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<td>Cytochrome P450</td>
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<td>Hepatic stellate cells</td>
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<td>Extracellular matrix</td>
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EGF receptor  
Transforming growth factor alpha  
Heparin binding EGF  
Basic fibroblast growth factor  
Urokinase-type plasminogen activator  
N-2-acetylaminofluorene  
Connective tissue growth factor  
Mesenchymal to epithelial transition  
Epithelial to mesenchymal transition  
Tissue inhibitor of metalloproteinases  
Smooth muscle cells  
Hepatocellular carcinoma  
Cell adhesion molecules  
Focal adhesion kinase  
Phosphoinositol 3-kinase  
Protein kinase B  
Crk-associated substrate  
c-Jun N-terminal kinases  
Mitogen-activated protein kinase 1  
Mitogen-activated protein kinase 3  
Ras homolog family member  
Ras-related C3 botulinum toxin substrate  
Growth factor receptor-bound 7  
Ras homolog family member A  
Actin-related protein  
EGFR  
TGFα  
HB EGF  
bFGF or FBF2  
u-PA  
2-AAF  
CTGF  
MET  
EMT  
TIMP  
SMC  
HCC  
CAM  
FAK  
PI3K  
AKT  
CAS  
JNK  
MAPK1  
MAPK3  
Rho  
Rac  
GRB7  
RhoA  
Arp 2/3
Cell division control protein 42	CDC42
Neural Wiskott-Aldrich syndrome protein	N-WASP
Engulfment and Cell Motility 2	ELMO2
Integrin-linked kinase	ILK
Rho-associated protein kinase	ROCK
Polyethylene glycol	PEG
Polyethylene glycol diacrylate	PEGDA
Magnetic Resonance Elastography	MRE
Loss modulus	G''
Storage modulus	G'
Atomic force microscopy	AFM
Superior vena cava	SVC
Inferior vena cava	IVC
Hepatocyte nuclear factor 4 alpha	HNF4α
The extracellular matrix is a complex environment of mechanical and chemical cues vital to individual cell growth, tissue formation, and ultimately complete organ function. The field of regenerative medicine aims to recreate the unique biological properties found in specific tissue types and to incorporate these properties into substrates that support long term cell function for applications including tissue engineering, cell therapies, and disease models. Because of the complexity of the extracellular matrix, difficulties exist in characterizing and utilizing components of the matrix that contribute to the long term function and viability of cells. Primary liver epithelial cells, or hepatocytes, are easily isolated in large quantities. However, these cells quickly lose viability and function once removed from the native organ. In order to support hepatocyte phenotype in culture, decellularized liver matrix was incorporated into transplantable hyaluronic acid hydrogels. These substrates were shown to bind growth factors, support hepatocyte attachment, and promote cell junction formation. In liver tissue, the physical property of matrix stiffness affects a myriad of cell behaviors including; cell attachment, viability, function, growth factor utilization, motility, and cytoskeletal organization. Gels were crosslinked at different stiffnesses in order to mimic and test a variety of mechanical environments. The hepatocyte substrates were formulated within a narrow physiologic range from slightly above to slightly below that of native liver. Primary human hepatocyte attachment, viability, and functional output increased with stiffness. Stiffness influenced hepatocyte morphology, cell signaling, and expression of important liver specific markers dependent on duration in culture and presence or absence of matrix in the gel. In conclusion, data demonstrates that inclusion of extracellular matrix in primary
hepatocyte cell culture substrates affects cell phenotype, and these effects are influenced by small changes in stiffness of the substrates. This thesis work suggests that substrate formulation and stiffness can be optimized for liver cell culture and different regenerative medicine applications.
# CHAPTER 1

Introduction to Liver Architecture and the Role of the Extracellular Matrix on Liver Cellular Function

Daniel B. Deegan

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1. Function and Gross Anatomy

1.1 Liver Function

The liver is the largest internal organ of the human body and performs over 500 unique functions (1). It is a major site of plasma protein biosynthesis, energy storage, metabolism, and detoxification. The liver produces important compounds including clotting factors, heparin, and albumin that give circulating blood many of its important physical properties (1). It also produces bile, which aids the digestion of lipids into fatty acids that are used throughout the body (2, 3). The liver is the main storage site for glycogen and serves as a buffer for blood glucose levels through activation of glycogenolysis, glycogenesis, or gluconeogenesis (4, 5). The organ also stores fat soluble compounds, vitamins, and minerals for use by cells throughout the body.

The liver is not only crucial to the production and storage of many of the building blocks used within the body, but is also vital for the removal of toxic compounds from the system, such as cellular waste products and xenobiotics (6). The liver aids in the filtration of damaged or dying red blood cells from the circulation. Bilirubin, formed from breakdown of hemoglobin, causes jaundice unless processed and excreted by the liver (7). The liver also metabolizes and excretes alcohol and toxic drug compounds like acetaminophen. These compounds are detoxified by a two-phase process where electrophilic intermediate metabolites are conjugated by substrates. This results in a detoxified polar product that may be excreted in the bile or urine (8).
1.2 Liver Vasculature

The importance of the liver is evidenced by the fact that this organ receives ¼ of the entire cardiac output. 25% of the blood moves directly from the heart through the hepatic artery to the liver. The other 75% of input enters the liver though the portal vein and is lower in oxygen content but contains nutrient rich blood from the intestines (9). This portion of the blood supplies the liver with needed factors, while also allowing the liver to filter and detoxify xenobiotic compounds entering the circulation through the digestive tract.

1.3 Macroscopic Structures

The human liver is composed of four main lobes; the left, right, caudate, and quadrate lobes. These lobes are further divided into subunits called lobules. Lobules are hexagonal shaped arrangements that contain discontinuous fenestrated endothelium, or sinusoids (10). These sinusoids are in intimate proximity to the liver parenchyma. The unfettered diffusibility through the sinusoids allows for greater interactions of parenchymal cells with compounds carried in the blood, as compared to endothelium within other tissue types (11). Each liver lobule is fed by 6 portal triads positioned at the periphery of the lobule. This structure is composed of a portal vein and hepatic artery, as well as a bile duct. Blood flows out of the lobule through the central vein that is positioned in the middle of the lobule. Bile canaliculi run parallel to the liver sinusoids but flow in the opposite direction of the sinusoidal circulation (12). The canaliculi merge with the biliary tree at structures called the Canals of Hering, which flow to the common bile duct that terminates in the gall bladder (13).
2. Cell Types of the Liver

The liver contains least 15 unique cell types (10). These cells types can be narrowed down to 5 main cell types found within and around the parenchyma, sinusoids, and bile ducts of the liver. Within these liver regions, cells communicate through autocrine and paracrine signaling to support various cell phenotypes. Together, these cells function to affect liver homeostasis and maintain a healthy organism.

