recovery solution (354253. Corning, NY) following the manufacturer's instructions. RNA was isolated and quantitated using the QIAGEN RNeasy Plus Kit. Quality of the RNA was confirmed by agarose gel-based methods (29). ddPCR was done according to standard protocols (30). Expression levels are normalized to GAPDH. Sequence for villin primer is F, 5'-GCAGCATTACCTGCTCTACGTT-3' ; R, 5'-GCTTGATAAGCTGATGCTGTAATTT-3'.

Sequence for MUC 2 primer is F,5'-ACCACGTAGTTGATGCCAGA-3'; R, 5'-GTGGCATGCCCTACAAGA-3'. Sequence for GAPDH primer is F, 5'-GTTCACCCATGACGAAACA'; R,'5'GTTCACACCCATGACGAAACA-3'. Used probes (Roche) 45, 71 and 19 to detect GAPDH, villin and MUC2 droplets, respectively.

Extracellular matrix (ECM) protein extraction and western blot

Decellularized tissues were homogenized in Laemmli buffer. The homogenates were left on ice for 1 h with vortexing every 15 min. The lysate was then spun at 16,000 g for 5 min at 4°C. The supernatant was discarded and the pellet (insolubilized ECM proteins) was resuspended in 8 M urea buffer and agitated at 1400 rpm for 2 h at 37°C. Protein concentration was determined by measuring UV absorbance at 280 nm with a nano-drop spectrophotometer. 30 µg of ECM proteins were loaded for western blot experiments using previously described protocols [22]. Primary antibodies used for western blot experiments were Vitronectin SC74484 (Santa Cruz) (1:500) and ECM1 SC365335 (Santa Cruz) (1:500).

Mass spectrometry

Extracted ECM proteins (30 µg) were loaded into the resolving area of a pre-cast SDS PAGE gel, stained with Coomassie blue, cut into small cubes and sent to the UTSW proteomics core for analysis by mass spectrometry. Protein gel pieces were reduced and alkylated with DTT (20 mM) and iodoacetamide (27.5 mM). A 0.1 µg/µL solution of trypsin in 50 mM triethylammonium bicarbonate (TEAB) was added to completely cover the gel, allowed to sit on ice, and then 50 µL of 50 mM TEAB was added and the gel pieces were digested overnight (Pierce). Following solid-phase extraction cleanup with an Oasis HLB µelution plate (Waters), the resulting peptides were

reconstituted in 10 uL of 2% (v/v) acetonitrile (ACN) and 0.1% trifluoroacetic acid in water. 2 uL of this were injected onto an Orbitrap Fusion Lumos mass spectrometer (Thermo Electron) coupled to an Ultimate 3000 RSLC-Nano liquid chromatography systems (Dionex). Samples were injected onto a 75 μm i.d., 75-cm long EasySpray column (Thermo), and eluted with a gradient from 1-28% buffer B over 90 min. Buffer A contained 2% (v/v) ACN and 0.1% formic acid in water, and buffer B contained 80% (v/v) ACN, 10% (v/v) trifluoroethanol, and 0.1% formic acid in water. The mass spectrometer operated in positive ion mode.

MS scans were acquired at 120,000 resolution in the Orbitrap and up to 10 MS/MS spectra were obtained in the ion trap for each full spectrum acquired using higher-energy collisional dissociation (HCD) for ions with charges 2-7. Dynamic exclusion was set for 25 s after an ion was selected for fragmentation. Raw MS data files were analyzed using Proteome Discoverer v2.2 (Thermo), with peptide identification performed using Sequest HT searching against the mouse protein database from UniProt. Fragment and precursor tolerances of 10 ppm and 0.6 Da were specified, and three missed cleavages were allowed. Carbamidomethylation of Cys was set as a fixed modification and oxidation of Met was set as a variable modification. The false-discovery rate (FDR) cutoff was 1% for all peptides.

The abundance of each protein was normalized to total ECM protein abundance of the sample by dividing the abundance of each protein in adjacent or polyp sample with total protein abundance of that sample. i.e

$$\frac{protein\ 'A'\ abundance\ in\ polyp\ 1}{total\ abundace\ of\ ECM\ proteins\ in\ polyp\ 1}$$

- Then, the normalized abundance was averaged i.e

$$\frac{normalized\ protein\ 'A'\ abundance\ in\ polyp1 + normalized\ A\ in\ polyp\ 2}{2}$$

- Then polyp/wt abundance ratios were obtained by dividing normalized abundance average of each protein in polyps by normalized abundance average of each protein in adjacent normal tissue. i.e

$$\frac{normalized\ abundance\ average\ of\ protein\ 'A'\ in\ polyp}{normalized\ abundance\ average\ of\ protein\ A\ in\ adjacent\ normal\ tissue}$$

Invasion assay

Decellularized mouse colons (DMC) from wild type (WT) mice or colons from the genetically engineered colorectal cancer (CC) mice models (CPC;Apc) were flash frozen then lyophilized overnight. Colons were then ground into a powder, added to media and 125ug is mixed with 125ug of Matrigel^{TM} (at a total volume of 100 ul) and used to coat 24 well 8um invasion assay inserts (Corning, NY). Human colonic epithelial cells (HCEC) or HCT116 colorectal cancer cells were then added to the top of the coated insert in media without FBS. At the bottom of the invasion chamber media with chemoattractant (serum and growth factors) were added. The assay was allowed to progress for 48hrs and then the invasive cells at the bottom of the insert stained with a differential quik stain kit (Polysciences Inc, PA) and counted. The cells were counted at 100X magnification.

Statistical Methods

For statistical testing, GraphPad Prism version 8.0.0 for Mac, GraphPad Software, San Diego, California USA, www.graphpad.com was used. The Mann Whitney unpaired T-test was used for determining statistical significance between two samples. One-way ANOVA was used to test for

statistical significance among more than 2 samples and Dunn's multiple comparison test was used to identify significances between multiple samples. A test was considered significant if $p<0.05$. One star (*) was given if p-value was less than 0.05. If p-value was less than 0.01 then it was flagged with two stars (**) and if it was less than 0.001, it was flagged with three stars (***). Error bars represent the standard deviation of the mean.

Figures

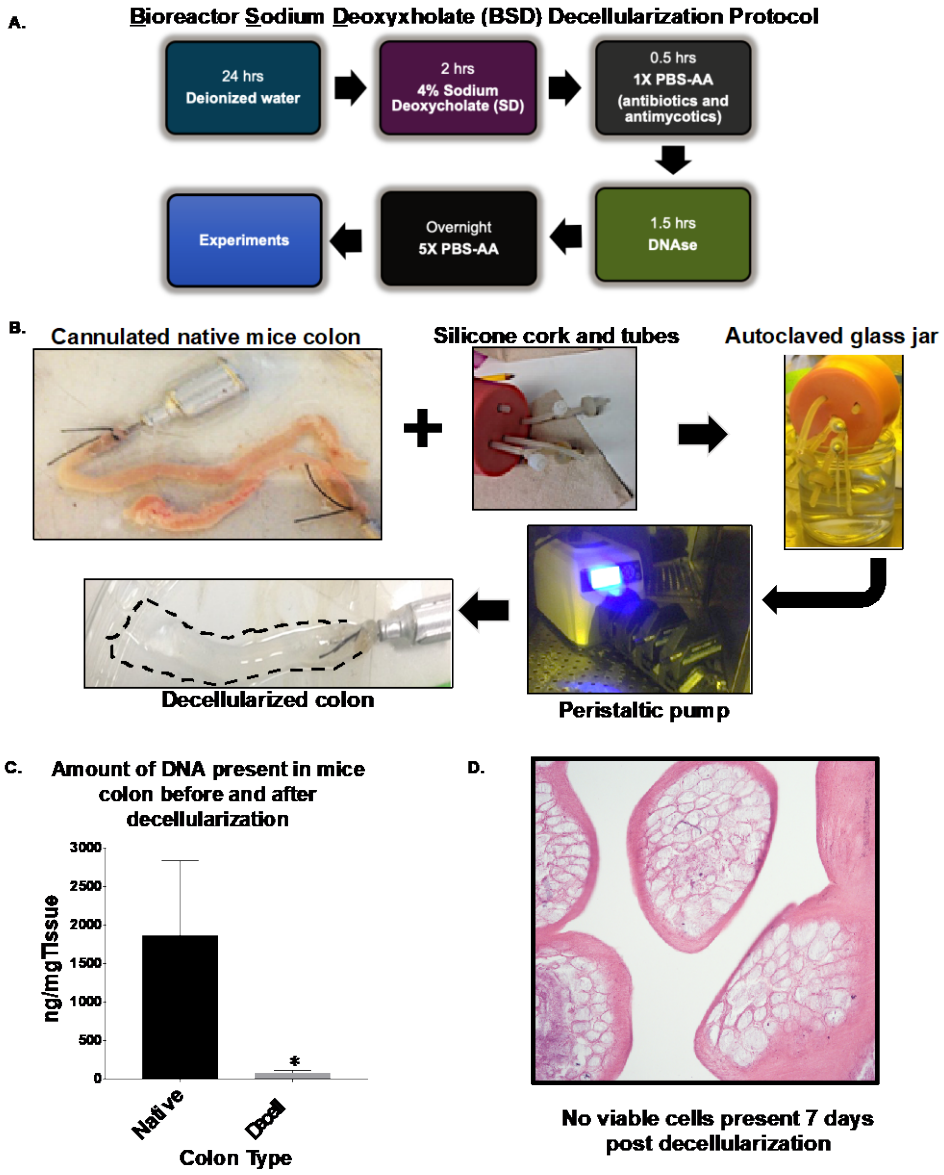


Figure 2. 1: **Decellularization process, DNA quantitation and tensile testing post decellularization of mice colons.**
A. Bioreactor Sodium-Deoxycholate DNAse (BSD Decellularization protocol. **B.** Snapshot of decellularization process. **C.** Quantitation of DNA present in colon with pico green assay using BSD protocol before and after decellularization. n= 4. Mann Whitney U-Test was used to test for statistical significance**.** $p<0.05$**. D.** H&E staining of un-reconstituted colonic tissue, 7 days after decellularization.

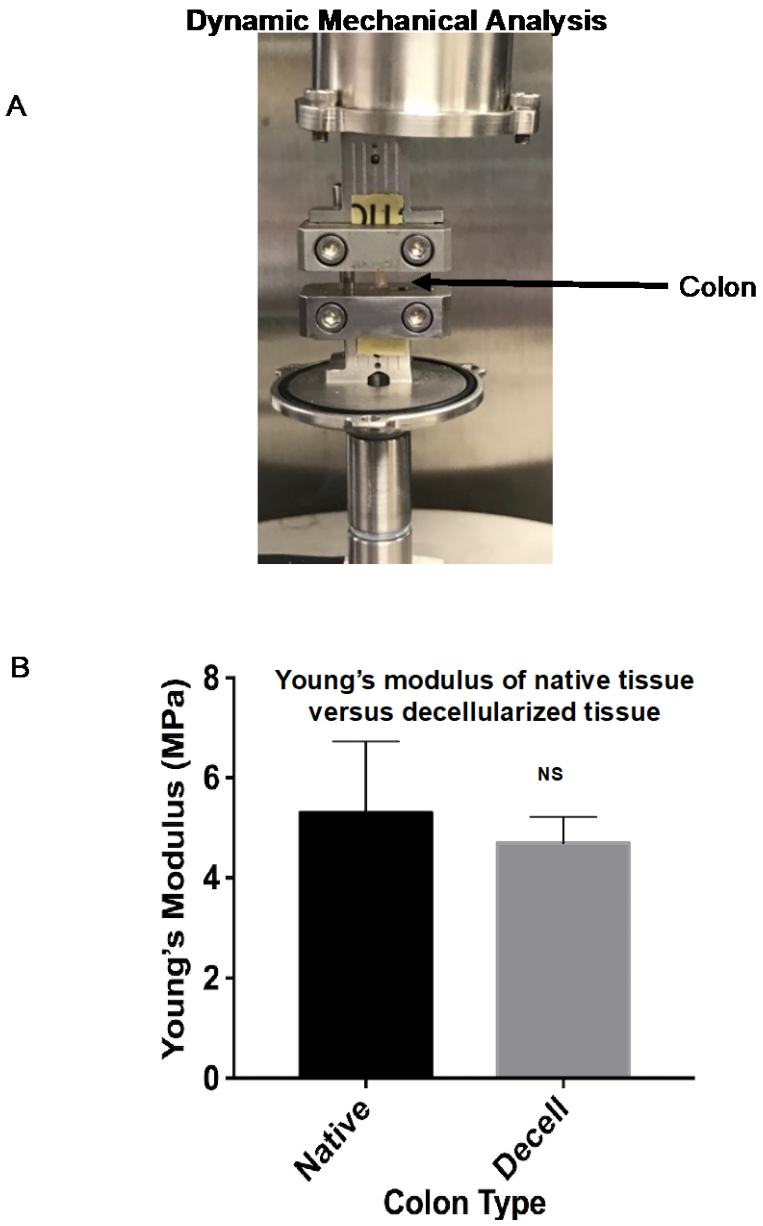


Figure 2. 2: **Decellularization process, DNA quantitation and tensile testing post decellularization of mice colons.**
A. Bioreactor Sodium-Deoxycholate DNAse (BSD Decellularization protocol. **B.** Snapshot of decellularization process. **C.** Quantitation of DNA present in colon before and after decellularization using pico green assay. n= 4. Mann Whitney U-Test was used to test for statistical significance**.** $p<0.05$**. D.** Dynamic analysis testing of colons before and after decellularization. $n\geq3$. $p<0.05$. Mann Whitney U-Test was used to test for statistical significance**.**

A.

Bioreactor or Agitation on Petri dish	Agent 1	Agent 2	Protocol Name
Bioreactor	1% Sodium dodecyl sulfate	1% Triton X-100	BST
Petri Dish	1% Sodium dodecyl sulfate	1% Triton X-100	PST
Bioreactor	4% Sodium deoxycholate	Deoxyribonuclease (DNAse)	BSD
Petri dish	4% Sodium deoxycholate	DNAse	PSD

B.

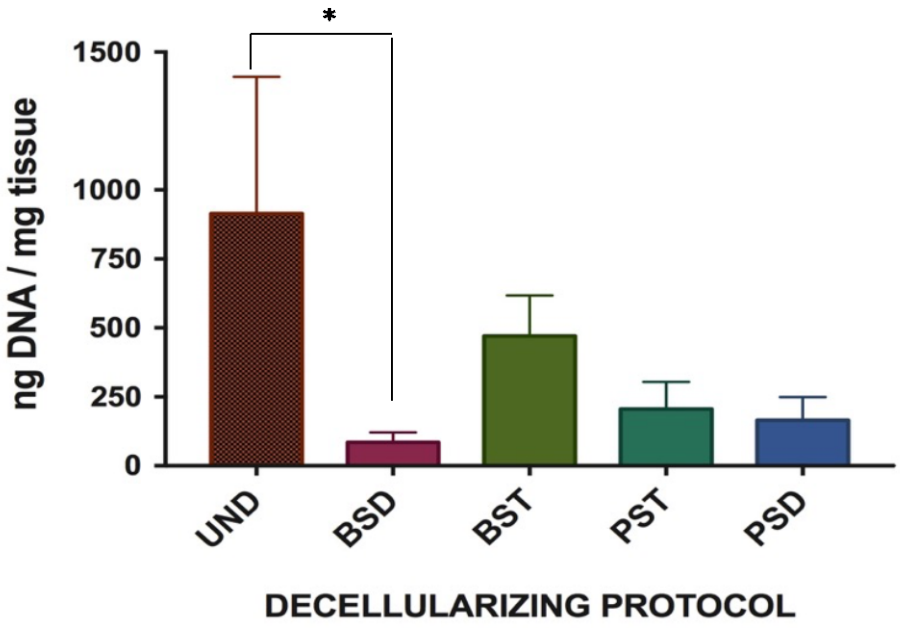


Figure 2. 3: **All decellularization protocols tested and their corresponding DNA quantitation.**
A. Table of the 2 different decellularization agent pairs and 2 decellularization methods, bioreactor or agitation incubation shaker. **B.** Quantitation of DNA present in colon before and after decellularization using pico green assay. n=>3. Significance difference ($p<0.05$) observed between DNA content in native colon and tissue decellularized via the BSD protocol. ANOVA and Dunn's multiple comparison test were used.

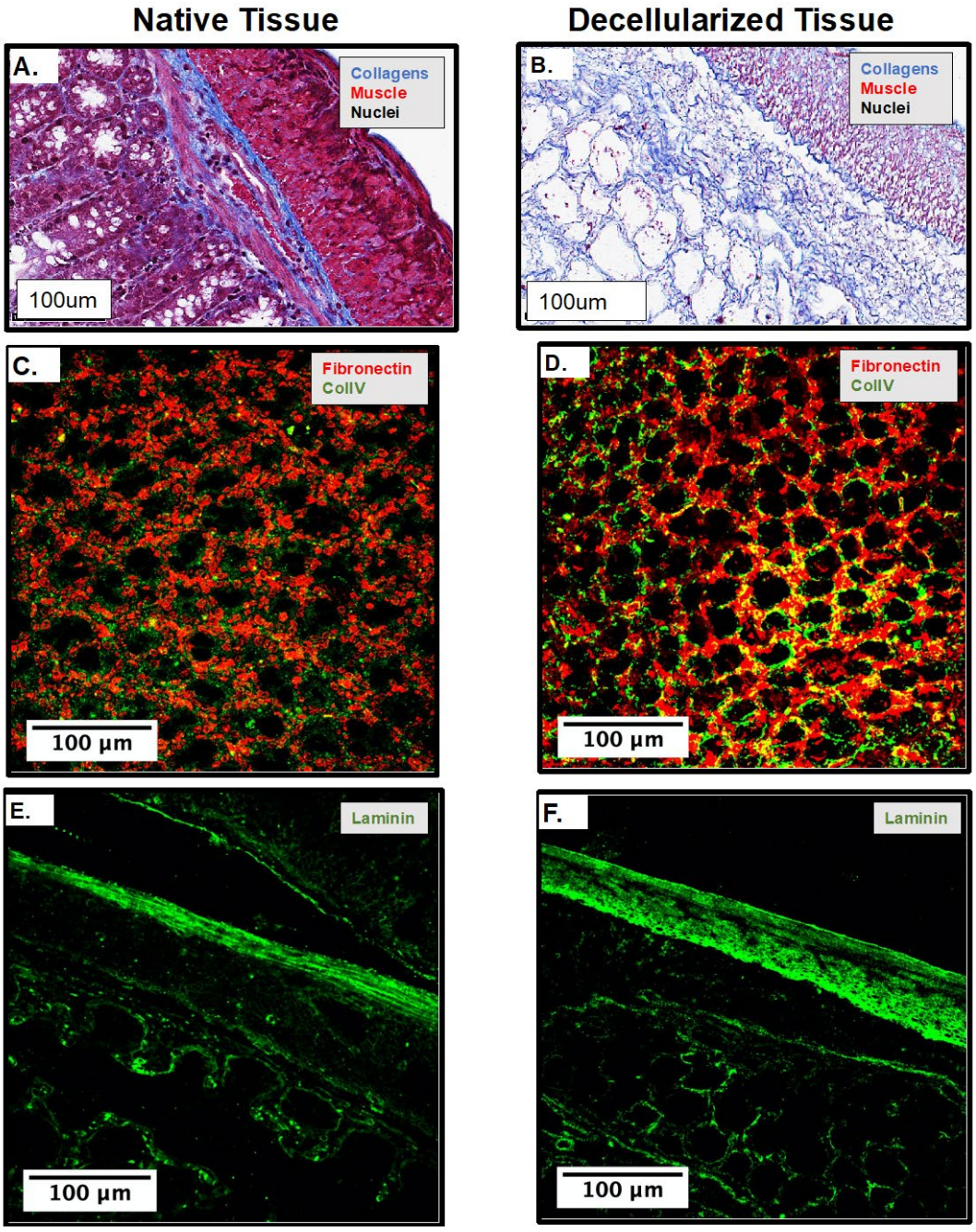


Figure 2. 4: **Identification of extracellular matrix proteins present post decellularization.**
A and **B.** Masson's Trichrome stain for collagens before and after decellularization. **C** and **D**. Immunofluorescence staining (IF) for Collagen IV and fibronectin. **E** and **F.** IF stain for laminin