2.1 Hepatocytes

Hepatocytes are the main cell type of the liver parenchyma and can carry out all major functions of the liver. Chief products include bile, cholesterol and albumin (15). Hepatocytes constitute 60% of the cell number and 80% of the actual volume of the liver (10, 16). They are polarized cells arranged in two-dimensional cords with one side of the cell communicating with the hepatic sinusoid, while the opposing side communicates
with the bile canaliculus (17, 18). The surface of the hepatocyte is covered with small projections known as microvilli which extend into the perisinusoidal area between the hepatocyte and the endothelium, called the space of Disse. These structures greatly expand the surface area of the cell, facilitating interactions with blood plasma that diffuses through the sinusoidal wall (19). Hepatocytes attach to each other by tight and adherens junctions on their lateral surfaces (20). These junctions isolate the apical surface, forming the canaliculus through which bile flows. These bile canaliculi provide complex linkages surrounding the cells that allow bile to drain into the bile ducts of the liver (17).

The functional roles of hepatocytes vary according to their location within a lobule (21). The lobules can be divided into three zones according to differences in oxygen, nutrient, and metabolite concentrations (22). Zone 1 is an oxygen and nutrient rich area closest to the portal triads at the periphery of the liver lobules. In this zone, hepatocytes have greater numbers of mitochondria which provide higher rates of oxidative phosphorylation that drive processes requiring great amounts of energy such as gluconeogenesis. Cells within this zone are also more frequently affected by viral infections like hepatitis because of their proximity to the portal area (23). Zone 2 is a transition region of lower oxygen content. Zone 3 surrounds the central vein and has lowest oxygen and nutrient concentration. Hepatocytes in zone 3 contain less mitochondria and a greater amount of smooth endoplasmic reticulum (SER) (24). They carry out processes such as glycolysis, lipid production, and xenobiotic detoxification that remove harmful compounds before they reenter the circulation (25, 26). These cells are also vital for the detoxification of bilirubin. Because of their roles in filtration and
excretion, these cells highly express cytochrome P450 (CYP) enzymes and are the cells most negatively impacted by toxin intake (27). The low oxygen content in zone 3 also makes hepatocytes of this area most susceptible to ischemic damage resulting from cirrhosis (26).

Upon liver injury, hepatocytes rapidly proliferate to regenerate liver tissue. The proliferative capacity of hepatocytes allows the liver to repair itself, despite ongoing contact with toxins or loss of up to 75% of tissue mass (28, 29). This unique ability is most likely the result of evolutionary changes and adaptation to the liver’s role in the body as a site of detoxification and waste processing.

2.2 Hepatic Stellate Cells (HSC)

Hepatic stellate cells are the architects of the liver and comprise 5-8% of the total liver cell number (30). These cells exist in two states in the space of Disse, a quiescent fat storing cell or an activated myofibroblast (31). In their normal quiescent state, stellate cells have a spindle-like morphology with elongated processes that extend between the sinusoids and hepatic cords and enable them to sense changes in the microenvironment (32). These cells store fat droplets and fat soluble compounds such as vitamin A. Even if not fully activated, cells in the less studied quiescent state have been reported to influence the extracellular matrix (ECM) by balancing the production of ECM degrading matrix metalloproteinases (MMPs) with low levels of matrix protein biosynthesis molecules (33, 34). Quiescent cells have also been shown to secrete soluble growth factors such as hepatocyte growth factor (HGF) (31).
Hepatic stellate cells become activated when they sense physical cues in the matrix or chemical signals released from immune cells and dead or dying hepatocytes resulting from toxicity, metastatic tumor cells, or viral infections. Upon activation, these cells express genes for alpha-smooth muscle actin (α-SMA) and collagen-1 (Col1) (32, 33). They adopt a myofibroblastic morphology, proliferate, and secrete various pro-inflammatory and mitogenic cytokines. HSCs travel to sites of injury and remodel the damaged tissue matrix by the production of MMPs, hepatocyte-influencing growth factors, and ECM molecules (35). The majority of ECM molecules generated are collagens. However, HSCs also produce several other ECM components including fibronectin, laminin, and glycosaminoglycans (GAGs) (36). Although vital to repair and acute injury response, HSCs can become overactive in a chronic liver injury state. This imbalance causes excess deposition and accumulation of ECM in the liver, eventually leading to fibrosis and cirrhosis (37, 38).

Like hepatocytes, HSC morphology and composition change between three zones of the lobules. In the periportal region, stellate cells have a smaller cytoplasmic volume and project numerous extensions into the space of Disse. In the middle zone, cells are larger, longer, and contain greater reserves of lipids and desmin. Moving into the pericentral zone, desmin levels in stellate cells decrease, while vitamin A amounts increase (32).

2.3 Kupffer Cells

Kupffer cells are star shaped immune cells located within the sinusoidal barrier. Although they only represent a small percentage of all cells within the liver, they
represent 80-90% of all macrophages in the body (39). Upon activation, Kupffer cells secrete inflammatory cytokines, growth factors, and reactive oxygen species (ROS) in response to various stimuli. These macrophages phagocytose large particles and eliminate dead or damaged red blood cells (RBCs), cancer cells, and hepatocytes, as well as bacteria and other foreign entities (40). Kupffer cells process millions of RBCs per minute and breakdown their main component, hemoglobin, into reusable polypeptides, iron, and bilirubin. Toxic bilirubin attaches to albumin within the blood and eventually diffuses through the sinusoidal lining. In the space of Disse, hepatocytes conjugate bilirubin for excretion out of the body (41).

2.4 Sinusoidal Endothelial Cells

Sinusoidal endothelial cells (SECs) constitute 20% of the total liver cell number. Unlike normal endothelial cells, hepatic sinusoidal cells form a fenestrated endothelium that lies on a discontinuous basal lamina in the liver lobules. These sinusoids control passive diffusion of substances into the parenchyma through sieve-like action and filter entering plasma as it passes from the blood into the space of Disse. Porosity of the endothelium increases from the portal triad to the central vein (42). This property allows for greater diffusion and interaction of waste products and metabolites with hepatocytes and HSCs in zone 3. Besides passive diffusion, SECs also scavenge waste products and cell remnants through phagocytosis or actively transport compounds from the sinusoidal lumen to perisinusoidal space through transcytosis (16).

SECs secrete a wide variety of cytokines, growth factors, and other compounds into the circulation. SECs contribute to the generation of fibronectin, collagen IV, and
participate in the activation of transforming growth factor beta (TGF-β) to the active form (26, 43). These cells also produce autocrine vasoactive compounds, such as endothelin-1 (ET-1), to affect blood flow and uptake from the sinusoidal lumen (44). Presence and long term exposure to certain drugs or toxins such as nicotine, endotoxin, and ethanol decrease fenestration size and reduce the ability of molecules like cholesterol to pass through the sinusoids for hepatocyte processing (10). This build-up in cholesterol can lead to atherosclerosis. Defenestration also blocks retinol metabolism and activates HSCs within the space of Disse (45). This activation exacerbates fibrosis of the perisinusoidal space, eventually resulting in cirrhosis.

2.5 Cholangiocytes (Biliary Epithelial Cells)

Cholangiocytes are epithelial cells that represent an alternate differentiation lineage of liver progenitor cells. Cholangiocytes vary in size and morphology and line extrahepatic and intrahepatic bile ductules (46). Unlike hepatocytes which produce bile-acids that must be actively transported to the canaliculus, cholangiocytes secrete components of bile such as electrolytes that enter the bile ducts through passive ionic exchange processes (39). Cholangiocytes modify bile by secretion and adsorption of ions and other molecules as it passes through the biliary tree. Cholangiocytes ultimately traffic bile through the liver, which exits to both the common bile duct and the gall bladder (47).

3. ECM components

As has been shown in multiple cell types, availability of nutrients and oxygen controls cell morphology and functional capacity. Another factor which greatly determines cell activity and viability is the underlying ECM. Liver cells are organized
and maintained by a complex extracellular microarchitecture. Unlike other organs, the ECM composes less than 10% of the liver volume (48). Despite this small percentage, ECM molecules play a tremendous role in liver function regulation, and direct repair and regeneration processes within tissue (49). When out of balance, excess ECM deposition causes dysfunction and disease states of the liver. Fibrillar collagens constitute the majority of the liver, but non-fibrillar collagens, glycoproteins, and glycosaminoglycans also play important roles in cell physiology.

3.1 Fibrillar Collagen

Fibrillar collagens, including collagens I, III, and V, comprise the largest percentage of ECM proteins in the liver. These collagens contain rigid triple helix amino acid structures and form heterogeneous fiber bundles that give the liver its strength and mechanical properties (49). Collagen I is the thickest and most prevalent type of fibrillar collagen. It is a highly ubiquitous protein but can be found in the highest concentrations in the periportal and pericentral regions (50). It contains several binding sites per peptide that attach α1β1 and α1β2 integrins. Collagen I also possesses several non-specific low affinity binding sites for cell attachment (51). Many other ECM components can complex with collagen I to interact with cells including other collagens, fibronectin, and proteoglycans.

Collagen III is structurally similar to collagen I and is found in all areas of the liver. Greater amounts are present in the periportal regions (22). This collagen type also comprises a large percentage of developing tissue. In adult liver, collagen III associates strongly with collagen I bundles. Collagen V is the last major fibrillar collagen found
throughout liver. Their thin fragile fibers link multiple collagen types to each other to form cohesive units at both ends of the sinusoids (50).

3.2 Non-Fibrillar Collagens

Non-fibrillar collagens are a category of basement membrane molecules with fragmented triple-helical structures. Their flexible structures create networks between other ECM components (49). Collagen IV is the main constituent of the basement membrane and secures other basement membrane components like perlecan and laminin (52) to the underlying structure. This collagen is chiefly secreted by endothelial cells. It forms the discontinuous sinusoids of liver lobules that allow for blood plasma flow into the space of Disse (49).

Other collagens of the basement membrane include collagen VI and VIII. Collagen VI forms networks with different collagen types and assembles collagen bundles, including collagens I and III fibers. Collagen VI affixes smaller basement membrane molecules as well as larger structures, such as blood vessels, by attachment and connection to collagen IV subunits (50). This collagen also functions in tissue repair through the binding and release of soluble factors that regulate mitogen-activating protein kinases (MAPKs). Collagen VIII also binds cytokines that may be liberated to initiate wound healing, especially during angiogenesis (49). This collagen type connects elastic fibers of the vasculature and contributes to the physical properties of vessels.

3.3 Non-Collagenous Glycoproteins

Fibronectin and laminin are the most widespread glycoproteins of the liver. Both glycoproteins have numerous roles in development, injury repair, and normal cellular
activity. Fibronectin (FN) is a high molecular weight protein dimer that encompasses numerous variants with two principal forms; plasma and cellular fibronectin. Plasma fibronectin is the most abundant glycoprotein found in the liver and is synthesized primarily by hepatocytes (53). Cellular fibronectin is an insoluble form that exists at low levels in normal liver tissue. The highest concentrations of this form are found in the pericentral area (22). It is secreted by a variety of cell types, but the main producer is the stellate cell. Cellular FN binds several other ECM components including collagen I, perlecan, and fibrin (54). It concentrates in bundles attached to hepatocyte microvilli and collagens of the space of Disse (55). It also occupies the pericellular spaces and basal membranes in the liver’s portal regions (56, 57).

During tissue repair, plasma FN enters the site of injured tissue and initiates ECM degradation and remodeling by activation of Kupffer cells and hepatic stellate cells. In this process, activated stellate cells produce MMPs that degrade plasma FN. Hepatocytes replace plasma FN with insoluble cellular FN for incorporation into the ECM (55). Fibronectin contains the Arginine-Glycine-Aspartic acid (RGD) cell binding sequence that interacts with several integrin types. With these high affinity binding sites, cellular FN acts as a chemoattractant, cell adhesion molecule, and growth factor for migrating cells.

Laminin is another RGD-containing ligand found chiefly in the basement membrane of the portal region and the sinusoidal wall. It is also present in certain liver growth and developmental states in the perisinusoidal space (58). Laminin attaches cells of the sinusoids through integrin interactions and can induce cell migration and motility. This glycoprotein secreted by hepatic stellate cells is vital in vascular structural
maintenance, but is also important in the regulation of development and differentiation in the liver (49, 59).

3.4 Glycosaminoglycans

GAGs are another important group of molecules that promote cell adhesion and viability within the ECM. GAGs are long polysaccharide chains of various sizes and sulfation states (60). There are six total GAG types that are classified into four structurally distinct families; heparan sulfate (HS)/heparin, chondroitin (CS)/dermatan sulfate (DS), keratan sulfate (KS), and hyaluronan (61). Most of these polysaccharides covalently attach to distinct protein cores to form proteoglycans and integrate with the ECM. The exception is HA, which does not directly covalently bond to proteins to form proteoglycans, but directly and independently attaches to the ECM (62).

GAGs bind and affect enzymes, enzyme inhibitors, cell attachment molecules, ECM proteins, and growth factors of the liver and other organ systems. These proteins contain positively charged amino acids that form ionic and hydrogen bonds to negatively charged sulfates and carboxylates of GAGs (63, 64). Specificity and strength of these interactions are determined by protein confirmation and how certain amino acid residues align with the arrangement of sulfated sites (62, 63). Through these interactions, GAGs influence the mechanical properties of the ECM, enzyme activation, and activity of a vast array of tissue specific growth factors.

GAGs greatly increase the half-life of growth factors compared to soluble growth factors that remain free in plasma. GAG binding protects these proteins from proteolytic degradation (65). This interaction also enhances presentation and utilization of growth
factors by clustering bound growth factors close to cell receptors of attached cells (66). With the capacity to control periodic release, cells easily exploit housed growth factors when needed for growth and functional maintenance (19). The ability to preserve and display growth factors reduces the need of tissues to generate large quantities of growth factors in a short period of time, which might produce adverse systemic effects.

The most common proteoglycan in the liver is perlecan, which is mainly produced by hepatocytes and is comprised of the GAG, heparan sulfate (67). HS proteoglycans compose 60% of all proteoglycans in the liver and are normally found in the ECM of the sinusoids, perisinusoidal space, and sinusoidal walls (49). HS has a similar structure to heparin but is generally longer in length and more varied in disaccharide and sulfated configurations (63, 68). This complexity and flexibility in arrangement allows HS to interact with a wide variety of biologically active proteins, growth factors, and cellular receptors (69). This GAG binds to collagen, fibronectin, and laminin in the ECM to support tissue structure and cell adhesion. HS binding to collagen organizes collagen bundles. Interactions with fibronectin and laminin control their confirmation and biological activity (70). HS is also a receptor for several different growth factors that affect cell growth and function, which includes a high specificity for HGF in the liver (62, 71, 72).