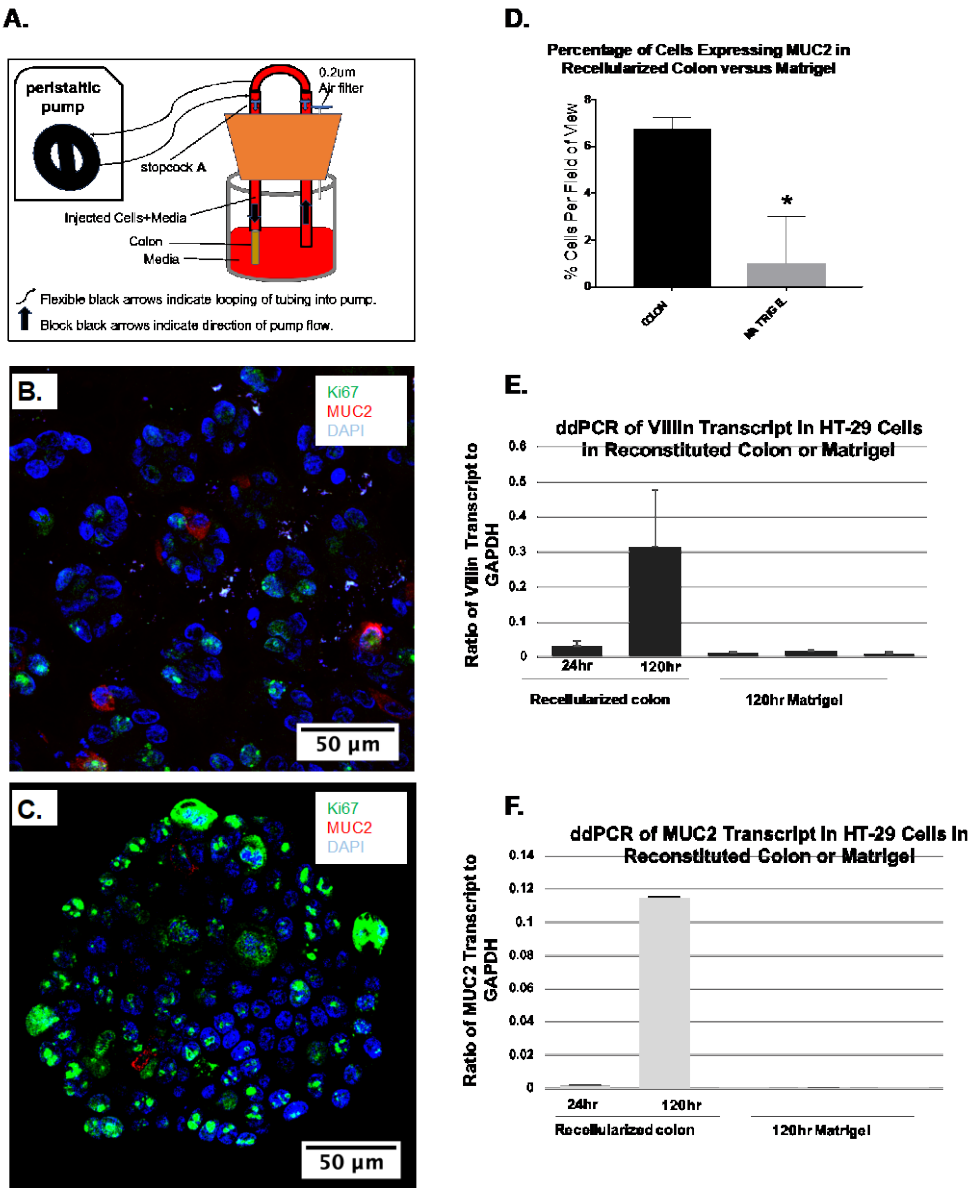


Figure 2. 5: **Significant presence of differentiated cells in decellularized matrices compared to Matrigel™.**
A Recellularization schematic. **B.** Immunofluorescence staining of proliferating (Ki67) and **C.** differentiated (MUC2) cells in decellularized matrix versus Matrigel ™ respectively. **D.** Quantitation of MUC2 expressing cells in decellularized matrix (colon) versus Matrigel™ . **E.** Digital droplet PCR (ddPCR) of Villin and **F.** MUC2 transcript of HT29 cells in colon or matrigel™. $p<0.05$. Mann Whitney U-Test was used to test for statistical significance**.**

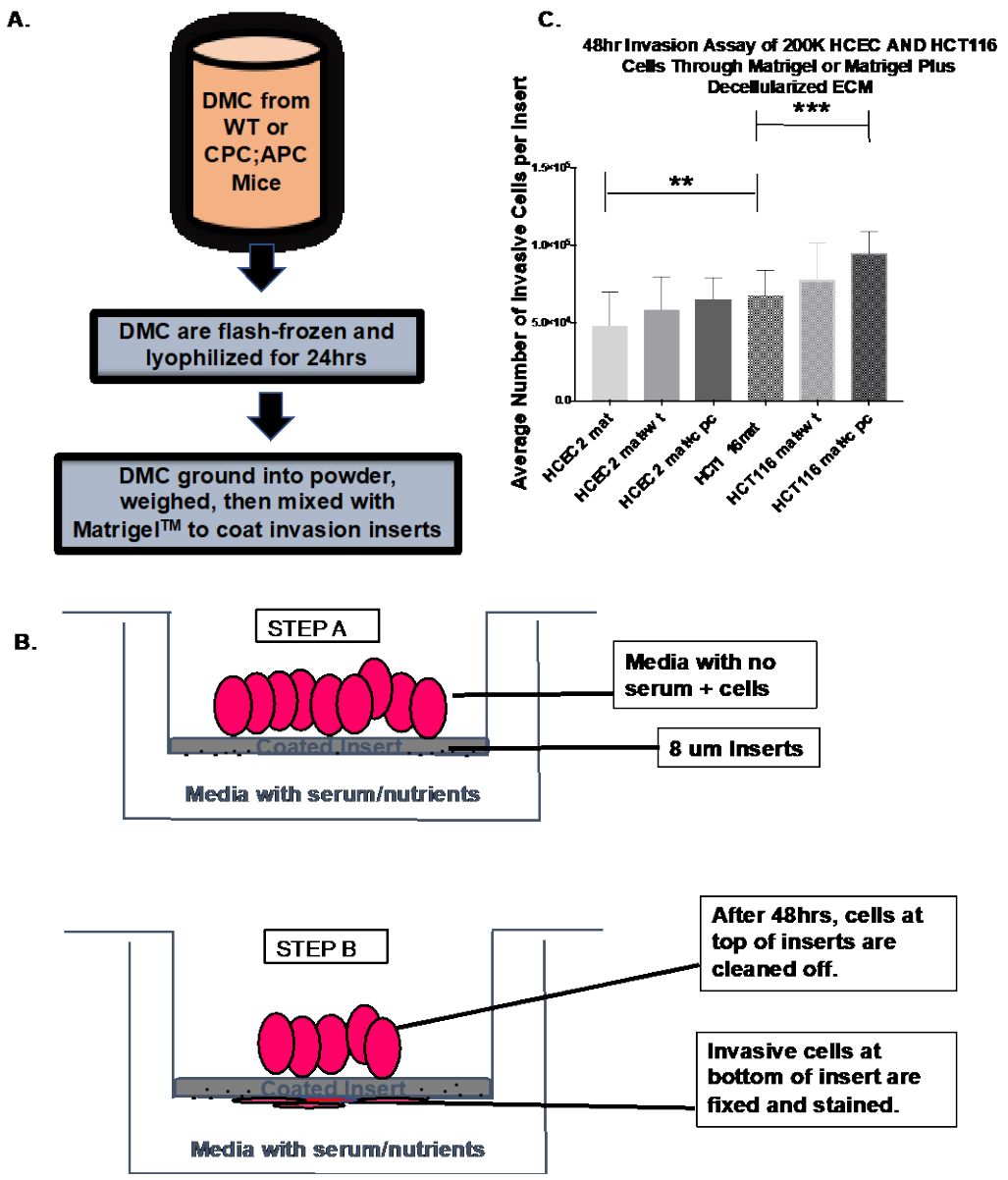


Figure 2. 6: **Invasion assay, identification and validation of ECM proteins with greater abundance in polyps**.
A. Schematic for decellularized tissue processing**. B**. Invasion assay schematic. **C.** Invasion assays of HCT116 and HCECs through invasion inserts coated with only Matrigel™ (mat) or Matrigel™ and normal DMC (HCT116 mat+wt) or Matrigel™ and DMC from mice with polyp (mat+cpc). $p<0.05$. ANOVA and Dunn's multiple comparison test were used.

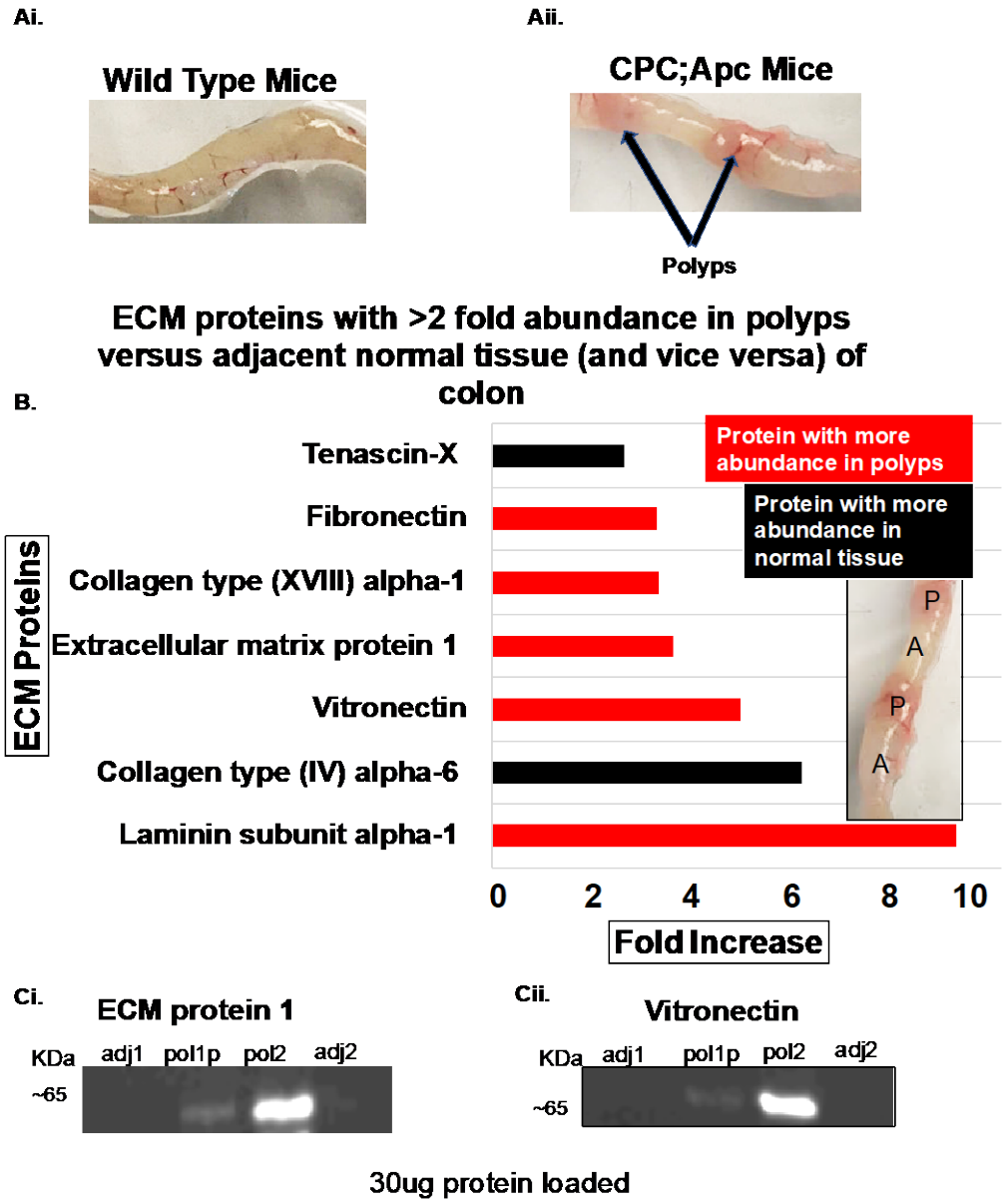
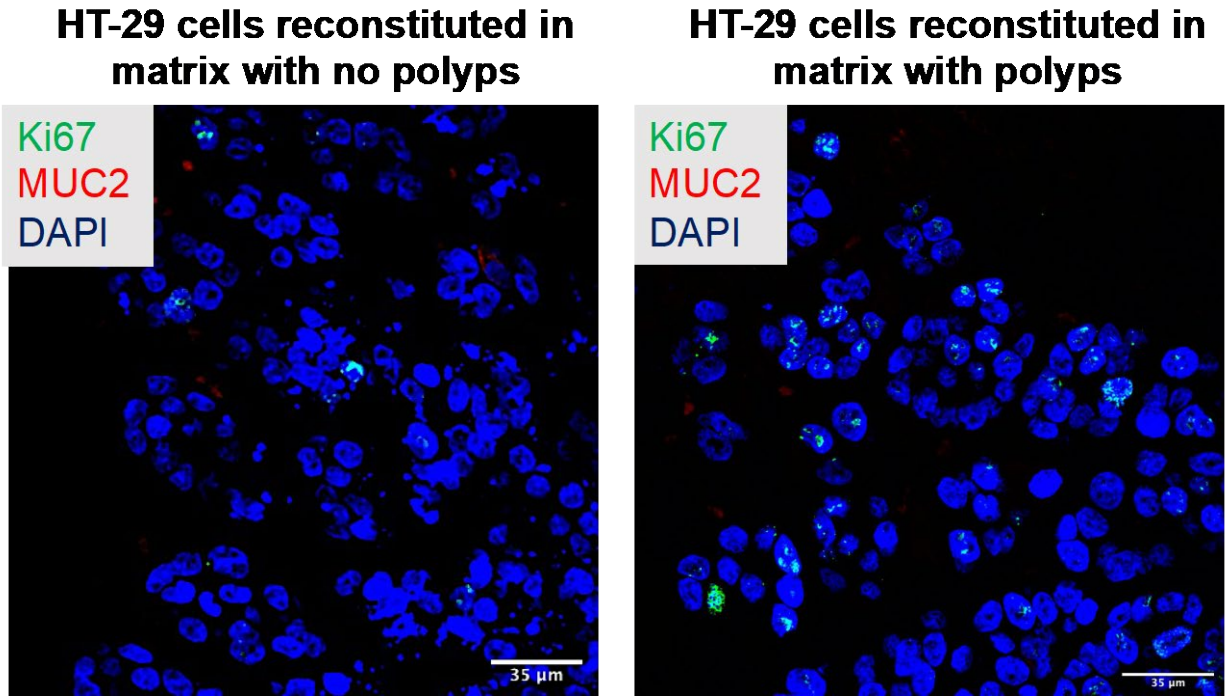
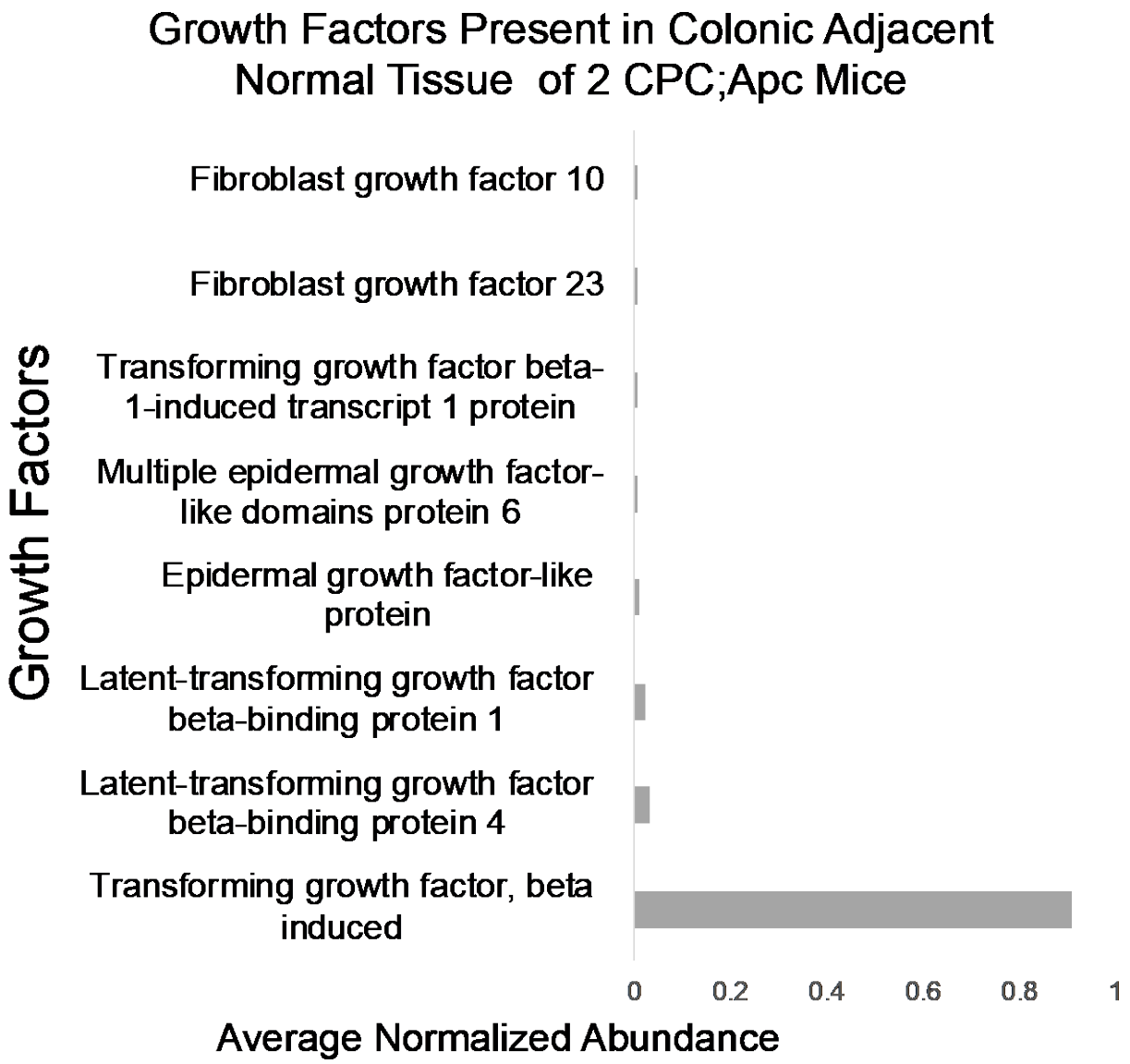


Figure 2. 7: **Invasion assay, identification and validation of ECM proteins with greater abundance in polyps**.
Ai Wild type mice at >200 days old and **Aii. CPC;Apc** mice at >200 days old. **B.** ECM proteins with greater fold abundance in polyp (red bars) or adjacent (black bars) regions of two 220 day old decellularized CPC;Apc male mice. Inset shows polyp (p) and adjacent (a) regions of CPC;Apc mice model. **Ci and Cii.** Western Blot shows expression of ECM protein 1 (**Ci)** and Vitronectin (**Cii**) in polyp region versus adjacent region. 30ug protein loaded



Supplementary Figure 2. 1 **More proliferative HT-29 cells found in reconstituted matrix of CPC;Apc mice with polyps compared to matrix without polyps after 5 days in culture.**



Supplementary Figure 2.2: **Growth factors and growth factor binding proteins present post decellularization in adjacent normal tissue.**

CHAPTER 3

Abstract

There is a need for the use of cell culture methods to model different aspects of cell behavior and develop therapeutics for diseases such as cancer. Methods such as 2D monolayer culture only partially reflect the in vivo physiology of cells in tissues. Placing cultured cells in 3D organoid culture is an improvement but still lacks most of the extracellular matrix components that occur in a tissue. The use of decellularized tissues may aid the evolution of ideal model systems to more closely reflecting the *in vivo* conditions. When cells are removed from a tissue without compromising tissue integrity, the decellularized tissue may provide a more physiologically relevant environment for cell growth and function. Decellularized tissues may also serve as excellent models for identifying genes important for cancer progression. In a previous study we reported that HT-29 human colon cancer cells have the ability to differentiate in decellularized tissue, similar to *in vitro,* and that decellularized tissue may provide information about extracellular matrix proteins that are important for colon cancer progression. In this study, I examined cell behavior of the human colon adenocarcinoma cell line, HCT116, and a cell line from a mice model of colon cancer. I also validated ECM proteins from our previous mass spectrometry data by using immunofluorescence staining and *in vitro* cell-culture assays. I observed that HCT116 cells were unable to differentiate in the decellularized tissue but instead migrated into adjacent tissue areas. To observe the effects of transcription factor Aryl hydrocarbon receptor (AhR) in tissue repopulation I knocked down the gene from a mice colon cancer cell line and used the cells to reconstitute decellularized mice colons. Knockdown of Aryl hydrocarbon receptor (AhR) reduced the repopulation ability of the mice colonic cancer cell lines. Finally, by analyzing and synthesizing mass spectrometry data, I observed that ECM

protein signatures are patterned: ECM proteins with greater abundance in mice polyps are also less abundant in young mice (that will still eventually develop polyps and later on tumors) while ECM proteins with more abundance in adjacent tissues were also more abundant in these young mice.