Hyaluronic acid (HA) comprises only a minor fraction of the liver ECM and total proteoglycan volume but still has significant roles in the physical properties of liver tissue. HA sequesters water to maintain hydration of the ECM and also provides lubrication to enable cell motility and migration (73). HA is important during new liver tissue formation. Concentrations of HA increase during development and tissue
regeneration to promote activity of proliferating cells (74). These properties and its ability to avoid immune reactions upon transplantation make HA a suitable substrate for cell culture and eventual use for in vivo treatments (75).

3.5 ECM Bound Growth Factors

Growth factors have a wide range of functions that vary from cell growth and proliferation to phenotypic determination and maintenance. Glycoproteins of the ECM bind these growth factors non-covalently through hydrogen binding and electrostatic interactions to protect them from degradation, concentrate them in specific areas of the tissue, and regulate interactions with cell receptors (76-78). Following attachment to nearby ECM molecules, cells utilize proteolytic cleavage to free bound growth factors, making them available to interact with membrane bound receptors (79). Numerous growth factors are important to the regulation of liver function. Three of these proteins with the most significant impact in the liver are hepatocyte growth factor (HGF), epidermal growth factor (EGF), and fibroblast growth factor (FGF).

HGF is a unique heterodimer chiefly secreted by mesenchymal cells, especially HSCs in the liver. It is also produced in lower levels by SECs in the liver (80, 81). After activation of this growth factor by serine protease, HGF acts as a powerful mitogen and stimulates cell proliferation by binding to the c-Met receptor of epithelial and endothelial cells to activate tyrosine kinase signaling (82, 83). HGF strongly affects both liver progenitor cells and differentiated hepatocytes (84-88). Studies with mesenchymal stem cells showed that the presence of HGF on surrounding ECM drives differentiation toward adult hepatocytes (89). HGF is vital to both progenitor cell and hepatocyte-mediated liver
regeneration. Spikes in HGF release, up to 20-fold normal plasma levels, correlate with increased liver mass production (29, 90, 91). This protein also controls morphology, migration, and tissue organization during reparative processes in the liver (84, 92, 93). In animal models, HGF treatment accelerated recovery from cirrhosis and enhanced regeneration (94). Because of its mitogenic properties, increased HGF levels have also been implicated in aiding development of certain types of cancer (95).

HGF binds with a high affinity to the liver matrix through the GAGs, heparin and heparan sulfate. HS mediates HGF use by cells and increases its half-life from 4 minutes in blood plasma to several hours to match usage rates of surrounding cells (96-98). HGF also has the ability to bind to areas of the matrix without HS through low affinity interactions with several collagen types (99). These properties of HGF show its importance in maintaining hepatocyte viability and function and demonstrate how substrates could be designed to maximize HGF utilization in vitro or during cell therapies.

While the importance of HGF in the maintenance of hepatocytes is well established, other factors greatly affect and support liver cells. EGF is an important growth factor that affects growth, viability, and differentiation of cells. EGF is primarily produced in the Brunner’s gland of the duodenum (100). In the liver, it enters through the portal vein of the portal triad and stimulates G1 to S phase transition and activates cell division of hepatocytes. Unlike HGF, EGF acts through the tyrosine kinase EGF receptor (EGFR) (101). It is vital for normal liver regeneration and induces Cyclin D1 (102, 103). Other ligands of the EGFR also have similar mitogenic effects to EGF, like transforming growth factor alpha (TGFα) and heparin binding EGF (HB EGF). They are manufactured
by endothelial cells and Kupffer cells in the liver and have both autocrine and paracrine roles in proliferation (29, 104).

Another highly mitogenic growth factor family is the fibroblast growth factor family. Like with HGF, heparin and heparan sulfate have a strong affinity for FGF. There are 22 members of FGF family, but the most studied and recognized member is basic fibroblast growth factor (bFGF or FBF2) (91, 105). FBF2 is secreted by HSCs in the perisinusoidal region and, similar to HGF and EGF, is vital to wound healing and angiogenesis in the liver (106).

4. Liver Regeneration

The roles of all of these tissue components show the importance of interplay between cells, ECM structural molecules, and growth factors in the liver. These relationships are not only important for normal function but also for development and regeneration. Liver regeneration is a well-studied growth process which gives insights into ontogeny of all organs (107). During liver regeneration, there is a complex step-wise process that creates coordination between all liver components in order to grow and organize new tissue. The exact mechanisms that mediate liver regeneration and the cell phenotypes involved in the process are still hotly debated.

4.1 Normal Liver Repair and Regeneration

Loss of liver tissue, either by physical removal of tissue mass or acute chemical toxicity, initiates the liver regenerative process. In the case of acute liver failure, dying cells stimulate an immune response by Kupffer cells to clear dead cellular material (108). Kupffer cells then activate HSCs and hepatocytes to begin regeneration of liver tissue
According to one model, liver regeneration is primarily accomplished by proliferation of adult hepatocytes of the parenchyma. An alternate theory states that regeneration is a delicate balance between epithelial to mesenchymal transitions (EMT) and mesenchymal to epithelial transitions (MET), similar to the lineage transitions that occur during development. This theory claims that following injury, epithelial cells within the liver undergo EMT and migrate to the periportal zone. These fibroblasts initiate repair and replicate before reverting back to hepatocytes or cholangiocytes in order to repopulate the liver parenchyma.

During liver restoration, one of the first molecules secreted to regulate cells is a proteinase called urokinase-type plasminogen activator (u-PA). It is released as rapidly as 1 minute following partial hepatectomy and correlates with an increase in its receptor u-PAR. Besides releasing plasminogen, u-PA frees and activates ECM bound HGF for immediate use and stimulation of hepatocytes. A peak in the release of HGF occurs just one hour post-partial hepatectomy. During this time, HSCs, Kupffer cells, and SECs are activated to release HGF, EGF, and other growth factors to promote further hepatocyte proliferation, as well as autocrine mediated proliferation. Peaks in growth factor production occur at 24 hours and 48 hours post-injury.

MMPs are also produced to degrade old or damaged matrix and allow for unrestricted hepatocyte replication. As a result, fibronectin and other cellular attachment molecules decrease in the periportal regions until after the cellular mass is restored. Hepatocytes proliferate starting in the periportal area. Proliferation proceeds into the mid-lobular area, before finally initiating in the pericentral region. Full cellular mass is replaced by 5-7 days in assays with rat models and around 3 months in humans.
In normal intact ECM, HSCs simultaneously remodel matrix as new cells proliferate. HSCs fully transdifferentiate into myofibroblasts and migrate to damaged areas to lay down ECM, starting with collagen I and FN in the perisinusoidal space (18). However, in an injury where whole tissue is lost, HSCs delay ECM production until new hepatocyte clusters are created. HSCs infiltrate newly proliferated masses and secrete laminin, collagen, and fibronectin to align hepatocytes into cords (59). This action attracts endothelial cells to form new bile ducts and fenestrated sinusoids. Hepatocyte division ceases when newly synthesized glycoproteins sequester HGF (115). Feedback mechanisms between HSCs and hepatocytes also increase production of TGF-β1, which halts new HGF secretion and returns proliferating hepatocytes to their resting states (35). In the days that follow, further ECM is produced to organize cells into proper lobule structures and restore full function in the liver.

4.2 Progenitor Cell Mediated Repair and Regeneration

In specific cases of liver repair, massive necrosis or chronic injury inhibits proliferation of the resident hepatocyte population. This scenario is modeled in rats by treatment of the chemical N-2-acetylaminofluorene (2-AAF) followed by partial hepatectomy (116). In these situations, the liver activates bipotential hepatic progenitor cells, to repopulate the tissue. In progenitor cell mediated regeneration, HSCs work in close proximity with progenitor cells to direct migration, proliferation, and differentiation (115, 117). HA deposits surround these two cell types during OC regeneration and provide lubrication to allow cells to more easily migrate (38, 73). HSCs create a provisional fibronectin rich matrix, which guides progenitor cells to damaged tissue with the aid of bound connective tissue growth factor (CTGF) (116). CTGF and HS-bound
HGF stimulate progenitor cells to attach and replicate (118). These bipotential cells either become differentiated hepatocytes or cholangiocytes to replenish lost tissue. Evidence also exists that stellate cells themselves could undergo mesenchymal to epithelial transition in order to bolster the compensatory hyperplasia (119). Following attachment and differentiation, fibronectin and HA concentrations diminish, and a normal matrix composition replaces the provisional matrix of the liver. This alternative method of regeneration proves that nature has more than one method of repair in the body to overcome different scenarios.

5. Liver Disease

Repair or regeneration is a delicate balance between matrix production, matrix degradation, and cell proliferation. Despite the extraordinary abilities of the liver to repair itself from acute trauma, problems arise when imbalances exist. Persistent stresses of toxins, alcohol, or viral hepatitis cause fibrosis and advanced cirrhosis, which lead to health complications or mortality in humans (52). Autoimmune responses, metabolic disorders, and simple genetics can also initiate liver dysfunction in both infants and adults (120). Many hallmarks of liver disease can be attributed to either the overactivation of fibroblasts and/or dedifferentiation of hepatocytes. In certain disease states, loss of function in cholangiocytes is also evident.

5.1 Metabolic Disease

Inheritance of faulty genes can cause specific enzyme deficiencies which result in disorders of the liver. These deficiencies can occur in several different cell organelles and impair various metabolic functions. Lysosomal and peroxisomal disorders can lead to
buildup of excess toxins and wastes that damage liver tissue (121). Specific diseases related to these organelles include Tay-Sachs disease, Gaucher disease, and Zellweger syndrome (122). Hemochromatosis is a deficiency in metabolism of metals in the body which creates an accumulation of toxic levels of iron in the liver. Problems with metabolism of galactose (galactosemia) or glycogen storage disease can lead to impairment in regulation blood glucose levels. These imbalances can cause energy loss and swelling of the liver (123). All of these metabolic diseases are currently incurable but can be managed with proper nutrition or enzyme replacement therapies.

5.2 Cholangiopathies

Chronic liver disease is the progressive loss of liver function which can result in end-stage liver disease and death. One of the cell types potentially targeted during the development of chronic liver disease is the cholangiocyte. Cholangiopathies were responsible for 16% of liver transplants from the years 1988 to 2014 (124). These diseases include primary sclerosing cholangitis, biliary atresia, cystic fibrosis, and biliary cirrhosis. During progression of these disorders, apoptosis of cholangiocytes, inflammation, and excess collagen deposition results in obstruction or destruction of bile ducts. This biliary degeneration can lead to increased levels of lipids and cholesterol, portal hypertension, and liver scarring ultimately resulting in high mortality rates (124, 125).

5.3 Progression of Liver Fibrosis

One of the chief orchestrators of chronic liver disease and patient morbidity is HSCs. Constant trauma and damage triggers release of inflammatory mediators and pro-
fibrotic cytokines that activate HSCs and portal fibroblasts (126). These activated cells deposit excess quantities of ECM leading to hallmarks of fibrosis. Hepatocytes, SECs, and cells that have undergone EMT also contribute to increased matrix and cytokine production (43).

Collagen I, the most prevalent ECM component, increases throughout the liver tissue and initiates further HSC activation. Cells in the space of Disse of the periportal region deposit abnormal amounts of laminin, type IV collagen, and perlecan. Collagen IV can increase up to 10-fold, while perlecan increases 8-times the normal liver concentration (49). This build-up of matrix increases endothelial cell attachment and eventually closes off the fenestrated sinusoids (42). Creating a continuous basement membrane seals off the sinusoids and has several detrimental effects on the liver. Most importantly wastes, toxins, and xenobiotics no longer easily interact with hepatocytes and instead, move through the liver into the peripheral circulation. If left unprocessed, excess bilirubin, the byproduct of hemoglobin catabolism, causes jaundice (127). Closed sinusoids also prevent lipids from being metabolized in the parenchyma and leads to atherosclerosis in patients (42). With increased fibrosis, sinusoids and liver vessels constrict and block, which restricts blood flow and causes portal hypertension in patients (128).

Changes during fibrosis also occur in the ECM of the centrilobular region. This area accumulates irregular levels of fibronectin, type III collagen, and dermatan sulfate. Elevated amounts of insoluble cFN activate HSCs and increase SEC attachment. Increased plasma FN causes aberrant Kupffer cell activation and increased inflammation
in the liver. Without corrections in matrix deposition, cirrhosis leads to complications and total loss of liver function.

Fibrosis is not only the result of increased matrix synthesis but additionally from reduced matrix degradation. During normal liver repair, HSCs maintain an equilibrium between production of MMPs and tissue inhibitor of metalloproteinases (TIMPs) (57). However, during fibrosis, TIMPs, especially TIMP1 and TIMP2, are secreted at a greater rate and block any action of MMPs (49). This inhibition, along with increases in matrix production, worsens liver health and function. To treat liver disease, therapies need to both revert HSCs and other ECM secreting cells to quiescent states and block the release of TIMPs. Through these actions, balance and normal liver function can be restored.

6. Stiffness in Tissue Microenvironments

Increased collagen deposition and crosslinking during fibrosis cause changes in the mechanical properties of the ECM. Mechanical properties of tissue microenvironments contribute to the overall function and health of the organ systems they comprise. These forces include shear stress induced by laminar flow in the vasculature, tension from attachment to adjacent cells, and compression or bending of tissue in response to external stimuli (129). One of the physical factors with the greatest impact on cell behavior is stiffness. Stiffness is the mechanical property that represents rigidity or the resistance to deformity following an applied force (130, 131). Variations in stiffness are found in both the cells and extracellular matrix (ECM) that constitute tissue.
6.1 ECM Stiffness

ECM stiffness varies with tissue or organ type. Brain and fat are two of the softest tissue types, while bone has the most rigid matrix composition and is several-fold stiffer (132). The mechanical properties of ECM directly correlate to tissue function. Physical characteristics of the matrix are determined by its composition of structural molecules, the tension generated by cell attachment and migration, and exogenous physiological forces such as blood flow. (133, 134) Stiffness also depends on location within the tissue or organ. Gradients of bone and skeletal muscle stiffness can be found in the body. ECM composition in the liver changes from the periportal to the pericentral regions of lobules resulting in changes in cell phenotype, even among cells of the same lineage (22, 135). Because of differences in the molecular arrangement throughout these liver microstructures, one can infer that variations in stiffness also exist. A clinical study supported this concept by measuring stiffness of liver sections in separate locations. Results showed differing measurements between lobes of same liver and among smaller areas within each lobe (136). Organ and tissue physiology show that the slightest differences in mechanical properties can alter function of adult cells.