Introduction

Since, Hayflick explained that cells have a finite capacity to divide, there have been number of techniques and advances to increase cell life span, differentiation capacity and molecular function of cells *in vitro or ex vivo* (J. W. Shay & W. E. Wright, 2000). Over the years, some of the techniques to grow cells long-term include overexpression of viral oncogenes, including SV40 and B cell Moloney murine leukemia retrovirus-specific integration site 1 (Bmi1), and human telomerase reverse transcriptase by retroviral transfection (J. R. Peters-Hall et al., 2018). While these have been successful in helping cells continue to divide in vitro, there have been some drawbacks. These cells often lose their ability to differentiate and make some functional proteins that they are normally able to make *in vivo*. One example is the immortalization of human bronchial epithelial cells (HBECs) with retroviral transfected cyclin dependent kinase 4 (Cdk4) and hTERT (Peters-Hall, Coquelin et al. 2018). Although these cells grow for a long time in vitro, >100 PD, they exhibit genomic instability, lose differentiation ability and are unable to make functional CFTR (cystic fibrosis transmembrane receptor) (J. R. Peters-Hall et al., 2018). Another way that cells are able to be grown long-term is by expanding them as spheroids in Matrigel™. Matrigel™ contains basement membrane components from a mouse sarcoma cell line that enables it to support the proliferation and differentiation of multiple different primary

cell lines. Primary cells from different tissues of human and mice origin have been successfully grown, expanded and differentiated in Matrigel™ (Hu et al., 2018; Roig et al., 2010). However, expensive additional factors such as Wnt, Noggin and R-spondin usually need to be added to sustain the stem cell capacity and hence, the proliferative and differentiation capacity of the cells.

Where primary cells have proven expensive or difficult to expand or keep function ability, some cancer cells have historically been used to study cell behavior or in functional assays for drug testing because of their simplicity and reproducibility (Lea, 2015). For example, HT-29 colon cancer cells have been shown to have multipotent differentiation capacity *in vitro and ex vivo* (Alabi, LaRanger, & Shay, 2019; Lea, 2015). CacCo-2 cells are another colon cancer cell line with demonstrated differentiation capacity *in vitro* (Sambuy et al., 2005). The three pathways by which orally administered drugs can be absorbed into the intestine, including passive diffusion, carrier mediated/limited transport and vesicular transport, can be modeled and observed with the CaCo-2 cells (van Breemen & Li, 2005). Therefore, CaCo-2 cells are used in an FDA approved permeability assay to determine the absorption of orally administered drugs and xenobiotics being developed for clinical use (Larregieu & Benet, 2013). During this assay, the cells grown as monolayers on transmembrane inserts for 21 days to allow them to polarize and differentiate into intestinal absorptive cells with microvilli on the apical end and form tight junctions with each other (Sambuy et al., 2005). The resistance of the tight junctions can be measured with transepithelial/transendothelial electrical resistance (TEER) to confirm that the cells are fully differentiated. TEER is used to quantitatively measure resistance provided by tight junctions of epithelial or endothelial cells monolayers in cell culture models (Srinivasan et al., 2015). HT-29, another adenocarcinoma cell line also has this ability to form tight junctions with one another,

however, CaCo-2 cells have higher trans epithelial resistance. Further, CaCo-2 cells express transport proteins and various metabolizing enzymes on the apical membrane (Sambuy et al., 2005). These enzymes are not normally secreted by adult colonocytes but are secreted by enterocytes. in the body and are important for drug absorption including aminopeptidase, and sulfatase (Sambuy et al., 2005). However, one of the major differences of CaCo-2 cells is that, unlike normal enterocytes, they do not normally express the most common drug metabolizing enzyme-cytochrome p450 isozymes, including CYP3A4 (van Breemen & Li, 2005). Nonetheless, vitamin D can be used to induced these cells to express CYP3A4 (van Breemen & Li, 2005). During culture of the CaCo-2 cells, the basolateral chamber layer of the transmembrane set up is filled with media and changed every 4 days until a set resistance is reached. Then, the drug is incubated with the cells at 37^{o}C on a rotary shaker for about 1 to 2 hrs. Some volume of the media (plus drug solution) on the apical surface and media from the basolateral chamber is then removed and analyzed using liquid chromatography Mass Spectrometry (LC-MS) to calculate the rate of drug transport from the apical to the basal chamber. The drug or compound may also be placed in the basolateral chamber and the rate of its active transport or efflux across the CaCo-2 monolayer is measured (van Breemen & Li, 2005).

An example of another cell line model that has proven helpful in understanding the role of genes involved in colorectal cancer is the AKP (ApcΔ/ Δ;KrasG12D/+;Trp53Δ/ Δ (AKP) cell line . These cells can be grown as organoids or as monolayers in 2D. The cells that make up the AKP organoids are isolated from genetically modified mice with conditional *KrasLSL-G12D/+;Trp53fl/fl* mutations under the control of the CRE recombinase. The epithelial cells from the crypts are then established in Wnt, Rspondin and Noggin (WRN) medium before being

transfected with a pSECC plasmid that contains Cre recombinase, Cas9 nuclease and single guide RNA to target APC (Roper et al., 2017). Cre recombinase, adopted from bacteriophage P1, is an enzyme that allows for conditional insertion of DNA cassettes into eukaryote chromosomes and mutagenesis of transgenes (Van Duyne, 2015). Cas9 is an enzyme adopted from bacteria, that allows for targeted genome editing with the aid of a 20-nt targeting sequence called the guide RNA (Ran et al., 2013). Upon transfection of cells with the pSECC plasmid, CRE is transiently expressed and will cause the expression of the KRAS and TP53 mutations as well as the CRISPR-Cas9 gene editing of APC. Correctly edited cells are selected by growing them in media without Wnt/Rspo (APC deletion), Nutlin-3 addition (p53 deletion) and EGF withdrawal (KRAD ^{G12D}).

Recently, researchers further genetically edited these AKP cells by knocking down the gene AhR to study its role in modulating colon cancer regulation in Myc (avian myelocytomatosis viral oncogene homolog) regulated colon cancer cells (Lafita-Navarro et al., 2018). Aryl hydrocarbon Receptor (AhR) is a highly conserved ligand-activated transcriptional factor largely known for regulating the response of the body to xenobiotic agents (Cauchi et al., 2001; Ye et al., 2018). Although expressed in all tissues, AhR is expressed higher in organs such as the skin, liver, gut and lungs-the organs that provide first line defense to the body (Guerrina, Traboulsi, Eidelman, & Baglole, 2018). In the absence of ligands, AhR is present in the cytoplasm where it is stabilized by a core complex of proteins that consists of heat shock protein 90 (HSP90), immunophilin-like X-associated protein 2 (XAP2) and co-chaperone p23 (**Figure 3.1**) (Guerrina et al., 2018). It can be activated by both endogenous ligands such as environmental toxicant 2,3,7,8-tetrachlorodibenzo-p-dioixn (TCDD) or exogenous ligands including cigarette smoke and

tryptophan metabolites such as kynurenine (Guerrina et al., 2018). Upon activation by ligands, in the canonical signaling pathway, AhR and the core complex of proteins translocate into the nucleus. AhR will then dissociate from the core protein complex, heterodimerize with the aryl hydrocarbon receptor nuclear translocator (ARNT) and then bind to the dioxin response element (DRE) (**Figure 3.1**). This complex will in turn bind to target genes and facilitate their expression (Guerrina et al., 2018). *Cypa1a1*, a xenobiotic metabolizing enzyme (XME) is one of such gene (Guerrina et al., 2018). One of the ways AhR exerts its xenobiotic sensing function is by regulating the expression of CYPIA1 (Cauchi et al., 2001). CYPIA1 and other XMEs can metabolize xenobiotics and endogenous substances such as prostaglandins by catalyzing addition and unmasking reactions that make the chemicals water soluble, inactive and more easily excreted (Guerrina et al., 2018).

However, the role of AhR extends beyond chemical sensing as it has been found to be directly and indirectly important for progression of breast and gastric cancers, organ development and immunity (Guerrina et al., 2018). In fact, alterations in the CYPIA1 gene as well as over-expression of the AHR gene have been linked with lung cancer (Cauchi et al., 2001; Guerrina et al., 2018; San Jose et al., 2010; Ye et al., 2018). Other functions of AhR that have been more recently uncovered are its roles in activating transcription of genes needed for ribosome biogenesis and protein translation such as OGFOD1 and NOLC1 (Lafita-Navarro et al., 2018). It has been shown that oncogenic-Myc, a transcription factor that is frequently amplified in ~75% of human cancers is associated with, regulates, and hijacks this function of AhR to sustain the increased proliferation rate of cancer cells (Carabet, Rennie, & Cherkasov, 2018; Lafita-Navarro et al., 2018). In the case of colon cancer cells, it does this by increasing expression of

intracellular tryptophan transporters, SLC1A5 and SLC7A5 and also increasing expression of the enzyme AFMID that converts tryptophan to kyneurine, an activating ligand of AhR. (**Figure 3.2**) (Lafita-Navarro et al., 2018). Developing inhibitors against this pathway may provide the needed therapeutic cancer treatment that can indirectly target Myc transformed cancer cells. This is important because although Myc is a universal oncogene, it has been hard to target directly (Carabet et al., 2018). One reason for this is that Myc does not have an effective binding pocket on its surface and is instead, highly disordered. (Carabet et al., 2018). This makes it harder to characterize its interactions or employ structure based approaches to design Myc inhibitors. (Carabet et al., 2018). Furthermore, Myc is important for normal physiological functions including regulating growth of normal cells (Carabet et al., 2018). Consequently, there no clinically approved inhibitors of Myc (Carabet et al., 2018). Design of novel cell models to better study role of AhR in colon cancer cells is therefore pertinent.

Since drug failure is also linked to the environment in which the cells are being studied or drugs are being tested, design of therapeutics to target Myc that would be approved clinically, may require alternate cell culture systems. One such cell culture system is that provided by decellularized tissues (DTs). DTs, when properly made, are excellent models to study cell behavior because they still contain extracellular matrix proteins found in native tissue that typically influence cell behavior and drug action. Hence cells studied in DTs may better model cell function and response. Additionally, DTs also contain spatial-temporal tissue specific architectural properties, that may make it possible to seed multiple types of cells, seen in native tissue into the colon. For example, a decellularized human colon was reconstituted with multiple cell types, stepwise, including colonic epithelial cells, endothelial cells and myofibroblasts

(Chen, Wei et al. 2016). Specifically, a microinjection pipette was used to reconstitute the mucosa with endothelial cells (Chen, Wei et al. 2016). After attachment of the endothelial cells, the crypts were reconstituted with human colonic epithelial cells (HCECs) via gravity precipitation of the cells seeded on top of the crypts (Chen, Wei et al. 2016). Finally, the muscularis mucosa was manually separated from the submucosa so that myofibroblasts could be seeded into the exposed muscularis mucosa. This system was used to identify novel driver genes of colon cancer progression (H. J. Chen et al., 2016). Further, work that I have done, shows the usefulness of an automated perfusion bioreactor system to reconstitute colonic tissue with human colon cancer cells (Alabi et al., 2019). When multipotent cells were seeded into this scaffold, they had more populations of differentiated cells than proliferative cells compared to when they were seeded in Matrigel (Alabi et al., 2019). This is likely because this system better mimics *in vivo* conditions.

In normal tissue, homeostasis is tightly regulated even as the ECM is constantly being remodeled, when the tissue is degraded or synthesized to provides cues to cells (Filipe et al., 2018). However, in cancer, tissue homeostasis is dysregulated. This loss supports some of the hallmarks of cancer progression such as cancer cell invasion and metastasis (Filipe et al., 2018). Because of this and the role of the ECM in cell signaling, the extracellular matrix is a potential source for developing novel drug therapies to treat cancer. Therefore, there have been a number of efforts to identify tumor-microenvironment specific ECM proteins. Upon colonic tissue decellularization, I have identified 6 ECM proteins and 8 growth factors specific to the tumor microenvironment or normal adjacent tissue (Alabi et al., 2019). Others have looked into other cancer tissue such as breast, to identify ECM proteins that are dysregulated in tumor

microenvironment compared to normal tissue (Naba et al., 2014). Consequently, there have been a number of efforts to develop drugs that target ECM proteins impaired during colorectal cancer progression. Examples of such efforts include drugs to target the proteoglycan components of the extracellular matrix system such as JG3 (oligomannurarate sulfate) and RK-682 which targets heparanase. (Ishida et al., 2004).

In this study I reconstituted decellularized tissue with HCT116, an aggressive human colon cancer cell line that has not been shown to have differentiation ability (Yeung, Gandhi, Wilding, Muschel, & Bodmer, 2010). In comparison to HT-29, which I have shown in previous work to be able to differentiate *ex vivo,* the HCT116 cells, expectedly did not differentiate *ex vivo*. Interestingly, the cells also seemed to migrate into nearby muscle tissue unlike HT-29 cells. I also used the decellularized tissue as a model to visualize the role of the transcription factor, AhR in the tissue repopulation capacity of isogenic cell lines of AKP. I found that knockdown of AhR reduced repopulation of AKP cells in decellularized tissue. Next, mass spectrometry of young colon cancer mice model, CPC;Apc mice compared to mass spectrometry data of older progressed CPC;Apc mice revealed patterning of microenvironments. Specifically, ECM proteins that were more abundant in tumors of older and progressed CPC;Apc mice were less abundant in young mice and vice versa, for the adjacent normal tissue. Additionally, I validated mass spectrometry results of ECM proteins via *in vitro* assays and western blotting. I showed that fibronectin, an ECM protein that by mass spectrometry is more abundant in the tumor microenvironment and less abundant in young mice, increased cell growth of AKP cells in *in vitro* assays. Finally, I showed that tenascin-X was more abundant in areas of colon without polyps versus adjacent normal tissue.

Results

HCT116 Colon Cancer Cells do not Differentiate *Ex Vivo.*

10 million HCT116 and Human Colonic Epithelial Cells (HCEC) were each used to reconstitute decellularized colonic tissue for 5 days using an automated bioreactor perfusion system (**Figure 3.3 A-B**). The cells were perfused at a flow rate of 40ul per min for 2hrs in a 1.2 ml cell suspension. H&E staining shows that HT-29 cells (**Figure 3.3A**) appear to have some differentiated cell type (MUC-2 producing cells) while HCT116 cells show no sign of differentiating into more specialized cell types of the colon (**Figure 3.3B**). However, as expected of aggressive cancer cell lines, they are more migratory and can be seen occupying areas in the muscle layer of the colonic tissue.

AhR loss Impacts AKP Cell Reconstitution Ability *Ex Vivo*

Cell population doublings (PD) are a better estimate of cell growth (Greenwood et al., 2004). Both AhR cells and AKP +shAhR (AKPH) had similar rates of population doublings in 2D *in vitro* assays (**Figure 3.4 A-C**). However, when 6 million AKP and shAhR AKP cells were each used to reconstitute decellularized colons for 3 days, there were on average, 80% more AKP cells in decellularized tissue compared to shAhR AKP cells (**Figure 3.4 D-F**). This indicates the importance of AhR in cell attachment or cell migration.

Microenvironment Signature Patterning

Mass spectrometry of young CPC;Apc mice showed relationships between normal and tumor microenvironments and ECM protein abundance (**Table 3.1**). ECM proteins such as laminin subunit alpha 1 and vitronectin were found to be highly abundant in the tumor microenvironment of old >220 day old CPC;Apc mice. At this stage many of the mouse have bloody stools and have low body weights compared to wild type mice or heterozygous counterparts. These are all hallmarks of colon cancer. These same proteins were present in lower abundance in young mice with no visible polyps. The converse was also the case. Tenascin-X and collagen type IV alpha 6 which were more abundant in the normal adjacent areas of the old CPC;Apc mice and were also more abundant in young CPC;Apc mice. Collagen type vi alpha 2 and collagen type iv alpha 1 served as control as they were present in almost equal abundance in young mice and in the polyps and normal adjacent tissue of old CPC;Apc mice.

Tenascin-X is More Abundant in Mice Normal Tissue Areas Compared to Tissue Areas with Polyps

To validate the mass spectrometry results that quantitatively showed that tenascin-X is less abundant in the tumor microenvironment, immunofluorescence staining (IF) of tenascin-X was done on progressed CPC;Apc mice (**Figure 3.5**). The IF analyses confirmed that tenascin-X is indeed more abundant in areas with no polyps versus areas with polyps of the same mice progressed CPC;Apc mice.

Colon Cancer Cells and not Normal Epithelial Cells have Increased Growth in the Presence of Fibronectin.

HCEC, AKP and AKPH were grown on tissue culture plates with and without fibronectin coating. While AKP and AKPH cells (**Figure 3.6 A-B**) resulted in a significant increase in cell growth as shown by population doubling increase, HCECs did not (**Figure 3.6C**). This suggests that intragenic signaling from mutated genes that regulate cancer development and progression, as well as ECM protein signaling are both needed for cancer progression.

Discussion

The decellularized culture system allowed the modeling and visualization of two important characteristics of aggressive cancer cell lines. The first is the inability to differentiate and the other is increased migration (Jogi, Vaapil, Johansson, & Pahlman, 2012). Both cancer hallmarks are usually difficult to model in *in vitro* assays at the same time. HT-29 cells, in contrast, have been found to be genetically similar to normal cells (Jogi et al., 2012); they did not migrate into nearby areas as HCT116 cells did. The differentiation ability of HT-29 cells was confirmed in a separate study (Alabi et al., 2019).