Studies have additionally shown the abilities of stiffness to direct stem cells towards specific cell lineages. In a previous study, mesenchymal stem cells (MSCs) were differentiated on varying substrate stiffnesses into three different cell lineages; neurogenic, myogenic, and osteogenic cells (137). A separate group’s analysis revealed that effects of growth factors on MSCs also changed with substrate stiffness. TGF-β differentiated MSCs into smooth muscle cells (SMCs) at a low stiffness while upregulating expression of chondrogenic markers at a higher stiffness level (138). By
simply changing this mechanical property, cell development and growth was directed to mimic cells of a certain tissue type.

Cells not only change function but also migrate according to stiffness gradients. In previous assays, MSCs under cell culture conditions traveled to areas of higher substrate stiffness. The hypothesis stated that the cells migrate toward an injury-related stiffness or a more scar-like site in order to aid in repair (139, 140). This phenomenon called durotaxis has been observed in several cell types and is thought to be related to mechanisms of development and wound healing (141). These studies show that physical cues are just as important as chemical stimuli in determining cell arrangement, activation, and phenotype.

6.2 Cellular Stiffness

Cell stiffness is directly correlated to the mechanical properties of the substrate to which it is attached. This material could be the underlying ECM, a tissue culture biomaterial, or a neighboring cell. Cell rigidity also depends on the cell lineage type, the cell’s maturity, and the functional state or health of the cell (142). Cell stiffness ranges from softer epithelial cells to stiffer smooth muscle cells and rigid osteocytes. Stem cells and progenitor cells adapt their physical structures as they mature and differentiate (143, 144).

Certain disease and repair conditions can direct mechanical properties. Cells adapt to dynamic physical environments and can change their activation states accordingly. During the development of atherosclerosis, endothelial cells increase in stiffness which, along with plaque build-up, limits flow of oxygen-rich blood (145, 146). Valvular
interstitial cells activate and become fibroblastic in diseased valves. They return to quiescent states following repair and remodeling (147). In cancer, cells with decreased stiffness have the highest metastatic potential (148). This concept holds true in ovarian cancer cells where stiffness can be used as a biomarker for invasiveness (142).

Mechanical properties regulate cell-cell and cell-matrix interactions. Stiffness of cells and the ECM can have independent or cumulative effects that cause systemic imbalances and disease. Cells and matrix can also interact to balance physical conditions and maintain normal tissue homeostasis and function. Determining methods to achieve a healthy stiffness state in tissue will lead to effective treatments to correct disorders or diseases in patients.

7. Liver ECM Stiffness during Developmental or Repair States

Stiffness is not a static property in liver tissue but fluctuates with organ development, disease, and repair. In early liver development, a low stiffness environment maintains progenitor cells in their undifferentiated form in the endoderm. As ECM structures are created, cytokine and environmental cues eventually initiate cell migration and differentiation of these cells into mature parenchymal liver cells (149, 150). These observations are supported by in vitro studies which have shown the ability of low stiffness substrates to maintain expression of stem cell markers in hepatic progenitor cells (151).

Normal liver repair or regeneration is also initiated by elements of stiffness. Matrix-producing hepatic stellate cells and endothelial cells are activated by damage or increases in stiffness and migrate to these areas for repair (59). In these situations, MMPs
are required to degrade proteolytic-resistant collagens. These MMPs break-up or remove damaged or excess ECM to allow formation of new matrix structures (37, 152). Physical properties of the ECM as well as cell signaling enable new cells to repopulate these repaired sites to restore normal tissue function (58).

In the instance of tissue resection such as a partial hepatectomy, the lost ECM mass is fully regenerated de novo with the help of the activated matrix-producing cells. The provisional matrix produced during early stages of repair or regeneration contains uncrosslinked collagen I (58). In progenitor cell-mediated regeneration, this softer composition initially maintains the hepatoblast phenotype of cells until ECM structures are fully formed with the addition of collagen IV and the crosslinking of collagen I (153). Regeneration initiated by proliferating hepatocytes occurs simultaneously to matrix production by HSCs. However, physical cues provided by the ECM contribute to control of the initiation and cessation of cell propagation (31, 35, 154).

Imbalances in these reparative processes can cause increases in stiffness and liver dysfunction. Liver stiffness is an important parameter in the prognosis of liver diseases including cirrhosis, hepatitis, and hepatocellular carcinoma (HCC) (155, 156). During a fibrotic state, HSCs undergo uncontrolled activation and deposit excess amounts of laminin and collagen, especially collagen IV (43, 59). Fibrosis also occurs because of a loss of MMP production and increase in collagen crosslinking by lysyl oxidases (LOX) (157). All these factors contribute to an increase in stiffness and lead to a loss of hepatocyte phenotype and cell death (151). The liver is typically able to repair itself if the causative stresses are eliminated. However, prolonged fibrosis can cause worsening levels of stiffness and cellular impairment which eventually lead to irreversible cirrhosis.
and liver failure (43, 130, 151). Elevated liver stiffness is also a precursor for the development of HCC (158). It can predict both the appearance and metastatic potential of tumors. Increased stiffness correlates with increased proliferation of cancer cells and greater resistance to chemotherapeutic agents (151).

Novel research and treatments for liver disease attempt to address the mechanisms that cause changes in tissue stiffness. Therapeutic strategies include inhibition of activated stellate cells to limit ECM accumulation and developing agents to block LOX to block collagen crosslinking. Groups are also investigating methods to better control the release and action of a variety of MMPs to enable better manipulation of matrix degradation to soften fibrotic tissue. Studies have shown that once cells are returned to a normal stiffness environment, full function and health can be restored. Obtaining better control over ECM stiffness levels will lead to greater management of liver disease.

8. Effects of Stiffness on Mechotransduction and Focal Adhesion Signaling

8.1 Cell Adhesion Molecules

Cells sense mechanical properties of their microenvironment through cell attachment initiated by integrins, syndecans, or other glycoproteins (159). Integrins are the most common cell adhesion molecules (CAMs) and are composed of both an alpha and beta subunit (160). These structural components determine the integrin’s specificity to certain ECM molecules including collagens, fibronectin, laminin, or vitronectin (161). Integrin engagement is vital for survival and normal phenotype of all epithelial cell types (162). These transmembrane structures initiate signal transduction by formation of focal adhesions that connect the ECM with the cytoskeleton. This mechanical force-induced
signaling allows cells to sense and interact with the surrounding substrate and affects cell proliferation, motility, and function (163, 164).