Since AhR and AhRH are isogenic cell lines with the only difference between them being the knockdown of AhR, I attribute the loss in reconstitution ability to the loss of AhR. Although, AhR has not been directly implicated in cell migration by others, its role in biogenesis and protein translation indirectly links it to cell migration. Some publications have highlighted the contribution of protein translation and ribosome biogenesis to cell migration (Prakash et al.,

2019). In the future, I plan to reconstitute CPC;Apc mice colon with AhR cells to further highlight the importance of the contribution of the extracellular matrix to cell adhesion/migration. My hypothesis is that AhR cells will regain ability to better repopulate decellularized colon, especially in regions where polyps are found, because of the abundance of extracellular matrix proteins to enhance cell signaling for proliferation and migration.

Abundance of tenascin-X (gene-TNXB) in normal regions of CPC;Apc mice colonic tissue compared to tumor region confirms the previous mass spectrometry experiments that quantitatively established this feauture. Tenascin-X is important for making connective tissue within joints and skin (Petersen & Douglas, 2013). The deficiency of TNXB is well known as one of the common causative agents of Ehlers Danlos syndrome (Petersen & Douglas, 2013). Interestingly, there have been reports linking Ehlers-Danlos syndrome and gastrointestinal disorders including constipation, rectal prolapse, abdominal pain, diverticulosis and hiatal hernia and impaired colon-neurons sensorimotor functions that leads to attenuated colonic movements (Aktar et al., 2018). It is possible that some of these gastrointestinal disorders are linked to the colon and could contribute to tumor development especially when there are other cell genetic alterations. In a very recent study where *in silico* analysis was done based on information obtained from the gene expression omnibus, cancer genome atlas and immunohistochemistry of tissue microarrays, it was found that there is a significant loss of tenascin-X expression during progression of tumors including colon, lung, breast and liver tumors (Sophie Liot, 2020).

In the present studies, I also validated the role of fibronectin in modulating cell growth of colon cancer cells as well as normal colonic epithelial cells. This was based on data from mass

spectrometry that compared ECM proteins from polyps versus adjacent tissue and showed that fibronectin is more abundant in areas of mice colonic polyps. Past research has also established the link between colon cancer progression and fibronectin alteration as we have described in chapter 2. Therefore, the ability of fibronectin to increase growth of colon cancer cells was predicted. Interestingly, the normal human colonic epithelial cells did not have a significant increase in cell growth. Cell growth rates remained about the same when they were cultured on fibronectin coated plates. This suggests that inhibitors specific to fibronectin may be a potential therapeutic target. However, further tests, both *in vitro* and *ex vivo* still need to be done especially since fibronectin is expressed by the ECM of normal tissues, as well and is important for normal tissue physiological functions.

Methods

Decellularization

Colonic tissue was harvested from BL6 mice under UTSW IACUC approved protocol number 2016-101375. The essential components of the colonic decellularization system include; a peristaltic pump (Cole Parmer: Master flex EW-07522-30) to perfuse decellularization agents, an autoclavable glass jar and a rubber stop-cork that houses silicone tubings (Cole Parmer: Masterflex EW-96410-14) and covers the glass jar (**Figure 2.1**). The tubings are connected to the peristaltic pump and serve as conduits for aseptically transporting decellularization agents perfused in a circular motion by the bioreactor from the glass jar, through the colon and back into the glass jar. After the colon was isolated from each mouse, a male luer lock connector was inserted into one end of the colon. Next, the male luer lock was tied to the colon with black silk

suture spools (Surgical Specialties, PA). The colon was then flushed with 1X PBS + 5% antibiotics and antimycotics (5XPBSAA) (Gemini Bio Products, CA) using a 30 ml syringe (BD: 309650) to remove bacteria and fecal matter up to three times. A male luer lock was also inserted and tied to the other end of the colon. Colon was flushed again with 5X PBSAA. Then, an autoclaved glass jar was filled with deionized water (DW). One of the male luer locks was next carefully connected in a clockwise fashion to the female luer lock of a decellularization tubing in the bioreactor jar to connect the colon to the peristaltic pump and also set it in place, in the glass jar filled with DW. Bubbles were removed from the colon and tubings using a clean 30 ml syringe and the colon was filled with DW water, from the jar, with the same 30ml syringes. Next, DW was perfused through the lumen of the colon, with the peristaltic pump, overnight at 4^{o}C. The next day 4% sodium deoxycholate (SD) was perfused through the colon for 2hrs at RT. This was followed by PBSAA (1% antibiotic and antimycotic solution) for 30mins at RT. Next, 2000 Kunitz of DNAse, diluted in 1M NaCl, was perfused though the colon for 1.5hrs. Colons were stored overnight at 4^{o}C in the bioreactor jar containing PBSAA without perfusion and then used for downstream experiments. Throughout the decellularization experiment flow was always generated by the peristaltic pump at a flow rate of 1ml/min.

Decellularized Tissue Reconstitution

Decellularized colon was conditioned in media for 1hr in a 37^{o}C and 5% CO_2 incubator. Decellularized colon was coated with fibronectin for 1hr by closing off both ends of the colon using male and female luer locks and manually injecting 1mg/ml human fibronectin into the lumen of the decellularized colon from one end of it (Thermofisher Scientific, MA, 33016015). Then the colon was placed in a petri dish and inserted into a 37^{o}C and 5% CO_2 incubator.

Afterwards, the bioreactor jar was filled with ~150mls of medium. The colons were connected to the perfusion pump by connecting the female luer lock of the tubing, inside the bioreactor jar, to the male luer lock that was inserted and tied to the colon. Next, air was removed, and the colons were filled with media by using a 30 ml syringe to remove air and draw media from the jar into the colon . Approximately 1.2 ml of medium with single cell suspensions were manually injected into the upper 3-way stopcock towards the direction of the colon using a 30 ml syringe (**Figure 2.5**). The bioreactor jar and pump were then placed in an incubator (37^0 C, 5% CO_2). The tubings of the bioreactor jar were connected to the Masterflex pump and the cell suspension was perfused through the colon lumen at a flow rate 0.04 ml/min for 2hr to allow twice the volume of cell suspension to perfuse through the colon, thereby ensuring that all the cells go through the colon. then static flow (no perfusion) overnight. Colons were re-filled with media every other day until the culture was completed by using a 30ml syringe to draw media from the jar into the colon.

Immunofluorescence

After removal from the bioreactor, tissue was fixed and embedded. Then sections were de-paraffinized in xylene, placed in 100%, then 95% ethanol, and finally rehydrated in deionized water. The tissue sections were then unmasked in a microwave in 10mM sodium citrate buffer pH 6 at power 5 for 20 minutes then allowed to cool for 30 minutes. Tissue was then blocked with 1X PBS+5% normal serum + 0.3% Triton X-100 at room temperature. Tissue was then incubated with primary antibodies overnight. The next day the slides were washed in 1X PBS 3 times and then incubated with a secondary antibody for 1hr in the dark. Finally, the slides were washed with PBS for 5 mins 3 times then mounting medium H-1500 (Vector Laboratories, CA)

was added with DAPI and covered with coverslips. For immunofluorescence (IF) staining the following primary antibodies, Ki67: ab15580 (Abcam) (1:1000), MUC-2: sc-7314 (1:200). The secondary antibodies used were Alexa Fluor 488 goat anti-rabbit IgG A21206 (ThermoFisher) (1:400) and Alexa Fluor 586 goat anti-mouse IgG A11004 (ThermoFisher) (1:400).

2D cell culture

Cancer cells, HT29 and HCT116 obtained from American Type Culture Collection were cultured in basal medium (4 parts Dulbecco modified essential medium and 1part M199; HyClone) supplemented with 10% cosmic calf serum. AKP 2D cells were obtained from the Maralice Conacci-Sorrell lab at the University of Texas Southwestern Medical Center. The cells were grown in basal medium plus 5% cosmic calf serum and 5% antibiotics and antimycotics. Mycoplasma contamination was tested for using an e-Myco PLUS mycoplasma PCR detection kit (Fisher Scientific, MA). All cells were grown in a 37ºC and 5% CO_2 incubator.

Fibronectin culture assay

Human colonic epithelial cells (100,000), AKP (50,000) and HCT116 (50,000) cells were cultured in 6-well fibronectin pre-coated plates (Corning) (354402). A Bio-rad TC-20 automated cell counter was used for cell counts. Population Doublings (PD) was calculated using the formula (log (number of cells on day 5-number of cells on day 0))/0.3. Human Colonic Epithelial Cells (HCEC) were cultured on Primaria dishes (Corning, NY) with HCEC medium which is made up of basal medium plus 5nM sodium selenite, 2ug/ml apo-transferrin, 20ng/ml epidermal growth factor, 10ug/ml insulin, 1ug/ml hydrocortisone, 2% cosmic calf serum and 50ug/ml

gentamicin). HCEC culture plates were also placed in low-oxygen (2%) containers placed before incubation at a 37ºC and 5% CO_2

Extracellular matrix (ECM) protein extraction and western blot

Decellularized tissues were homogenized in Laemmli buffer. The homogenates were left on ice for 1 h with vortexing every 15 min. The lysate was then spun at 16,000 g for 5 min at 4°C. The supernatant was discarded and the pellet (insolubilized ECM proteins) was resuspended in 8 M urea buffer and agitated at 1400 rpm for 2 h at 37°C. Protein concentration was determined by measuring UV absorbance at 280 nm with a Nano-drop spectrophotometer. 30 µg of ECM proteins were loaded for western blot experiments using previously described protocols [22]. Primary antibodies used for western blot experiments were Vitronectin SC74484 (Santa Cruz) (1:500) and ECM1 SC365335 (Santa Cruz) (1:500).

Mass spectrometry

Extracted ECM proteins (30 µg) were loaded into the resolving area of a pre-cast SDS PAGE gel, stained with Coomassie blue, cut into small cubes and sent to the UTSW proteomics core for analysis by mass spectrometry. Protein gel pieces were reduced and alkylated with DTT (20 mM) and iodoacetamide (27.5 mM). A 0.1 µg/µL solution of trypsin in 50 mM triethylammonium bicarbonate (TEAB) was added to completely cover the gel, allowed to sit on ice, and then 50 µL of 50 mM TEAB was added and the gel pieces were digested overnight (Pierce). Following solid-phase extraction cleanup with an Oasis HLB µelution plate (Waters), the resulting peptides were reconstituted in 10 uL of 2% (v/v) acetonitrile (ACN) and 0.1% trifluoroacetic acid in water. 2 uL of this were injected onto an Orbitrap Fusion Lumos mass spectrometer (Thermo Electron) coupled

to an Ultimate 3000 RSLC-Nano liquid chromatography systems (Dionex). Samples were injected onto a 75 μm i.d., 75-cm long EasySpray column (Thermo), and eluted with a gradient from 1-28% buffer B over 90 min. Buffer A contained 2% (v/v) ACN and 0.1% formic acid in water, and buffer B contained 80% (v/v) ACN, 10% (v/v) trifluoroethanol, and 0.1% formic acid in water. The mass spectrometer operated in positive ion mode.

MS scans were acquired at 120,000 resolution in the Orbitrap and up to 10 MS/MS spectra were obtained in the ion trap for each full spectrum acquired using higher-energy collisional dissociation (HCD) for ions with charges 2-7. Dynamic exclusion was set for 25 s after an ion was selected for fragmentation. Raw MS data files were analyzed using Proteome Discoverer v2.2 (Thermo), with peptide identification performed using Sequest HT searching against the mouse protein database from UniProt. Fragment and precursor tolerances of 10 ppm and 0.6 Da were specified, and three missed cleavages were allowed. Carbamidomethylation of Cys was set as a fixed modification and oxidation of Met was set as a variable modification. The false-discovery rate (FDR) cutoff was 1% for all peptides.

The abundance of each protein was normalized to total ECM protein abundance of the sample by dividing the abundance of each protein in adjacent or polyp sample with total protein abundance of that sample. i.e

$$\frac{protein\ 'A'\ abundance\ in\ polyp\ 1}{total\ abundace\ of\ ECM\ proteins\ in\ polyp\ 1}$$

- Then, the normalized abundance was averaged i.e

$$\frac{normalized\ protein\ 'A'\ abundance\ in\ polyp1 + normalized\ A\ in\ polyp\ 2}{2}$$

- Then polyp/wt abundance ratios were obtained by dividing normalized abundance average of each protein in polyps by normalized abundance average of each protein in adjacent normal tissue. i.e

$$\frac{normalized\ abundance\ average\ of\ protein\ 'A'\ in\ polyp}{normalized\ abundance\ average\ of\ protein\ A\ in\ adjacent\ normal\ tissue}$$

Statistical Methods

For statistical testing, GraphPad Prism version 8.0.0 for Mac, GraphPad Software, San Diego, California USA, www.graphpad.com was used. The Mann Whitney unpaired T-test was used for determining statistical significance between two samples. One-way ANOVA was used to test for statistical significance among more than 2 samples and Dunn's multiple comparison test was used to identify significances between multiple samples. A test was considered significant if $p<0.05$. One star (*) was given if p-value was less than 0.05. If p-value was less than 0.01 then it was flagged with two stars (**) and if it was less than 0.001, it was flagged with three stars (***). Error bars represent the standard deviation of the mean. Automated counting of reconstituted AKP cells was done using color segmentation plug-in in Fiji software (Schindelin et al., 2012).

Figures

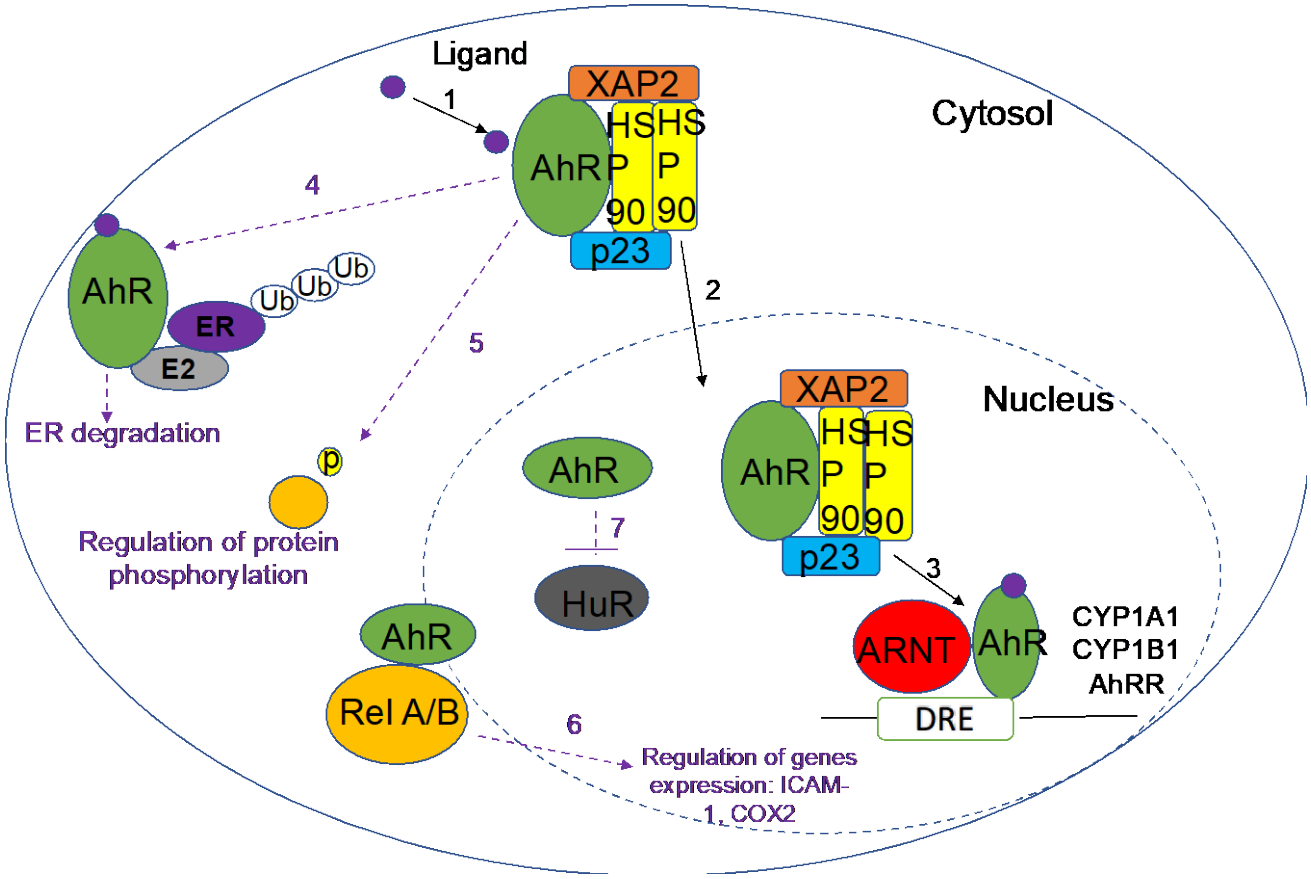


Figure 3.1: **Visual representation of Aryl Hydrocarbon Receptor (AhR) signaling AND degradation pathways**.
Adapted from Guerrina, Necola et al. 2018

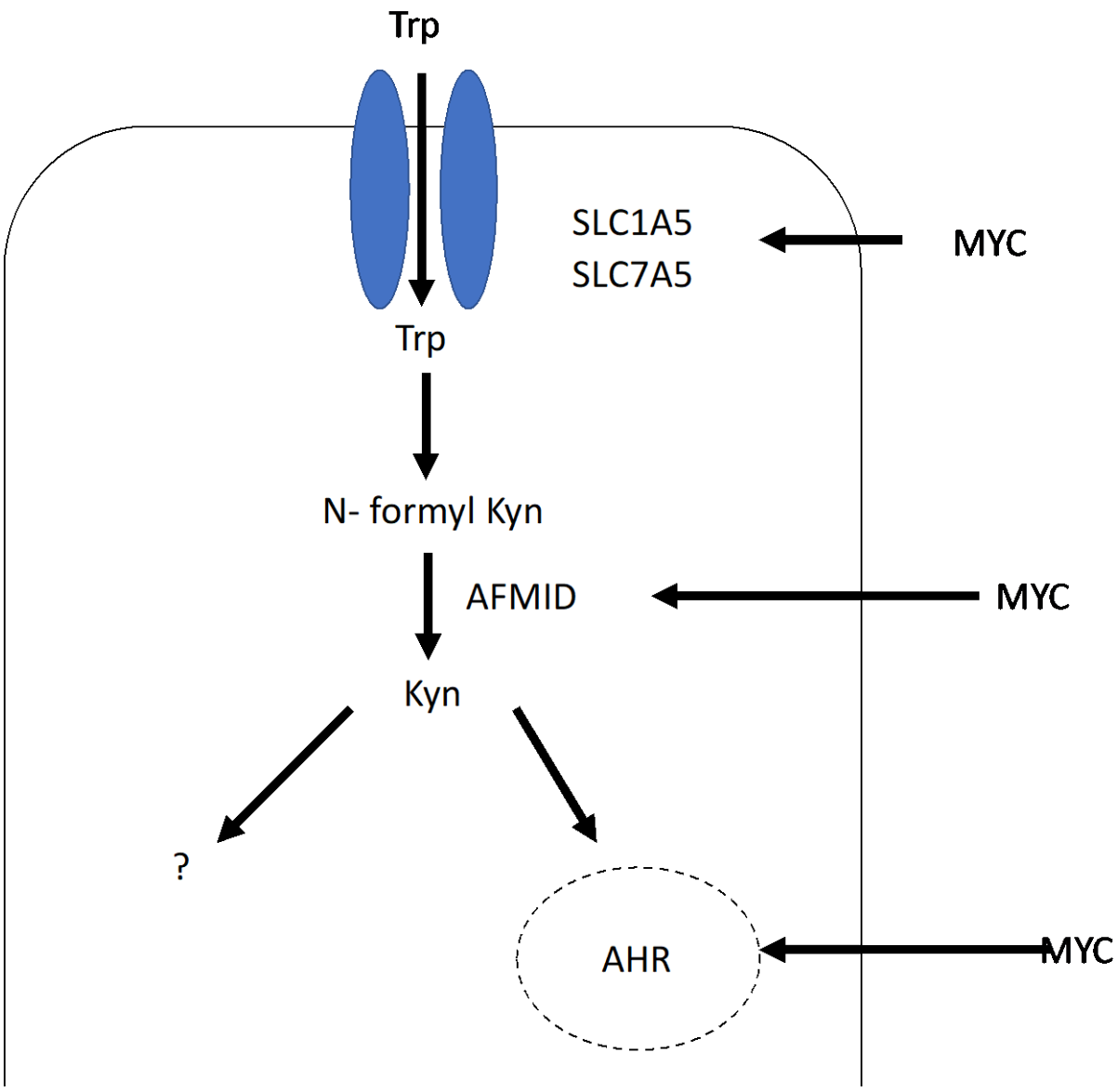


Figure 3.2: **Visual representation of Myc-regulated tryptophan and AhR signaling**.
Adapted from Lafita-Navarro, M.C. et al 2018.

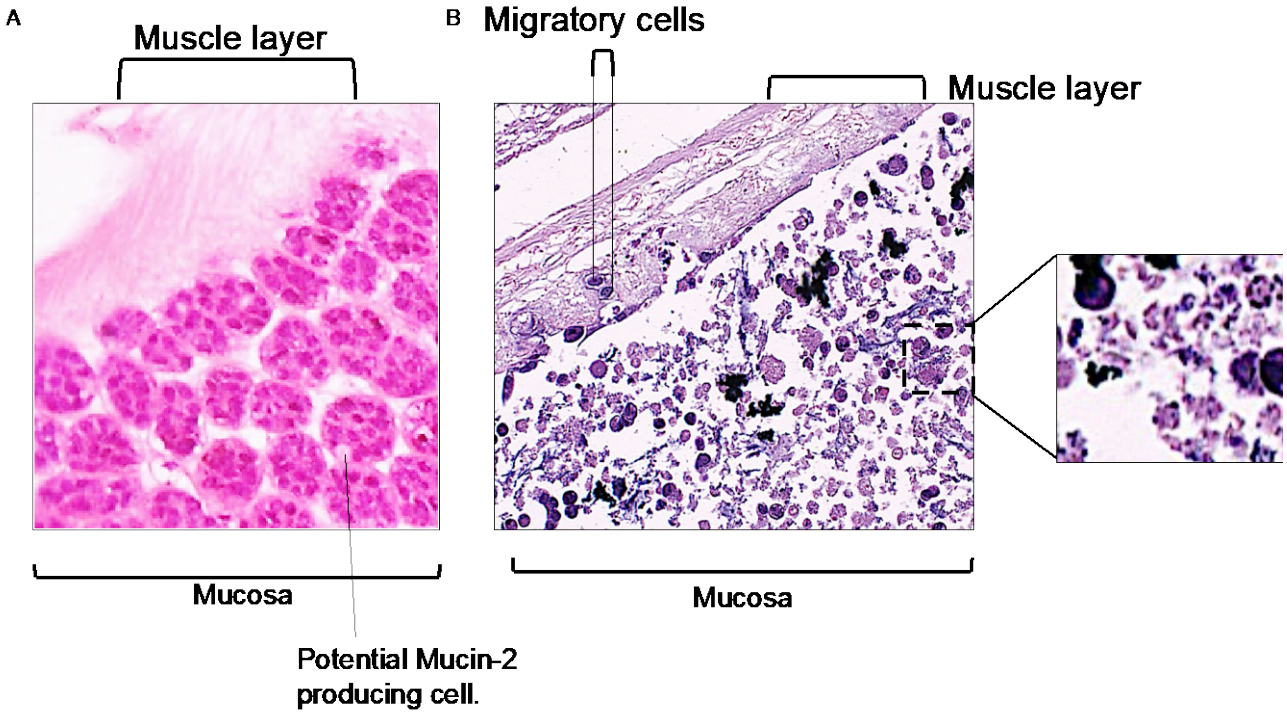


Figure 3.3: **H&E staining of decellularized tissue reconstituted with HT-29 (Fig 3A) and HCT116 cells (Fig 3B).**

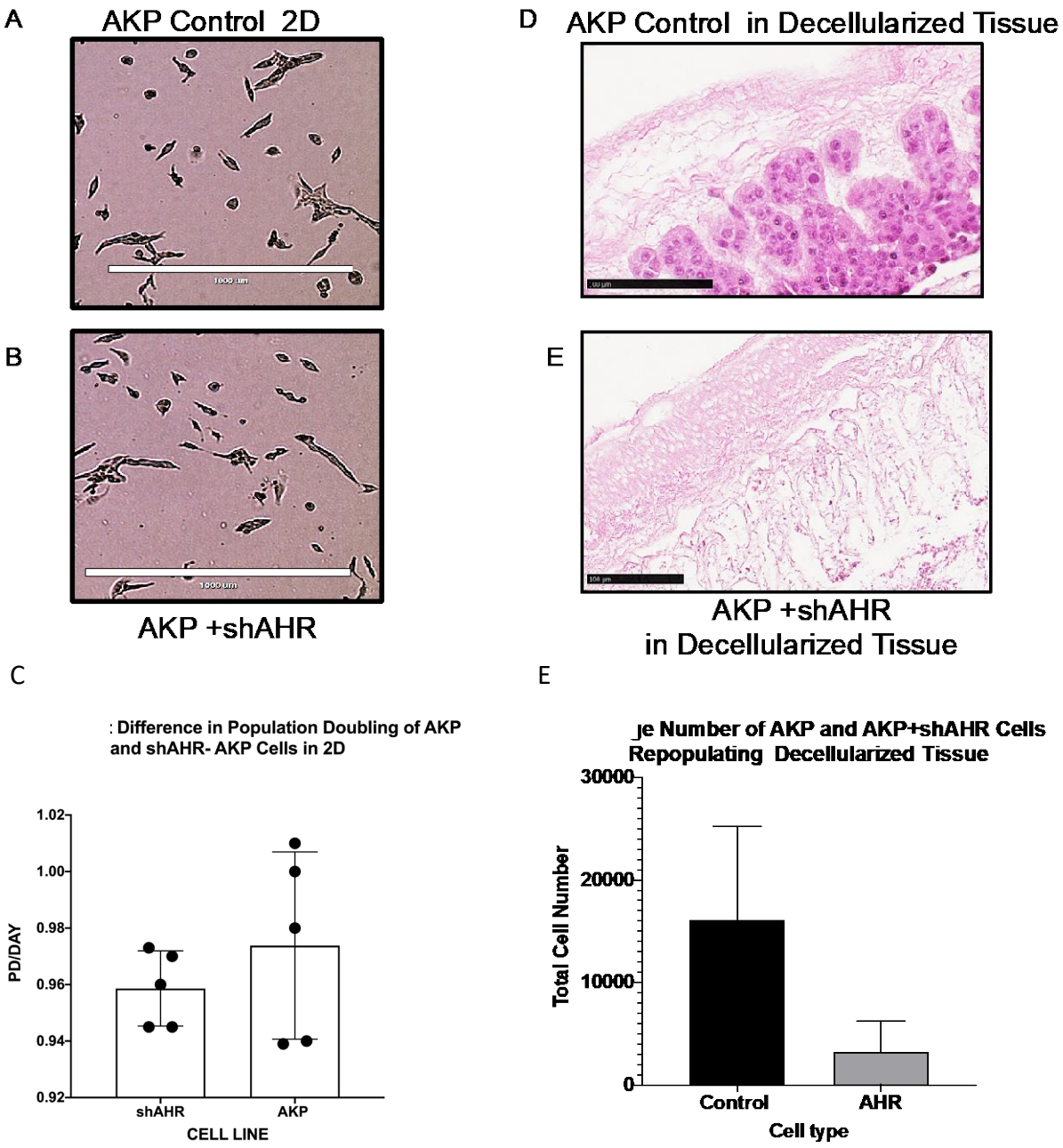


Figure 3.4: **AKP +shAHR maintain growth in 2D (Fig 4A-C) but lose ability to reconstitute decellularized tissue *ex vivo* (Fig 4D-F).**

ECM PROTEIN	% Abundance in polyps	% Abundance in adjacent tissue	% Abundance in young mice
Laminin subunit alpha 1	0.06	0.01	0
Vitronectin	0.20	0.04	0
ECM Protein1	0.07	0.02	0.01
Fibronectin	8.68	2.68	1.6
Tenascin-X	0.02	0.04	0.43
Collagen type IV alpha 6	0.01	0.04	0.22
Collagen type IV alpha 1	1.88	1.73	2.03
Collagen type VI alpha 2	3.82	4.85	5.28

Table 3. 1: **Table summarizing percentage abundance of selected ECM proteins in polyps, adjacent tissue and young CPC;Apc colon cancer mice model**.

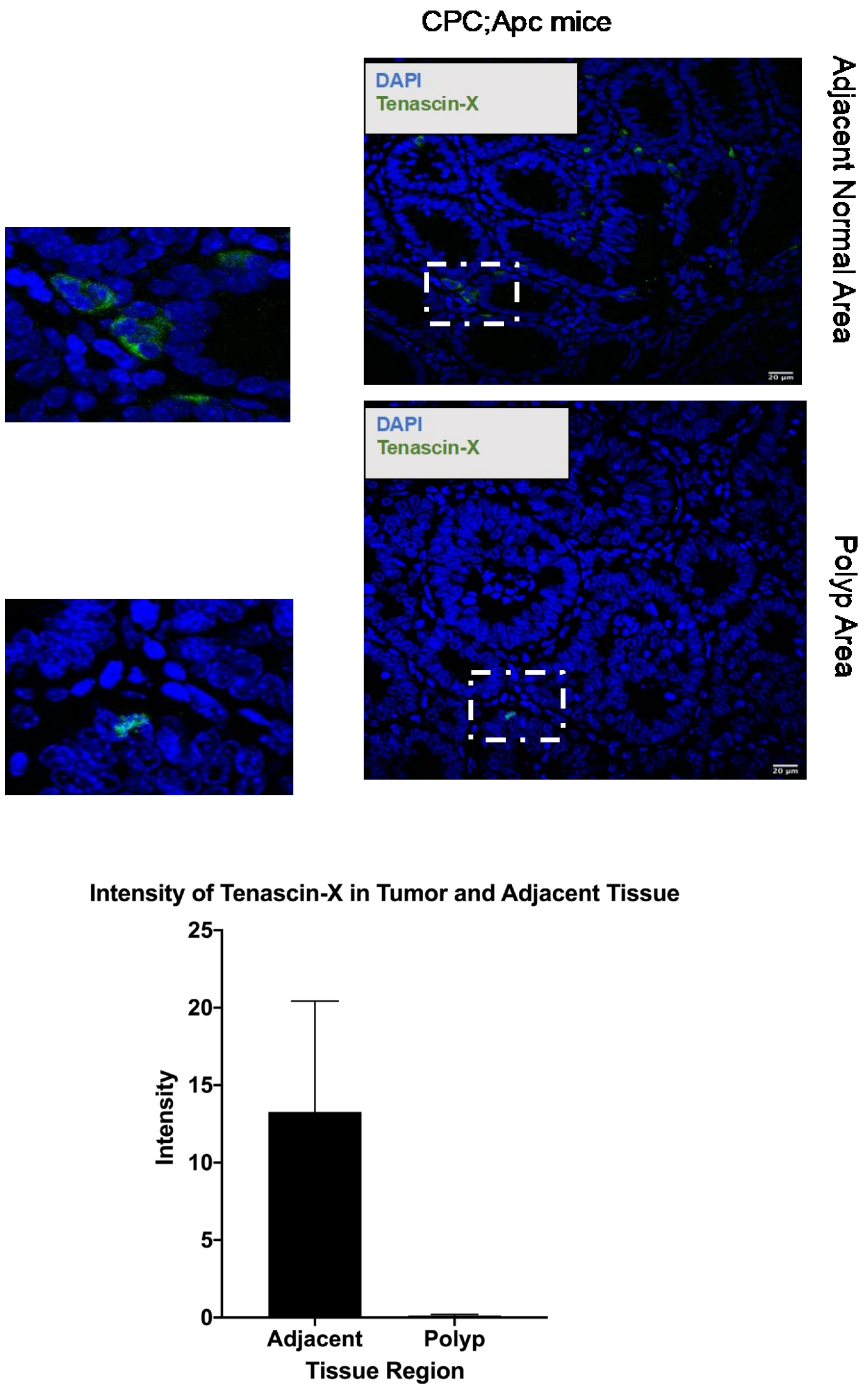


Figure 3. 4: **Tenascin-X is more abundant in adjacent normal tissue compared to normal tissue area as q previously quantitated by Mass spectrometry**.

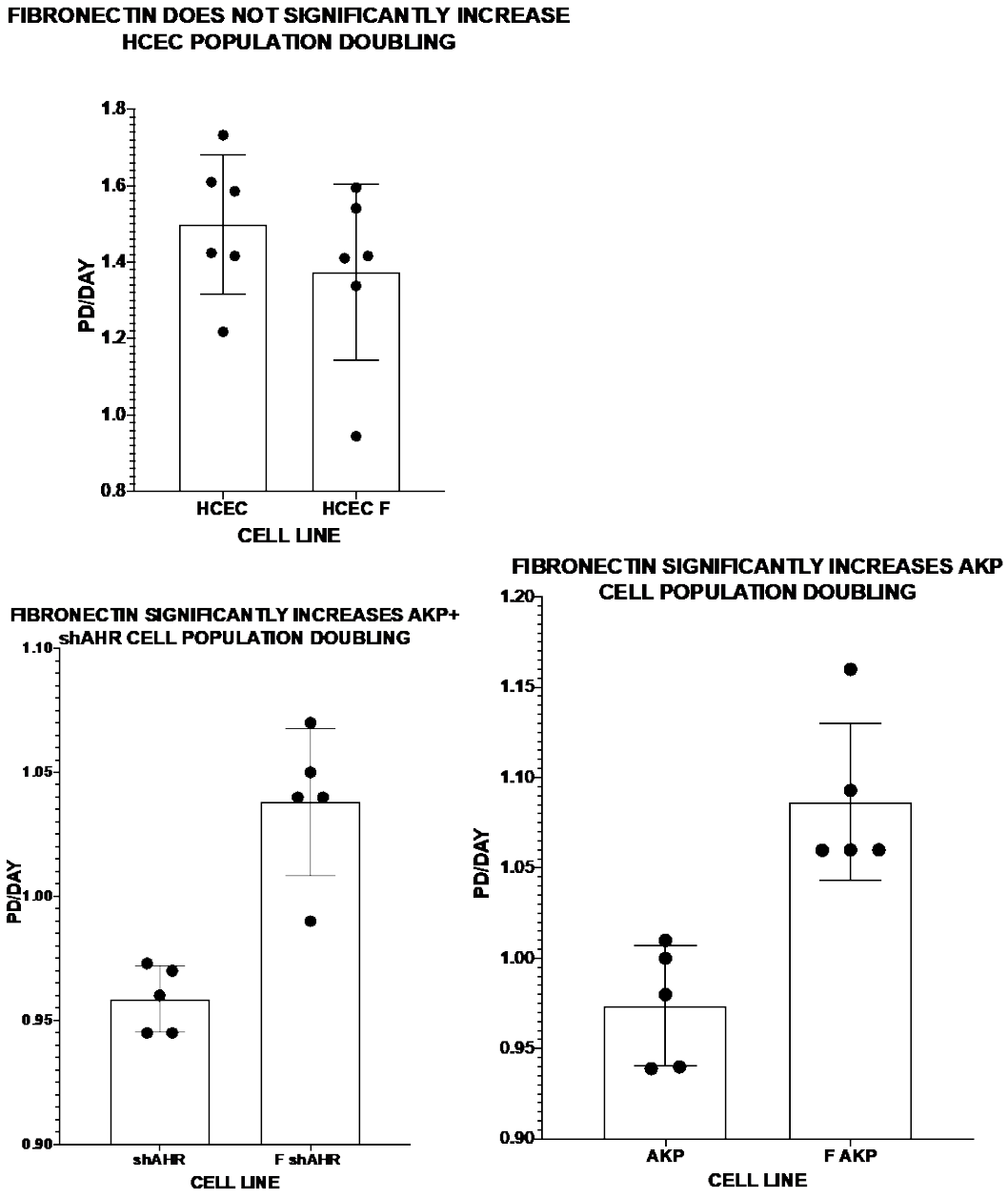


Figure 3.5: **Fibronectin increases growth of AKP cells (A-B) but not Human colonic epithelial cells (HCEC) in i*n vitro* cell culture assays**. PD = population doubling. 'F' in cell line = Fibronectin. Non-parametric T test (Mann Whitney U) was used to test for significance. $p<0.01$. N=5

CHAPTER FOUR

Future perspectives and conclusions

Whole organ decellularization is a relatively new field dating back only to the 2000s (**Figure 4.1**) (Garreta et al., 2017). The work I have described in this dissertation describes a methodology optimized to remove all viable cells from normal and cancer susceptible mice colonic tissue obtained from mice while still preserving tissue architecture, mechanical and biochemical properties. Preservation of tissue architecture was shown by H&E staining. Preservation of mechanical properties was demonstrated by mechanical dynamic analysis testing. Finally, preservation of biochemical properties was shown by persistence of extracellular matrix proteins, including collagens, post decellularization.

I have applied the biological scaffold derived from this decellularization process as a tool for culturing and comparing cell behavior. I have shown that multiple colonic cancer cell types including HT-29 cells remain proliferative in this system. Importantly, when compared to Matrigel ™, the decellularization system better maintains differentiated cell populations as normally observed *in vivo* under homeostatic conditions. I have also used the decellularized scaffold as an experimental model to identify extracellular matrix proteins potentially associated with colorectal cancer. Two of those proteins have been validated by testing *in vitro* .

Although, the decellularized system holds significant promise as a cell culture system and drug discovery tool, there are a number of issues that remain to be resolved. These include the

development of additional methodologies and optimization experiments to effectively harness the power of this decellularization system. In this chapter, I will provide an overview of the alternate uses of decellularized scaffolds beyond tissue culture. In addition, I will cover the disadvantages/pitfalls of this system and next steps/improvements that need to be made to aid in the implementation and widespread use of decellularized tissue as a viable cell culture and regenerative medicine research tool.

Next Steps and Outstanding Questions

Reconstitution with Multiple Cell Types

One of my future goals is to reconstitute the decellularized colonic tissue with multiple cell types that are normally found in the colon including endothelial cells, mesenchymal cells, intestinal stem cells, normal epithelial cells and, in the case of modeling cancer progression, mixtures of normal and colonic tumor cells. This should allow a better model *in vivo* of colonic tissue physiology. Reconstituted tissues as described in this dissertation may prove to be useful for regenerative-medicine purposes including whole-organ replacement. Some ways that cell reconstitution could be achieved include manually adding cells to a specific area of the tissue that they are normally found in, via processes such as immersion, using automated systems such as bioreactors to perfuse cells or a mixture of both methods (Alabi et al., 2019; H. J. Chen et al., 2016; Crapo et al., 2011).