Other adhesion molecules also have roles in regulating cell behavior. Syndecans are transmembrane proteins that are linked to GAG chains of heparan sulfate and chondroitin sulfate (165). Syndecans, with the aid of these GAGs, bind and concentrate important growth factors, such as the liver HGF and EGF (166, 167). Although not as prevalent as integrins, these proteoglycans also form anchoring junctions and can bind to collagens and fibronectin of the ECM to support cell to matrix attachment. In addition, syndecans can act alongside certain integrins to promote cell-cell adhesion (159).

Cadherins are a large family of glycoproteins that are the main effectors of cell-cell binding. They provide connections between actin filaments of various cells to help determine morphology and motility (168). Several classes of these calcium dependent structures exist. Expression of E-cadherin (epithelial-cadherin) is specifically vital to epithelial cell viability and polarity. E-cadherin forms homophilic cell-cell junctions between hepatocytes within the liver and is critical to tissue formation (168, 169). Loss of E-cadherin in vivo has been correlated with loss of cell phenotype and the development of metastases in patients (170).

Tight junctions are another type of intercellular complex important to epithelial cell adhesion, cell polarity, and function (171, 172). These structures prevent leaking of material between cells and allow for direct diffusion and active transport of molecules and ions between cells (173). Claudins, especially claudin-1, are required for structural maintenance of tight junctions (174). Although not an integral structural component, the
tight junction protein occludin is a central protein involved in cell polarity and directional migration. Occludin enables organization of actin filaments in response to stimuli that lead to formation of cell protrusions and cell movement (175).

8.2 Mechanotransduction and Cytoskeletal Regulation

Cell adhesion molecules are fundamental for sensing the physical characteristics of adjacent cells and the ECM or underlying cell culture material. Although all CAMs are important to normal cellular functions, integrins are the molecules that are essential for detecting substrate stiffness (176). Bound integrins develop small focal adhesion complexes and stimulate assembly of more mature focal adhesions through recruitment of kinases and adaptor proteins to enable cellular mechanotransduction (177, 178). Cytoplasmic proteins serve as mediators to amplify or modify the signals generated from integrin attachment. Signals are passed bidirectionally through either inside-out or outside-in activation to affect cell phenotype and create changes in the surrounding ECM microenvironment (179, 180).

During cell attachment, several different focal adhesion molecules are recruited to interact at the cytoplasmic domains of the integrins. One of the first proteins to bind to the integrin tail regions is talin. Talin is a key component of focal adhesions that functionally activates integrins and determines their affinity for specific ligands (181). Talin recruits the adaptor proteins paxillin and vinculin to focal adhesion complexes to regulate the actin cytoskeleton (182). These linkages generate strain between integrins and cytoskeletal structures to allow cells to sense substrate mechanical properties, which in turn direct morphology and motility (183). Vinculin localizes to focal adhesion sites as
well as cadherin junctions. It directly connects talin to actin filaments within the cell. Presence of vinculin is required for cell attachment, cell spreading, and filopodia formation (184-186). Paxillin is another scaffold component that joins with talin to control focal adhesion stability and turnover. It also interacts with different intracellular kinases to regulate assembly of focal adhesion complexes and organization of the cytoskeleton (183).

Focal adhesion kinase (FAK) is one of the main kinases that co-localizes with integrins and affects their activation (179, 183). FAK has an integral role in mechanotransduction. In previous studies utilizing FAK negative fibroblasts, mechanical stiffness did not initiate normal cellular responses, and cell migration tendencies were blocked (187). To initiate responses, FAK interacts with Src tyrosine kinase as well as adaptor proteins and dozens of other focal adhesion signaling molecules (164). FAK contains C-terminal and N-terminal domains that specify activity and transduction of signals. The C-terminal domain allows FAK to migrate, attach, and disrupt focal adhesion sites, while the N-terminus binds and regulates localization of ligands such as paxillin (188, 189). Integrin attachment activates FAK by inducing kinase clustering and autophosphorylation. This action subsequently allows Src to bind, activate, and further phosphorylate FAK at various amino acid residues (164, 190).

Depending on the site targeted, phosphorylation of FAK affects a wide variety of proteins and intracellular pathways to execute different and sometimes opposing functions. FAK is important in cell viability and stimulates phosphoinositide 3-kinase (PI3K) mediated upregulation of protein kinase B (AKT) (191). FAK also supports survival by serving as a scaffold for phosphorylation of Crk-associated substrate (CAS)
which activates c-Jun N-terminal kinases (JNK), mitogen-activated protein kinase 1 (MAPK1), and mitogen-activated protein kinase 3 (MAPK3) (192). Previous studies have shown the ability to prevent anoikis, or detachment related apoptosis, in epithelial cells through FAK expression (193, 194).

In addition to influencing viability and proliferation, activated FAK in combination with Src can phosphorylate talin and lead to the disassembly of focal adhesion complexes (195, 196). Under unique conditions, FAK has also been observed to strengthen integrin attachment and enhance focal adhesion formation (197, 198). Differences in results of these studies could be explained by the ability of FAK to stimulate at least four separate pathways of actin assembly and cell motility (199). PI3K and CAS pathways are not only FAK targets implicated in cell survival, but also stimulate cell spreading and migration (164, 200). Both PI3K and CAS serve as regulators of the downstream Ras homolog family member (Rho) GTPase, Ras-related C3 botulinum toxin substrate (Rac) (192, 201). FAK also regulates function of the adaptor protein growth factor receptor-bound 7 (GRB7) as well as neural Wiskott-Aldrich syndrome protein (N-WASP) (202). N-WASP controls actin polymerization and remodeling through the actin-related protein (Arp) 2/3 complex and another type of Rho GTPase, cell division control protein 42 (CDC42) (164, 199). Studies have also shown that FAK can actually suppress a third type of Rho GTPase, Ras homolog family member A (RhoA), to promote focal adhesion turnover (203).

FAK regulates Rho GTPases that are vital to focal adhesion assembly and disassembly, actin organization, and ultimately cell motility. The three main Rho GTPases (Rac1, CDC42, and RhoA) work in concert with each other to initiate cell
migration (204). However, cells seeded on varying ECM molecules or stiffnesses can produce activation profiles of the Rac1 and CDC42 that differ from RhoA in timing or expression levels. In research on fibroblasts seeded on fibronectin, Rac1 and CDC42 were stimulated at early time points post-seeding, while RhoA was activated much later \textit{in vitro} (199). These previous studies show that FAK activation occurs at various phosphorylation sites or affects different pathways to control the two sets of Rho GTPases (205).

Rac1 and CDC42 stimulate actin polymerization and extension of cellular processes for movement. Rac1 is directly involved in formation of lamellipodia at the leading edge of polarized cells (206, 207). Besides activation by FAK, paxillin can bind and stimulate Rac1 activity. CDC42 binds and activates n-WASP to create thin projections called filopodia past the frontal cellular boundary (183). Both GTPases and FAK are required for durotaxis and other forms of directional migration controlled by feedback signaling mechanisms (208). Lower substrate stiffness levels have been shown to support turnover of focal adhesion sites, easier alteration of cell morphology, and simpler cell detachment for motility (199).