Cell sources to reconstitute decellularized mouse colonic issue can include freshly isolated mice or human colonic epithelial cells which have intestinal Leucine Rich Repeat Containing G-

protein coupled receptor (LGR5) stem cell expressing population. LGR5 is part of the GPCR family and is a 7 transmembrane protein receptor and is expressed by intestinal cells that can differentiate into the full repertoire of differentiated intestinal cells (Morgan, Mortensson, & Williams, 2018). However, maintaining this stem cell population *in vitro* is expensive and technically challenging. This is because current protocols to sustain the LGR5 expressing stem cells involve culturing them in Matrigel^{TM} and in medium supplemented with growth factors such as R-spondin (Harnack et al., 2019; Sato et al., 2011). However, it is possible that decellularized matrices may also help to preserve stem cell population of intestinal cells. Our previous work provide evidence of this in the lung system. In these studies we have shown that human bronchial epithelial cells are able to not only differentiate in *ex vivo* lung decellularized tissue, into upper and lower airway epithelial cells, they also contain populations of basal stem-like cells (LaRanger et al., 2018). Basal cells of the lungs are referred to as stem or progenitor cells which are important for repair during lung epithelial injury. They also maintain airway homeostasis and essentially regenerate luminal populations of the human upper airway epithelium (Hynds & Janes, 2017). The surprising and novel findings from these lung reconstitution experiments is that human bronchial epithelial cells could not only reconstitute upper airway cells but also could reconstitute lower airway cells suggesting the multipotency of the human bronchial epithelial cells.

Embryonic stem cells are another potential cell source, however, there are some ongoing ethical issues to consider with using these cells (Fu et al., 2014). Alternate cell sources for whole organ reconstitution include cells that are made to constitutively express the catalytic component of the human telomerase (hTERT), a reverse transcriptase, or cyclin dependent kinase 4 (CDK4), or

both, to bypass replicative senescence (that may be *in vitro* cell-culture induced) (H. J. Chen et al., 2016; Roig et al., 2010; Jerry W Shay & Woodring E Wright, 2000). Telomerase solves the end-replication problem by adding to the ends of chromosomes, G rich repeats (TTAGG in humans) called telomeres. This prevents cells with very short telomeres from being recognized as DNA damage which will leads to induction of cell-cycle arrest (Roig et al., 2010; Jerry W Shay & Woodring E Wright, 2000). CDK4 phosphorylates the retinoblastoma protein (RB1) resulting in progression of cells from the G1 to S phase of the cell cycle (Schettini et al., 2018). Although, there is great utility for these cells immortalized with CDK4 and hTERT, especially as part of an *ex vivo* decellularized cell-culture system, however, for organ implantation or tissue regenerative purposes, preclinical and clinical safety studies will need to be done (Jerry W Shay & Woodring E Wright, 2000). Another cell source, which I have extensively discussed in chapters 1 and 2, are cells grown in improved culture conditions, such as with feeder layers and in low (more physiological) oxygen conditions, that allow them to grow for extended divisions *in vitro* while still maintaining differentiation potential, all without genetic alterations (LaRanger et al., 2018; Jennifer R Peters-Hall et al., 2018).

In order to reconstitute the decellularized tissue with endothelial cells, I plan to isolate the vasculature during harvesting of colonic tissue in my future experiments. Interestingly, reconstitution with endothelial cells has been found to improve organization of seeded cells (Gilpin & Yang, 2017). The vasculature is also a well-used access point to perfuse whole organs with decellularizing agents or cells (Crapo et al., 2011; Gilpin & Yang, 2017). However, isolating the vasculature can be difficult in mice (especially in young mice) because the blood vessels are really small, may be hard to find and easily punctured. Also, unlike some other

organs, such as the lung and small intestine, which have one major artery delivering oxygen and nutrients to it, there are multiple arteries that that deliver blood to the colon. The celiac artery delivers blood to the proximal portions of the colon while the inferior mesenteric artery delivers blood to the distal colon (Matheson, Wilson, & Garrison, 2000). All of these arteries will need to be isolated to use the vasculature tree of the colon for decellularization or reconstitution. Alternatively, the use of human vascular or umbilical cord endothelial cells may obviate this technical problem.

Measuring Viscoelastic Properties of Tissue

The constituent tissues of the body, including cells and the extracellular matrix, exhibit both viscous-like and elastic properties (Lakes, 2009). They are therefore referred to as viscoelastic. Elasticity is the ability of a material to recover or resist deformation after an external force that deforms it has been applied to it (De Vicente, 2012). Viscosity, on the other hand, is a property of a fluid to resist the force for flow (De Vicente, 2012). Viscous materials show differences in their response to deformation compared to elastic materials. Soft tissues, have both viscous and elastic components and so give both viscous and elastic responses to applied force or deformation (De Vicente, 2012). Because the relationship between stress and strain of viscoelastic materials depends on time, the stress-strain curve of a viscoelastic material will also include the element of time (Lakes, 2009). Viscous, elastic and viscoelastic materials have their typical stress-strain curves (**Figure 4.2**) (Özkaya & Nordin, 1999). Additionally, the loading and unloading behavior a viscoelastic material will be different compared to a perfectly linear or viscous materials (El-Amin, 2016).

It is important to characterize the viscoelastic properties of materials, especially those that will be used to study physiologic functions or used for implantations. This is because viscoelastic properties give us information about the change in tissue properties over time under physiologic conditions and with repeated application or force (Vizesi, Jones, Lotz, Gianoutsos, & Walsh, 2008). To measure viscoelastic properties, sinusoidal stress or strain load perturbations can be applied to a tissue and the corresponding strain or stress output is measured (El-Amin, 2016). Alternatively, viscoelastic properties can be quantified using stress-relaxation and creep experiments (El-Amin, 2016; Vizesi et al., 2008). During a typical creep experiment, the tissue will be subjected to constant load and time-dependent changes of strain will be recorded (El-Amin, 2016). Similarly, during stress-relaxation experiments a constant strain is applied, and time-dependent stress reduction is measured (El-Amin, 2016). Additionally, during measurements to quantify viscoelastic properties, the tissue is loaded and unloaded cyclically before the experiments progress. This is called preconditioning and allows for more consistent mechanical responses to be obtained (El-Amin, 2016).

The previous experiments I performed characterizing the mechanical properties of decellularized tissue did not delve into the viscoelastic nature of the decellularized colon or time-dependent regulation of mechanical properties including physiologic functioning of the decellularized tissue. Therefore, future experiments should include characterizing viscoelastic properties of normal mice colonic tissue, before and after decellularization. Although linear elasticity /stiffness parameters of mice colonic tissue, obtained via my protocol, did not change post-decellularization, viscoelastic properties of tissue may have changed post-decellularization. Therefore, I will also characterize the viscoelasticity of colonic tissue from normal mice and

from colon cancer susceptible mice before and after the formation of polyps or tumors. This may provide insights into how viscoelastic properties change over time in pre-cancerous tissues and/or during cancer progression.

Validation of Additional ECM Proteins and Growth factors

Our mass spectrometry data identified more than 9 ECM proteins that are more abundantly expressed in tumors or adjacent normal areas of the colon cancer susceptible mouse model, CPC;Apc. I confirmed and validated two of those extracellular matrix proteins in chapter 2 by *in vitro* testing and by using immunofluorescence staining of mice colonic tissue sections. Four of the ECM proteins that have not yet been identified including, alpha-6 collagen type IV, Laminin subunit beta 1 and alpha 1, are alpha or beta chains of that ECM protein. They cannot be tested alone *in vitro* or used to reconstitute decellularized tissue for *ex vivo* testing as they need to complex with each other to form a specific function ECM protein chain (Tanzer, 2006). Therefore, the ECM proteins I propose to validate for their involvement in cancer progression will include proteoglycan 3, vitronectin., and extracellular matrix protein 1. I will also be validating the 8 growth factors and growth factor associated proteins including latent-transforming growth factor beta-binding protein 3. These proteins were found to be associated or more abundant expressed in normal or tumor microenvironments of mice and their roles in colon cancer progression have been described in chapter 2.

I will test the influence of these proteins in cancer progression by using them to coat tissue culture plates in *in vitro* assays and determine how they modulate invasion ability (in wound-healing assays) and for their influence on cellular proliferative ability. I also plan to use these

extracellular matrix proteins to coat decellularized tissues, in increasing amounts during *ex vivo* cell culture, and see how they influence cell proliferation, repopulation and differentiation ability. Finally, I plan to add the identified growth factors to *in vitro* and *ex vivo* culture media to see how they also modulate cell behavior. For extracellular matrix proteins or growth factors that modulate cell behavior significantly, I plan to perform co-immunoprecipitation experiments to identify other proteins that are bound to them in order to begin to understand mechanistically, how the ECM regulates cell behavior.

Pitfalls of Decellularized Tissue Models

Time and Financial costs

The first pitfall associated with use of decellularized tissue as a cell culture system is the financial costs associated with starting it up as a culture system and during its continuous use. These costs may not be faced in a 2D culture or Matrigel ™ culture lab and may limit the widespread use or make it easier for labs not to switch to this *ex vivo* culture system despite its potential advantages. Specifically, costs include the initial cost to buy the peristaltic pump, which could range from $500 to more than $5000 and to obtain approval to buy and use mice. Ongoing costs including animal housing costs at an institution's animal facility could also be a deterrent.

When many multiple mice are used for studies and/or the mice need to be housed for long periods, these costs will add up. For example, for some of the genetically engineered mice model where the mice have to be kept for 6-8 months before they develop polyps and can be used for further experiments, each mouse will cost about a thousand dollars to house, on average, before it is used. Further, although Matrigel ™, is expensive to purchase, there are no 'housing cost'

associated with its use. Cell culture petri dishes which are used for 2D-cell culture are typically not expensive and have no other costs associated with them. Hence, it's easier for researchers to continue using these systems.

Two other disadvantages of using decellularized tissues are that it is tedious/time consuming compared to 2D or Matrigel ™ culture and a lot of cells are typically needed for reconstitution, particularly when an entire or most of an entire organ needs to be repopulated. My colon decellularization method takes 2 days to complete (Alabi et al., 2019). During this two day period, I need to change the decellularization agents and glass jars and clear out air from the bioreactor system. With Matrigel ™ culture, the Matrigel ™ can be thawed overnight or for a few hours and it is ready for cell culture. With petri dishes used in 2D culture, there is no wait time. Furthermore, I reconstitute the colon with 10 million cells; although this is enough to cover a significant portion of the tissue, there is definitely still room for more cells to be added. This would mean that if a functional decellularized tissue needs to be replanted there will need to be a large cell source available for it. As discussed in earlier chapters of this dissertation, maintenance of the proliferative and differentiation potential of cells can be quite challenging. In fact, current progenitor cell technology cannot support the creation of differentiated cells in numbers essential for the regeneration of human-sized organs (Fu et al., 2014).

Protocol Standardization

Importantly, many elements of the tissue decellularization protocols have come from different investigators. These elements have not yet been standardized which makes it challenging to reduce batch to batch variability and compare results between labs. These elements include the

specific type of detergents used for a whole organ/tissue, the time each detergent should be used and how the decellularization agents should be applied. All of these elements are critical to the final decellularized product. They could potentially affect downstream experiments as they are crucial to the preservation of tissue architecture, extracellular matrix proteins and tissue mechanical properties, all of which may be important components of the decellularized tissue depending on what the tissue is being used. For example, while I used a perfusate flow rate of 1ml per min during decellularization for the colonic tissue, others have used flow rates such as 0.001 ml/min, albeit for a different tissue-rat ileum (Totonelli et al., 2012). Some of the few standard quantitative minimum requirements of decellularized tissues include <50 ng dsDNA per mg ECM dry weight, <200 bp DNA fragment length, lack of visible nuclear material in H&E or in 4',6-diamidino-2-phenylindole (DAPI) stained tissues (Crapo et al., 2011).

Alternate Uses of Decellularized Tissues Beyond Cell Culture System

Aside from being used as a cell culture system for basic research or to identify extracellular matrix protein involved in colon cancer progression, decellularized tissues have utility in other areas. One important area where decellularized tissue might be applied is replacement of tissues or organs during organ failure (Crapo et al., 2011; Yu, Alkhawaji, Ding, & Mei, 2016). Post implantation, decellularized tissue can chemo-attract progenitor cells to the implantation site (Guruswamy Damodaran & Vermette, 2018). This is further supported by studies that have shown that tissue specific extracellular matrix can induce the differentiation of progenitor cells in their appropriate niches (Orlando, 2013). Therefore, implanted tissue could possibly be reconstituted with cells obtained directly from the patients. With the current donor transplant shortage problem, decellularized tissue, especially when their mechanical and biochemical

properties are preserved, are potential sources to address this shortage. There are many examples in the literature where decellularized tissues have already been used for allogenic transplantation in animals and human studies (Crapo, Gilbert et al. 2011(Garreta et al., 2017)). Because there are no endogenous cells, immunogenic effects, including M1 macrophage recruitment and subsequent fibrosis, which are huge risks of allogenic transplantation, can be avoided (Crapo, Gilbert et al. 2011 (Orlando, 2013)). Further, some tissues are also transplanted non-orthotopically. Some of them are commercially available such as IOPatch™ which is obtained from human pericardium but is used during ophthalmological repair (Crapo, Gilbert et al. 2011). Another example is CopiOs® which is used for dental surgery but is obtained from pericardium of pigs (Crapo, Gilbert et al. 2011). A longer list of commercially available clinical products derived from decellularized tissues are listed in **Table 4.1**

Another potential utility of decellularized tissue can be achieved when they are solubilized to form hydrogels. Hydrogels are hydrated polymers with structural integrity provided by physical and chemical crosslinks between polymer chains (Saldin, Cramer, Velankar, White, & Badylak, 2017). These polymer chains can be from synthetic sources including polyethylene oxide or natural sources such as collagen or complex mixtures of extracellular matrix proteins such as those found in Matrigel ^{TM} or decellularized tissue (Saldin et al., 2017). In the case of decellularized tissue, the extracellular matrix is made into hydrogels largely by grinding the tissue to powder, digesting the ECM powder with 1mg/ml pepsin enzyme dissolved in 0.01N HCL for 48hrs at room temperature, neutralizing with 0.1N NaOH base and gelation in a non-humidified 37^{o}C incubator for 1hr. The resulting decellularized matrix hydrogel can be used as a 3D organotypic cell culture model and to stimulate tissue growth in areas after injury (Saldin et

al., 2017). For applications specific to the colon, hydrogels have been used to deliver drugs to the colon to treat patients with ulcerative colitis. Solubilized decellularized colonic tissue could also potentially be used to stimulate colonic tissue repair or promote healing in cases of ulcer or ulcerative colitis or other gastrointestinal pathologies, injuries or defects which affect millions of people worldwide (Keane et al., 2017).

In conclusion, tissue decellularization provides a biomaterial and scaffold that can applied to a number of functions that ranges from cell culture to identification of novel targeted proteins to treat cancer including whole-organ transplantation. Universal or standardized decellularization protocols will further ease the application of decellularized scaffolds for these purposes. My work, described in these chapters, has highlighted the importance of the cellular environment in which cells are grown that influences their behavior and potential for colon cancer progression. I have also identified ECM proteins associated with colon cancer that may have pharmacological indications.

Figures

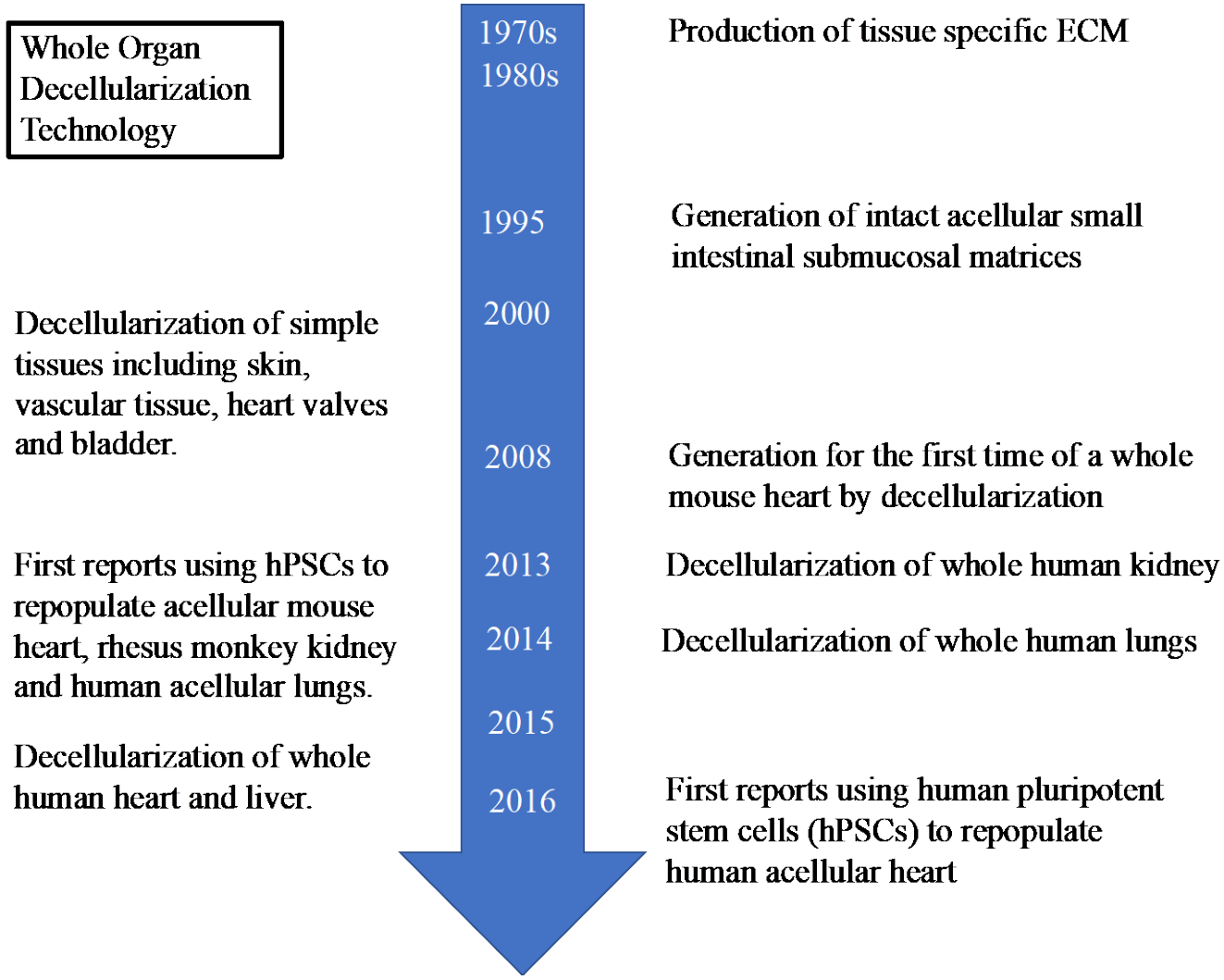


Figure 4. 1: Timeline (1970s to 2016) of events leading up to whole organ decellularization and key milestones in the organ/tissue decellularization afterwards.
Adapted from Garreta, Oria et al. 2017.

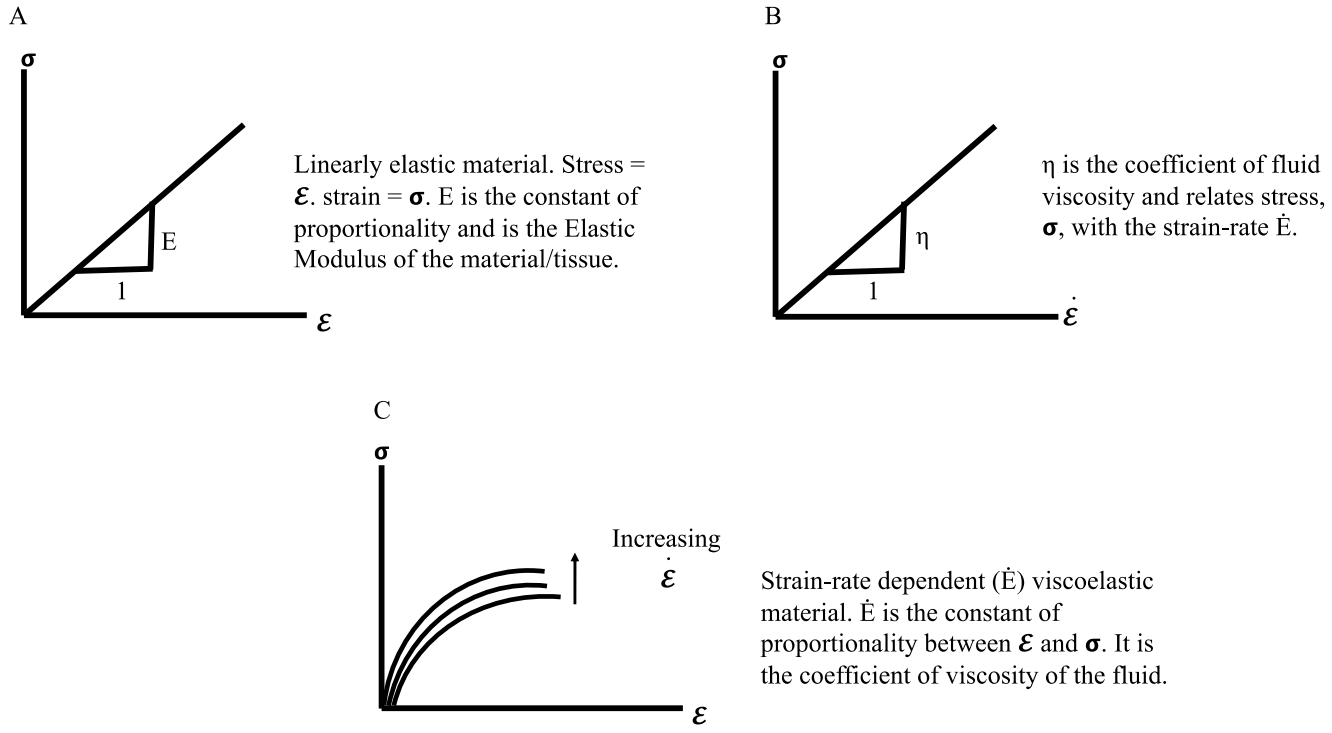


Figure 4. 2: Typical Stress-strain curves of **A.** linearly elastic materials, **B.** viscous liquids and **C.** viscoelastic materials.
Adapted from De Vicente 2012 and El-Amin 2016.

Product (Manufacturer)	Manufacturer	Tissue Source	Application Focus
AlloDerm®	LifeCell Corp	Human Dermis	Soft Tissue
NeoForm™	Mentor Worldwide LLC	Human Dermis	Breast
Lyoplant®	B. Braun Melsungen AG	Bovine pericardium	Dura mater
Prima™ Plus	Edwards Lifesciences LLC	Porcine heart valve	Valve replacement
MatriStem®, Acell Vet	Acell Inc	Porcine urinary bladder	Soft tissue
CorMatrix ECM™	CorMatrix®Cardio vascular Inc	Porcine small intestine	Cardia tissue

Table 4. 1: Table showing the mammalian sources and applications of commercially available decellularized tissues.
Adapted from Crapo, Gilbert et al. 2011.

Bibliography

Aggarwal, S. (2010). Targeted cancer therapies. *Nat Rev Drug Discov, 9*(6), 427-428. doi:10.1038/nrd3186

Aktar, R., Peiris, M., Fikree, A., Cibert-Goton, V., Walmsley, M., Tough, I. R., . . . Blackshaw, L. A. (2018). The extracellular matrix glycoprotein tenascin-X regulates peripheral sensory and motor neurones. *J Physiol, 596*(17), 4237-4251. doi:10.1113/JP276300

Alabi, B. R., LaRanger, R., & Shay, J. W. (2019). Decellularized mice colons as models to study the contribution of the extracellular matrix to cell behavior and colon cancer progression. *Acta Biomater, 100*, 213-222. doi:10.1016/j.actbio.2019.09.033

Bourgine, J., Billaut-Laden, I., Happillon, M., Lo-Guidice, J. M., Maunoury, V., Imbenotte, M., & Broly, F. (2012). Gene expression profiling of systems involved in the metabolism and the disposition of xenobiotics: comparison between human intestinal biopsy samples and colon cell lines. *Drug Metab Dispos, 40*(4), 694-705. doi:10.1124/dmd.111.042465

Burch, G. H., Gong, Y., Liu, W., Dettman, R. W., Curry, C. J., Smith, L., . . . Bristow, J. (1997). Tenascin-X deficiency is associated with Ehlers-Danlos syndrome. *Nat Genet, 17*(1), 104-108. doi:10.1038/ng0997-104

Carabet, L. A., Rennie, P. S., & Cherkasov, A. (2018). Therapeutic Inhibition of Myc in Cancer. Structural Bases and Computer-Aided Drug Discovery Approaches. *Int J Mol Sci, 20*(1). doi:10.3390/ijms20010120

Cauchi, S., Stucker, I., Solas, C., Laurent-Puig, P., Cenee, S., Hemon, D., . . . Massaad-Massade, L. (2001). Polymorphisms of human aryl hydrocarbon receptor (AhR) gene in a French population: relationship with CYP1A1 inducibility and lung cancer. *Carcinogenesis, 22*(11), 1819-1824. doi:10.1093/carcin/22.11.1819

Chaimov, D., Baruch, L., Krishtul, S., Meivar-Levy, I., Ferber, S., & Machluf, M. (2017). Innovative encapsulation platform based on pancreatic extracellular matrix achieve substantial insulin delivery. *J Control Release, 257*, 91-101. doi:10.1016/j.jconrel.2016.07.045

Chen, H., Zhong, J., Wang, J., Huang, R., Qiao, X., Wang, H., & Tan, Z. (2019). Enhanced growth and differentiation of myoblast cells grown on E-jet 3D printed platforms. *Int J Nanomedicine, 14*, 937-950. doi:10.2147/IJN.S193624

Chen, H. J., Wei, Z., Sun, J., Bhattacharya, A., Savage, D. J., Serda, R., . . . Shuler, M. L. (2016). A recellularized human colon model identifies cancer driver genes. *Nat Biotechnol, 34*(8), 845-851. doi:10.1038/nbt.3586

Cohen, E., Ophir, I., & Shaul, Y. B. (1999). Induced differentiation in HT29, a human colon adenocarcinoma cell line. *J Cell Sci, 112 (Pt 16)*, 2657-2666. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/10413674

Colony, P. C. (1996). Structural characterization of colonic cell types and correlation with specific functions. *Dig Dis Sci, 41*(1), 88-104. doi:10.1007/bf02208589

Crapo, P. M., Gilbert, T. W., & Badylak, S. F. (2011). An overview of tissue and whole organ decellularization processes. *Biomaterials, 32*(12), 3233-3243. doi:10.1016/j.biomaterials.2011.01.057

Crotti, S., Piccoli, M., Rizzolio, F., Giordano, A., Nitti, D., & Agostini, M. (2017). Extracellular matrix and colorectal cancer: how surrounding microenvironment affects cancer cell behavior? *Journal of cellular physiology, 232*(5), 967-975.

Cully, M. (2016). Anticancer drugs: Exploiting a weakness in colorectal cancers. *Nat Rev Drug Discov, 15*(12), 820-821. doi:10.1038/nrd.2016.247

De Arcangelis, A., Lefebvre, O., Mechine-Neuville, A., Arnold, C., Klein, A., Remy, L., . . . Simon-Assmann, P. (2001). Overexpression of laminin alpha1 chain in colonic cancer cells induces an increase in tumor growth. *Int J Cancer, 94*(1), 44-53. doi:10.1002/ijc.1444

de Santa Barbara, P., van den Brink, G. R., & Roberts, D. J. (2003). Development and differentiation of the intestinal epithelium. *Cell Mol Life Sci, 60*(7), 1322-1332. doi:10.1007/s00018-003-2289-3

De Vicente, J. (2012). *Viscoelasticity: From Theory to Biological Applications*: BoD–Books on Demand.

Dunn, S. J., Appleton, P. L., Nelson, S. A., Nathke, I. S., Gavaghan, D. J., & Osborne, J. M. (2012). A two-dimensional model of the colonic crypt accounting for the role of the basement membrane and pericryptal fibroblast sheath. *PLoS Comput Biol, 8*(5), e1002515. doi:10.1371/journal.pcbi.1002515

Edmondson, R., Broglie, J. J., Adcock, A. F., & Yang, L. (2014). Three-dimensional cell culture systems and their applications in drug discovery and cell-based biosensors. *Assay Drug Dev Technol, 12*(4), 207-218. doi:10.1089/adt.2014.573

El-Amin, M. F. (2016). Viscoelastic and Viscoplastic Materials.

Fakih, M. G. (2015). Metastatic colorectal cancer: current state and future directions. *J Clin Oncol, 33*(16), 1809-1824. doi:10.1200/JCO.2014.59.7633

Fang, Y., Liang, X., Jiang, W., Li, J., Xu, J., & Cai, X. (2015). Cyclin b1 suppresses colorectal cancer invasion and metastasis by regulating e-cadherin. *PLoS One, 10*(5), e0126875. doi:10.1371/journal.pone.0126875

Farsi, A., Pullen, A. D., Latham, J. P., Bowen, J., Carlsson, M., Stitt, E. H., & Marigo, M. (2017). Full deflection profile calculation and Young's modulus optimisation for engineered high performance materials. *Sci Rep, 7*, 46190. doi:10.1038/srep46190

Fernandez-Perez, J., & Ahearne, M. (2019). The impact of decellularization methods on extracellular matrix derived hydrogels. *Sci Rep, 9*(1), 14933. doi:10.1038/s41598-019-49575-2

Filipe, E. C., Chitty, J. L., & Cox, T. R. (2018). Charting the unexplored extracellular matrix in cancer. *Int J Exp Pathol, 99*(2), 58-76. doi:10.1111/iep.12269

Foty, R. (2011). A simple hanging drop cell culture protocol for generation of 3D spheroids. *J Vis Exp*(51). doi:10.3791/2720

Frantz, C., Stewart, K. M., & Weaver, V. M. (2010). The extracellular matrix at a glance. *J Cell Sci, 123*(Pt 24), 4195-4200. doi:10.1242/jcs.023820

Fu, R. H., Wang, Y. C., Liu, S. P., Shih, T. R., Lin, H. L., Chen, Y. M., . . . Lin, S. Z. (2014). Decellularization and recellularization technologies in tissue engineering. *Cell Transplant, 23*(4-5), 621-630. doi:10.3727/096368914X678382

Garreta, E., Oria, R., Tarantino, C., Pla-Roca, M., Prado, P., Fernandez-Aviles, F., . . . Montserrat, N. (2017). Tissue engineering by decellularization and 3D bioprinting. *Materials Today, 20*(4), 166-178.

Gerber, D. E. (2008). Targeted therapies: a new generation of cancer treatments. *Am Fam Physician, 77*(3), 311-319. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/18297955

Gilbert, T. W., Sellaro, T. L., & Badylak, S. F. (2006). Decellularization of tissues and organs. *Biomaterials, 27*(19), 3675-3683. doi:10.1016/j.biomaterials.2006.02.014
Gilpin, A., & Yang, Y. (2017). Decellularization Strategies for Regenerative Medicine: From Processing Techniques to Applications. *Biomed Res Int, 2017*, 9831534. doi:10.1155/2017/9831534
Ginsburg, G. S., & Willard, H. F. (2013). *Genomic and personalized medicine* (2nd ed.). London ; Waltham, MA: Elsevier/Academic Press.
Giobbe, G. G., Crowley, C., Luni, C., Campinoti, S., Khedr, M., Kretzschmar, K., . . . De Coppi, P. (2019). Extracellular matrix hydrogel derived from decellularized tissues enables endodermal organoid culture. *Nat Commun, 10*(1), 5658. doi:10.1038/s41467-019-13605-4
Gjorevski, N., Sachs, N., Manfrin, A., Giger, S., Bragina, M. E., Ordonez-Moran, P., . . . Lutolf, M. P. (2016). Designer matrices for intestinal stem cell and organoid culture. *Nature, 539*(7630), 560-564. doi:10.1038/nature20168
Goers, L., Freemont, P., & Polizzi, K. M. (2014). Co-culture systems and technologies: taking synthetic biology to the next level. *J R Soc Interface, 11*(96). doi:10.1098/rsif.2014.0065
Gout, S., Marie, C., Laine, M., Tavernier, G., Block, M. R., & Jacquier-Sarlin, M. (2004). Early enterocytic differentiation of HT-29 cells: biochemical changes and strength increases of adherens junctions. *Exp Cell Res, 299*(2), 498-510. doi:10.1016/j.yexcr.2004.06.008
Gouveia-Fernandes, S., Carvalho, T., Domingues, G., Bordeira-Carrico, R., Dias, S., & Serpa, J. (2016). Colorectal cancer aggressiveness is related to fibronectin over expression, driving the activation of SDF-1: CXCR4 axis. *International Journal of Cancer and Clinical Research, 3*(8), 72-81.
Greenwood, S. K., Hill, R. B., Sun, J. T., Armstrong, M. J., Johnson, T. E., Gara, J. P., & Galloway, S. M. (2004). Population doubling: a simple and more accurate estimation of cell growth suppression in the in vitro assay for chromosomal aberrations that reduces irrelevant positive results. *Environ Mol Mutagen, 43*(1), 36-44. doi:10.1002/em.10207
Gribble, F. M., & Reimann, F. (2016). Enteroendocrine Cells: Chemosensors in the Intestinal Epithelium. *Annu Rev Physiol, 78*, 277-299. doi:10.1146/annurev-physiol-021115-105439
Guenther, U., Herbst, H., Bauer, M., Isbert, C., Buhr, H. J., Riecken, E. O., & Schuppan, D. (2001). Collagen type XVIII/endostatin is differentially expressed in primary and metastatic colorectal cancers and ovarian carcinomas. *Br J Cancer, 85*(10), 1540-1545. doi:10.1054/bjoc.2001.2143
Guerrina, N., Traboulsi, H., Eidelman, D. H., & Baglole, C. J. (2018). The Aryl Hydrocarbon Receptor and the Maintenance of Lung Health. *Int J Mol Sci, 19*(12). doi:10.3390/ijms19123882
Guruswamy Damodaran, R., & Vermette, P. (2018). Tissue and organ decellularization in regenerative medicine. *Biotechnol Prog, 34*(6), 1494-1505. doi:10.1002/btpr.2699
Halim, S., Markert, E. K., & Vazquez, A. (2018). Analysis of cell proliferation and tissue remodelling uncovers a KLF4 activity score associated with poor prognosis in colorectal cancer. *Br J Cancer, 119*(7), 855-863. doi:10.1038/s41416-018-0253-0
Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell, 100*(1), 57-70. doi:10.1016/s0092-8674(00)81683-9

Handorf, A. M., Zhou, Y., Halanski, M. A., & Li, W. J. (2015). Tissue stiffness dictates development, homeostasis, and disease progression. *Organogenesis, 11*(1), 1-15. doi:10.1080/15476278.2015.1019687

Harnack, C., Berger, H., Antanaviciute, A., Vidal, R., Sauer, S., Simmons, A., . . . Sigal, M. (2019). R-spondin 3 promotes stem cell recovery and epithelial regeneration in the colon. *Nat Commun, 10*(1), 4368. doi:10.1038/s41467-019-12349-5

Hinoi, T., Akyol, A., Theisen, B. K., Ferguson, D. O., Greenson, J. K., Williams, B. O., . . . Fearon, E. R. (2007). Mouse model of colonic adenoma-carcinoma progression based on somatic Apc inactivation. *Cancer Res, 67*(20), 9721-9730. doi:10.1158/0008-5472.CAN-07-2735

Hinz, B. (2009). Tissue stiffness, latent TGF-beta1 activation, and mechanical signal transduction: implications for the pathogenesis and treatment of fibrosis. *Curr Rheumatol Rep, 11*(2), 120-126. doi:10.1007/s11926-009-0017-1

Hu, H., Gehart, H., Artegiani, B., C, L. O.-I., Dekkers, F., Basak, O., . . . Clevers, H. (2018). Long-Term Expansion of Functional Mouse and Human Hepatocytes as 3D Organoids. *Cell, 175*(6), 1591-1606 e1519. doi:10.1016/j.cell.2018.11.013

Huet, C., Sahuquillo-Merino, C., Coudrier, E., & Louvard, D. (1987). Absorptive and mucus-secreting subclones isolated from a multipotent intestinal cell line (HT-29) provide new models for cell polarity and terminal differentiation. *J Cell Biol, 105*(1), 345-357. doi:10.1083/jcb.105.1.345

Hynds, R. E., & Janes, S. M. (2017). Airway Basal Cell Heterogeneity and Lung Squamous Cell Carcinoma. *Cancer Prev Res (Phila), 10*(9), 491-493. doi:10.1158/1940-6207.CAPR-17-0202

Iivarinen, J. T., Korhonen, R. K., & Jurvelin, J. S. (2014). Experimental and numerical analysis of soft tissue stiffness measurement using manual indentation device--significance of indentation geometry and soft tissue thickness. *Skin Res Technol, 20*(3), 347-354. doi:10.1111/srt.12125

Ikeda, K., Iyama, K., Ishikawa, N., Egami, H., Nakao, M., Sado, Y., . . . Baba, H. (2006). Loss of expression of type IV collagen alpha5 and alpha6 chains in colorectal cancer associated with the hypermethylation of their promoter region. *Am J Pathol, 168*(3), 856-865. doi:10.2353/ajpath.2006.050384

Ishida, K., Hirai, G., Murakami, K., Teruya, T., Simizu, S., Sodeoka, M., & Osada, H. (2004). Structure-based design of a selective heparanase inhibitor as an antimetastatic agent. *Mol Cancer Ther, 3*(9), 1069-1077. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/15367701

Jacob, A., & Prekeris, R. (2015). The regulation of MMP targeting to invadopodia during cancer metastasis. *Front Cell Dev Biol, 3*, 4. doi:10.3389/fcell.2015.00004

Jayadev, R., & Sherwood, D. R. (2017). Basement membranes. *Curr Biol, 27*(6), R207-R211. doi:10.1016/j.cub.2017.02.006

Jogi, A., Vaapil, M., Johansson, M., & Pahlman, S. (2012). Cancer cell differentiation heterogeneity and aggressive behavior in solid tumors. *Ups J Med Sci, 117*(2), 217-224. doi:10.3109/03009734.2012.659294

Kapalczynska, M., Kolenda, T., Przybyla, W., Zajaczkowska, M., Teresiak, A., Filas, V., . . . Lamperska, K. (2018). 2D and 3D cell cultures - a comparison of different types of cancer cell cultures. *Arch Med Sci, 14*(4), 910-919. doi:10.5114/aoms.2016.63743

Keane, T. J., Dziki, J., Castelton, A., Faulk, D. M., Messerschmidt, V., Londono, R., . . . Badylak, S. F. (2017). Preparation and characterization of a biologic scaffold and hydrogel derived from colonic mucosa. *J Biomed Mater Res B Appl Biomater, 105*(2), 291-306. doi:10.1002/jbm.b.33556

Kenny, F. N., Drymoussi, Z., Delaine-Smith, R., Kao, A. P., Laly, A. C., Knight, M. M., . . . Connelly, J. T. (2018). Tissue stiffening promotes keratinocyte proliferation through activation of epidermal growth factor signaling. *J Cell Sci, 131*(10). doi:10.1242/jcs.215780

Kim, S. B., Bozeman, R. G., Kaisani, A., Kim, W., Zhang, L., Richardson, J. A., . . . Shay, J. W. (2016). Radiation promotes colorectal cancer initiation and progression by inducing senescence-associated inflammatory responses. *Oncogene, 35*(26), 3365-3375. doi:10.1038/onc.2015.395

Kleinman, H. K. (2001). Preparation of basement membrane components from EHS tumors. *Curr Protoc Cell Biol, Chapter 10*, Unit 10 12. doi:10.1002/0471143030.cb1002s00

Kretzschmar, K., & Clevers, H. (2016). Organoids: Modeling Development and the Stem Cell Niche in a Dish. *Dev Cell, 38*(6), 590-600. doi:10.1016/j.devcel.2016.08.014

Lafita-Navarro, M. C., Kim, M., Borenstein-Auerbach, N., Venkateswaran, N., Hao, Y. H., Ray, R., . . . Conacci-Sorrell, M. (2018). The aryl hydrocarbon receptor regulates nucleolar activity and protein synthesis in MYC-expressing cells. *Genes Dev, 32*(19-20), 1303-1308. doi:10.1101/gad.313007.118

Lakes, R. (2009). Viscoelastic Materials. *Viscoelastic Materials*, 1-461. doi:10.1017/Cbo9780511626722

Langhans, S. A. (2018). Three-Dimensional in Vitro Cell Culture Models in Drug Discovery and Drug Repositioning. *Front Pharmacol, 9*, 6. doi:10.3389/fphar.2018.00006

LaRanger, R., Peters-Hall, J. R., Coquelin, M., Alabi, B. R., Chen, C. T., Wright, W. E., & Shay, J. W. (2018). Reconstituting Mouse Lungs with Conditionally Reprogrammed Human Bronchial Epithelial Cells. *Tissue Eng Part A, 24*(7-8), 559-568. doi:10.1089/ten.TEA.2017.0114

Larregieu, C. A., & Benet, L. Z. (2013). Drug discovery and regulatory considerations for improving in silico and in vitro predictions that use Caco-2 as a surrogate for human intestinal permeability measurements. *AAPS J, 15*(2), 483-497. doi:10.1208/s12248-013-9456-8

Lea, T. (2015). Caco-2 Cell Line. In K. Verhoeckx, P. Cotter, I. Lopez-Exposito, C. Kleiveland, T. Lea, A. Mackie, T. Requena, D. Swiatecka, & H. Wichers (Eds.), *The Impact of Food Bioactives on Health: in vitro and ex vivo models* (pp. 103-111). Cham (CH).

Lee, S. J., & Yun, C. C. (2010). Colorectal cancer cells - Proliferation, survival and invasion by lysophosphatidic acid. *Int J Biochem Cell Biol, 42*(12), 1907-1910. doi:10.1016/j.biocel.2010.09.021

London, A. S., Mackay, K., Lihon, M., He, Y., & Alabi, B. R. (2014). Gel filtration chromatography as a method for removing bacterial endotoxin from antibody preparations. *Biotechnol Prog, 30*(6), 1497-1501. doi:10.1002/btpr.1961

Lu, P., Weaver, V. M., & Werb, Z. (2012). The extracellular matrix: a dynamic niche in cancer progression. *J Cell Biol, 196*(4), 395-406. doi:10.1083/jcb.201102147

Lu, X., Walker, T., MacManus, J. P., & Seligy, V. L. (1992). Differentiation of HT-29 human colonic adenocarcinoma cells correlates with increased expression of mitochondrial

RNA: effects of trehalose on cell growth and maturation. *Cancer Res, 52*(13), 3718-3725. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/1377597
Maddaluno, L., Urwyler, C., & Werner, S. (2017). Fibroblast growth factors: key players in regeneration and tissue repair. *Development, 144*(22), 4047-4060. doi:10.1242/dev.152587
Matheson, P. J., Wilson, M. A., & Garrison, R. N. (2000). Regulation of intestinal blood flow. *J Surg Res, 93*(1), 182-196. doi:10.1006/jsre.2000.5862
Meivar-Levy, I., & Ferber, S. (2003). New organs from our own tissues: liver-to-pancreas transdifferentiation. *Trends Endocrinol Metab, 14*(10), 460-466. doi:10.1016/j.tem.2003.10.006
Menard, K. P. a. M., N.R. (2015). Dynamic Mechanical Analysis in the Analysis of Polymers and Rubbers. In *Encyclopedia of Polymer Science and Technology* (pp. 1-33).
Mongiat, M., Andreuzzi, E., Tarticchio, G., & Paulitti, A. (2016). Extracellular Matrix, a Hard Player in Angiogenesis. *Int J Mol Sci, 17*(11). doi:10.3390/ijms17111822
Morgan, R. G., Mortensson, E., & Williams, A. C. (2018). Targeting LGR5 in Colorectal Cancer: therapeutic gold or too plastic? *Br J Cancer, 118*(11), 1410-1418. doi:10.1038/s41416-018-0118-6
Mouw, J. K., Ou, G., & Weaver, V. M. (2014). Extracellular matrix assembly: a multiscale deconstruction. *Nat Rev Mol Cell Biol, 15*(12), 771-785. doi:10.1038/nrm3902
Murakami, T., Kikuchi, H., Ishimatsu, H., Iino, I., Hirotsu, A., Matsumoto, T., . . . Konno, H. (2017). Tenascin C in colorectal cancer stroma is a predictive marker for liver metastasis and is a potent target of miR-198 as identified by microRNA analysis. *Br J Cancer, 117*(9), 1360-1370. doi:10.1038/bjc.2017.291
Naba, A., Clauser, K. R., Lamar, J. M., Carr, S. A., & Hynes, R. O. (2014). Extracellular matrix signatures of human mammary carcinoma identify novel metastasis promoters. *Elife, 3*, e01308. doi:10.7554/eLife.01308
Neve, A., Cantatore, F. P., Maruotti, N., Corrado, A., & Ribatti, D. (2014). Extracellular matrix modulates angiogenesis in physiological and pathological conditions. *Biomed Res Int, 2014*, 756078. doi:10.1155/2014/756078
Orlando, G. (2013). *Regenerative medicine applications in organ transplantation*: Academic Press.
Özkaya, N., & Nordin, M. (1999). Mechanical properties of biological tissues. In *Fundamentals of Biomechanics* (pp. 195-218): Springer.
Peters-Hall, J. R., Coquelin, M. L., Torres, M. J., LaRanger, R., Alabi, B. R., Sho, S., . . . Shay, J. W. (2018). Long-term culture and cloning of primary human bronchial basal cells that maintain multipotent differentiation capacity and CFTR channel function. *Am J Physiol Lung Cell Mol Physiol, 315*(2), L313-L327. doi:10.1152/ajplung.00355.2017
Peters-Hall, J. R., Coquelin, M. L., Torres, M. J., LaRanger, R., Alabi, B. R., Sho, S., . . . Shay, J. W. (2018). Long-term culture and cloning of primary human bronchial basal cells that maintain multipotent differentiation capacity and CFTR channel function. *American Journal of Physiology-Lung Cellular and Molecular Physiology, 315*(2), L313-L327.
Petersen, J. W., & Douglas, J. Y. (2013). Tenascin-X, collagen, and Ehlers-Danlos syndrome: tenascin-X gene defects can protect against adverse cardiovascular events. *Med Hypotheses, 81*(3), 443-447. doi:10.1016/j.mehy.2013.06.005

Prakash, V., Carson, B. B., Feenstra, J. M., Dass, R. A., Sekyrova, P., Hoshino, A., . . . Vincent, C. T. (2019). Ribosome biogenesis during cell cycle arrest fuels EMT in development and disease. *Nat Commun, 10*(1), 2110. doi:10.1038/s41467-019-10100-8

Prasad, S., Hogaboam, C. M., & Jarai, G. (2014). Deficient repair response of IPF fibroblasts in a co-culture model of epithelial injury and repair. *Fibrogenesis Tissue Repair, 7*, 7. doi:10.1186/1755-1536-7-7

Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., & Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nat Protoc, 8*(11), 2281-2308. doi:10.1038/nprot.2013.143

Robertson, I. B., Horiguchi, M., Zilberberg, L., Dabovic, B., Hadjiolova, K., & Rifkin, D. B. (2015). Latent TGF-beta-binding proteins. *Matrix Biol, 47*, 44-53. doi:10.1016/j.matbio.2015.05.005

Roig, A. I., Eskiocak, U., Hight, S. K., Kim, S. B., Delgado, O., Souza, R. F., . . . Shay, J. W. (2010). Immortalized epithelial cells derived from human colon biopsies express stem cell markers and differentiate in vitro. *Gastroenterology, 138*(3), 1012-1021 e1011-1015. doi:10.1053/j.gastro.2009.11.052

Roper, J., Tammela, T., Cetinbas, N. M., Akkad, A., Roghanian, A., Rickelt, S., . . . Yilmaz, O. H. (2017). In vivo genome editing and organoid transplantation models of colorectal cancer and metastasis. *Nat Biotechnol, 35*(6), 569-576. doi:10.1038/nbt.3836

Saito, N., & Kameoka, S. (2005). Serum laminin is an independent prognostic factor in colorectal cancer. *Int J Colorectal Dis, 20*(3), 238-244. doi:10.1007/s00384-004-0676-3

Saito, N., Nishimura, H., & Kameoka, S. (2008). Clinical significance of fibronectin expression in colorectal cancer. *Mol Med Rep, 1*(1), 77-81. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/21479381

Saldin, L. T., Cramer, M. C., Velankar, S. S., White, L. J., & Badylak, S. F. (2017). Extracellular matrix hydrogels from decellularized tissues: Structure and function. *Acta Biomater, 49*, 1-15. doi:10.1016/j.actbio.2016.11.068

Sambuy, Y., De Angelis, I., Ranaldi, G., Scarino, M. L., Stammati, A., & Zucco, F. (2005). The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biol Toxicol, 21*(1), 1-26. doi:10.1007/s10565-005-0085-6

San Jose, C., Cabanillas, A., Benitez, J., Carrillo, J. A., Jimenez, M., & Gervasini, G. (2010). CYP1A1 gene polymorphisms increase lung cancer risk in a high-incidence region of Spain: a case control study. *BMC Cancer, 10*, 463. doi:10.1186/1471-2407-10-463

Sato, T., Stange, D. E., Ferrante, M., Vries, R. G., Van Es, J. H., Van den Brink, S., . . . Clevers, H. (2011). Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology, 141*(5), 1762-1772. doi:10.1053/j.gastro.2011.07.050

Schalkwijk, J., Zweers, M. C., Steijlen, P. M., Dean, W. B., Taylor, G., van Vlijmen, I. M., . . . Bristow, J. (2001). A recessive form of the Ehlers-Danlos syndrome caused by tenascin-X deficiency. *N Engl J Med, 345*(16), 1167-1175. doi:10.1056/NEJMoa002939

Schettini, F., De Santo, I., Rea, C. G., De Placido, P., Formisano, L., Giuliano, M., . . . Del Mastro, L. (2018). CDK 4/6 Inhibitors as Single Agent in Advanced Solid Tumors. *Frontiers in Oncology, 8*(608). doi:10.3389/fonc.2018.00608

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., . . . Cardona, A. (2012). Fiji: an open-source platform for biological-image analysis. *Nat Methods, 9*(7), 676-682. doi:10.1038/nmeth.2019

Shay, J. W., & Wright, W. E. (2000). Hayflick, his limit, and cellular ageing. *Nat Rev Mol Cell Biol, 1*(1), 72-76. doi:10.1038/35036093

Shay, J. W., & Wright, W. E. (2000). The use of telomerized cells for tissue engineering. *Nature biotechnology, 18*(1), 22-23.

Siegel, R. L., Torre, L. A., Soerjomataram, I., Hayes, R. B., Bray, F., Weber, T. K., & Jemal, A. (2019). Global patterns and trends in colorectal cancer incidence in young adults. *Gut, 68*(12), 2179-2185. doi:10.1136/gutjnl-2019-319511

Sood, D., Tang-Schomer, M., Pouli, D., Mizzoni, C., Raia, N., Tai, A., . . . Kaplan, D. L. (2019). 3D extracellular matrix microenvironment in bioengineered tissue models of primary pediatric and adult brain tumors. *Nat Commun, 10*(1), 4529. doi:10.1038/s41467-019-12420-1

Sophie Liot, A. A., Valérie Hervieu. Naïma El Kholti, Joost Schalkwijk, Bernard Verrier, Ulrich Valcourt, EliseLambert. (2020). Loss of Tenascin-X expression during tumor progression: A new pan-cancer marker. *Matrix Biology*.

Sottile, J. (2004). Regulation of angiogenesis by extracellular matrix. *Biochim Biophys Acta, 1654*(1), 13-22. doi:10.1016/j.bbcan.2003.07.002

Srinivasan, B., Kolli, A. R., Esch, M. B., Abaci, H. E., Shuler, M. L., & Hickman, J. J. (2015). TEER measurement techniques for in vitro barrier model systems. *J Lab Autom, 20*(2), 107-126. doi:10.1177/2211068214561025

Strugari, A. F. G., Stan, M. S., Gharbia, S., Hermenean, A., & Dinischiotu, A. (2018). Characterization of Nanoparticle Intestinal Transport Using an In Vitro Co-Culture Model. *Nanomaterials (Basel), 9*(1). doi:10.3390/nano9010005

Tanzer, M. L. (2006). Current concepts of extracellular matrix. *J Orthop Sci, 11*(3), 326-331. doi:10.1007/s00776-006-1012-2

Thorne, J. T., Segal, T. R., Chang, S., Jorge, S., Segars, J. H., & Leppert, P. C. (2015). Dynamic reciprocity between cells and their microenvironment in reproduction. *Biol Reprod, 92*(1), 25. doi:10.1095/biolreprod.114.121368

Totonelli, G., Maghsoudlou, P., Garriboli, M., Riegler, J., Orlando, G., Burns, A. J., . . . De Coppi, P. (2012). A rat decellularized small bowel scaffold that preserves villus-crypt architecture for intestinal regeneration. *Biomaterials, 33*(12), 3401-3410. doi:10.1016/j.biomaterials.2012.01.012

Urbischek, M., Rannikmae, H., Foets, T., Ravn, K., Hyvonen, M., & de la Roche, M. (2019). Organoid culture media formulated with growth factors of defined cellular activity. *Sci Rep, 9*(1), 6193. doi:10.1038/s41598-019-42604-0

Urciuolo, A., & De Coppi, P. (2018). Decellularized Tissue for Muscle Regeneration. *Int J Mol Sci, 19*(8). doi:10.3390/ijms19082392

Valcourt, U., Alcaraz, L. B., Exposito, J. Y., Lethias, C., & Bartholin, L. (2015). Tenascin-X: beyond the architectural function. *Cell Adh Migr, 9*(1-2), 154-165. doi:10.4161/19336918.2014.994893

van Breemen, R. B., & Li, Y. (2005). Caco-2 cell permeability assays to measure drug absorption. *Expert Opin Drug Metab Toxicol, 1*(2), 175-185. doi:10.1517/17425255.1.2.175

Van Duyne, G. D. (2015). Cre Recombinase. *Microbiol Spectr, 3*(1), MDNA3-0014-2014. doi:10.1128/microbiolspec.MDNA3-0014-2014

Vehvilainen, P., Koli, K., Myllarniemi, M., Lindholm, P., Soini, Y., Salmenkivi, K., . . . Keski-Oja, J. (2011). Latent TGF-beta binding proteins (LTBPs) 1 and 3 differentially regulate transforming growth factor-beta activity in malignant mesothelioma. *Hum Pathol, 42*(2), 269-278. doi:10.1016/j.humpath.2010.07.005

Verhoeckx, K., Paul Cotter, Iván López-Expósito, Charlotte Kleiveland, Tor Lea, Alan Mackie, Teresa Requena, Dominika Swiatecka, and Harry Wichers, . (2015). In K. Verhoeckx, P. Cotter, I. Lopez-Exposito, C. Kleiveland, T. Lea, A. Mackie, T. Requena, D. Swiatecka, & H. Wichers (Eds.), *The Impact of Food Bioactives on Health: in vitro and ex vivo models*. Cham (CH).

Vizesi, F., Jones, C., Lotz, N., Gianoutsos, M., & Walsh, W. R. (2008). Stress relaxation and creep: viscoelastic properties of common suture materials used for flexor tendon repair. *J Hand Surg Am, 33*(2), 241-246. doi:10.1016/j.jhsa.2007.10.011

Wang, L., Johnson, J. A., Zhang, Q., & Beahm, E. K. (2013). Combining decellularized human adipose tissue extracellular matrix and adipose-derived stem cells for adipose tissue engineering. *Acta Biomater, 9*(11), 8921-8931. doi:10.1016/j.actbio.2013.06.035

Wang, S., Wang, X., Boone, J., Wie, J., Yip, K. P., Zhang, J., . . . Liu, R. (2017). Application of Hanging Drop Technique for Kidney Tissue Culture. *Kidney Blood Press Res, 42*(2), 220-231. doi:10.1159/000476018

Wang, W., Zhang, L., Morlock, L., Williams, N. S., Shay, J. W., & De Brabander, J. K. (2019). Design and Synthesis of TASIN Analogues Specifically Targeting Colorectal Cancer Cell Lines with Mutant Adenomatous Polyposis Coli (APC). *J Med Chem, 62*(10), 5217-5241. doi:10.1021/acs.jmedchem.9b00532

Wells, R. G. (2008). The role of matrix stiffness in regulating cell behavior. *Hepatology, 47*(4), 1394-1400. doi:10.1002/hep.22193

Wrighton, K. H. (2011). Mechanotransduction: YAP and TAZ feel the force. *Nat Rev Mol Cell Biol, 12*(7), 404. doi:10.1038/nrm3136

Xu, J., Sun, M., Tan, Y., Wang, H., Wang, H., Li, P., . . . Li, Y. (2017). Effect of matrix stiffness on the proliferation and differentiation of umbilical cord mesenchymal stem cells. *Differentiation, 96*, 30-39. doi:10.1016/j.diff.2017.07.001

Xu, R., Boudreau, A., & Bissell, M. J. (2009). Tissue architecture and function: dynamic reciprocity via extra- and intra-cellular matrices. *Cancer Metastasis Rev, 28*(1-2), 167-176. doi:10.1007/s10555-008-9178-z

Ye, M., Zhang, Y., Gao, H., Xu, Y., Jing, P., Wu, J., . . . Zhang, J. (2018). Activation of the Aryl Hydrocarbon Receptor Leads to Resistance to EGFR TKIs in Non-Small Cell Lung Cancer by Activating Src-mediated Bypass Signaling. *Clin Cancer Res, 24*(5), 1227-1239. doi:10.1158/1078-0432.CCR-17-0396

Yeh, Y. T., Hur, S. S., Chang, J., Wang, K. C., Chiu, J. J., Li, Y. S., & Chien, S. (2012). Matrix stiffness regulates endothelial cell proliferation through septin 9. *PLoS One, 7*(10), e46889. doi:10.1371/journal.pone.0046889

Yeung, T. M., Gandhi, S. C., Wilding, J. L., Muschel, R., & Bodmer, W. F. (2010). Cancer stem cells from colorectal cancer-derived cell lines. *Proc Natl Acad Sci U S A, 107*(8), 3722-3727. doi:10.1073/pnas.0915135107

Yu, Y., Alkhawaji, A., Ding, Y., & Mei, J. (2016). Decellularized scaffolds in regenerative medicine. *Oncotarget, 7*(36), 58671-58683. doi:10.18632/oncotarget.10945

Yue, B. (2014). Biology of the extracellular matrix: an overview. *J Glaucoma, 23*(8 Suppl 1), S20-23. doi:10.1097/IJG.0000000000000108

Yun, Y. R., Won, J. E., Jeon, E., Lee, S., Kang, W., Jo, H., . . . Kim, H. W. (2010). Fibroblast growth factors: biology, function, and application for tissue regeneration. *J Tissue Eng, 2010*, 218142. doi:10.4061/2010/218142

Zhang, L., Theodoropoulos, P. C., Eskiocak, U., Wang, W., Moon, Y. A., Posner, B., . . . Shay, J. W. (2016). Selective targeting of mutant adenomatous polyposis coli (APC) in colorectal cancer. *Sci Transl Med, 8*(361), 361ra140. doi:10.1126/scitranslmed.aaf8127