RhoA can act in opposition to cell movement by increasing the assembly and formation of mature focal adhesions and contractile actin stress fibers through stimulation of Rho-associated protein kinase (ROCK). Increases in RhoA correlate with increases in stiffness and create greater cytoskeletal organization, actin polarization, and stabilization and anchorage of cell structures (209). However, overexpression of RhoA can actually lead to build-up of stress fibers and EMT which promotes the development of cancer (210, 211). In contrast to formation of stable adhesions, RhoA is also essential to
generate traction force for migration on substrates. Cells can initiate stronger traction forces on stiffer or inflexible surfaces (209). The tradeoff is that greater stiffness in substrates creates more stable focal adhesions that make cell detachment more difficult (199). Therefore, an intermediate, substrate stiffness is optimal for traction and speed for cell motility.

In addition to FAK’s effects on Rho GTPase expression, integrin-linked kinase (ILK) can act in parallel to influence cell morphology and motility (212). ILK recruits PINCH1 to focal adhesions to regulate assembly and disassembly during cell migration. ILK also complexes with Engulfment and Cell Motility 2 (ELMO2) and RhoG for cell polarization (213). Similar to FAK, ILK can stimulate PI3K-induced activation of Rac1 to control cytoskeletal rearrangement and formation of cellular processes (214, 215).

As discussed, mechanical sensing and signaling involves a complex array of focal adhesion sites, intracellular kinases, and Rho GTPases. These various molecules act in conjunction to affect the actin cytoskeleton and form cellular protrusions. The outside-in signaling and resultant inside-out adjustments applied by the cell can affect morphology, motility, and ultimately the overall function of the cell. Understanding the mechanisms and actions of important effectors like FAK, ILK, and Rho GTPases in specific cell types under defined culture conditions will allow researchers to better model disease and more precisely manipulate substrates for use in testing or therapies.
Works Cited


132. Ma PX. Biomaterials and regenerative medicine. Cambridge, United Kingdom: Cambridge University Press; 2014. xvi, 703 pages p.


CHAPTER 2

Decellularization and the Incorporation of Liver Extracellular Matrix in Cell Culture Substrates

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In order to optimize cell culture conditions, substrates must contain the proper milieu of structural components and growth factors. These molecules also must be present in the correct ratios to grow and maintain specific cell types. The method of decellularization solves this problem by removing native cells from tissue while preserving the natural ECM arrangement. The byproduct is a non-immunogenic scaffold that can be directly seeded or indirectly incorporated into tissue culture substrates and cell therapies. In turn, these substrates can be utilized to test the effects of certain substrate properties on cell signaling and function. Our own assays used the versatile nature of liver ECM hydrogels in order to study the effects of mechanical stiffness on primary hepatocyte function.

1. ECM Isolation through Decellularization Methods

A wide variety of approaches were developed to decellularize tissue or whole organs for ECM isolation and eventual use in cell culture. Methods are dependent upon the tissue and animal type being processed. Variations exist in the mechanical or chemical procedures used. Protocols utilize different physical forces, compounds, and reagents for cellular removal. Incubations in solutions and subsequent washing steps vary in duration. These diverse procedures for decellularization have experienced a wide range of success and failure.

Each decellularization technique possesses a set of advantages and disadvantages. One of the first methods developed in this field ruptured cells through utilization of distilled water and salt solutions in combination with mechanical force (1). Use of hypertonic and hypotonic solutions to lyse cells does minimum harm to the underlying
ECM structure but on the other hand, does not remove smaller cellular components from the tissue (2-8). Addition of physical disruption and agitation of tissue promotes full clearance, however, it can also lead to damage of the intact architecture and unintended removal of ECM constituents (9).

Another early decellularization technique implemented the use of freezing and thawing to eliminate cells from tissue constructs (10-14). This simple method does not require creation of solutions or the involvement of physical force. Repeated freeze thaw effectively kills and lysed cells but can ultimately degrade certain proteins of the ECM, especially growth factors which exhibit limited half-lives (15). Because of the sensitivity of specific molecules and the unknown extent of ECM damage due to this process, freeze thaw cycles should be kept to a minimum.

Newer methods incorporated varying concentrations of enzymatic reagents and detergents into decellularization protocols. Enzymes including trypsin and DNase are often used to treat scaffolds for cellular removal (16-24). These compounds are highly successful in liberating cells from the ECM and break down immunogenic cellular components such as nucleic acids (16). During natural processes in the body, apoptotic cells trigger caspase-activated DNases which degrade DNA into 180 base pair fragments. These nucleic acids are further degraded by DNase II of macrophages to avoid initiation of innate immune reactions (25). For these reasons, important criteria established for decellularization include degrading any residual DNA fragments to below 200 base pairs (bp) in length and reducing DNA levels to below 50 ng of DNA per mg of dried tissue (9). These actions are vital in avoiding immune responses when these substrates are implanted into in vivo systems. Despite abilities to reduce immunoreactivity, lengthy
enzymatic treatments can also lead to destruction of ECM proteins and glycosaminoglycans (GAGs) (26). Difficulties also exist in subsequent removal of enzymes from the scaffold. Remnant enzymes could be harmful to animal models or human patients and induce their own immune reactions.

An alternative option to enzymes for elimination of cellular material is detergents. Previous publications have reported advantages of detergents over enzymatic methods in maintenance of mechanical and elastic properties of the ECM (27). Detergents vary in structure and strength, which both dictate concentrations applied and length of time utilized. The two main types of detergents used in decellularization are ionic and non-ionic detergents. Ionic detergents contain a charged head group and hydrophobic tail group. This structure allows for rapid and effective disruption of the cellular membrane and full removal of all cellular components from tissue (16, 24, 28-31). However, because of its strength, this compound can easily denature or remove ECM molecules such as GAGs and basement membrane proteins if not limited in concentration or duration of use (32, 33). In comparison, non-ionic detergents are gentle on the underlying matrix. Their hydrophilic groups are uncharged but are still able to disrupt lipid bonds (28, 34-38). However, their mild nature means greater detergent concentrations and longer washes are required for full cellular removal. Non-ionic detergents are often combined with other reagents to ensure complete and timely decellularization (7, 39, 40).

The decellularization method designed by our research group and chosen for use in this thesis project involves use of both ionic and non-ionic detergents. The decellularization protocol has been optimized for use with livers of rat models. The process takes advantage of the rat’s intact vasculature in order to perfuse solutions
throughout the organ. This approach enables solutions to infiltrate all areas of the tissue for maximal contact with cells. The impact is faster and more efficient decellularization as compared to simply immersing tissue surfaces. These developed methods allow preservation of structural and biologically active components vital for effective growth and maintenance of cells. For whole organ engineering, perfusion also allows for later use of the vasculature during reseeding of cells. Through this established protocol, decellularized liver tissue or substrates supplemented with the isolated ECM improve liver cell viability and long term function in the cell culture assays of this thesis work.

**Fig.1** Rat Liver Decellularization, adapted from Shupe et al (1)
A) PBS washing removes blood from the liver. B) Triton-X 100 detergent solubilizes lipid membranes, while C) SDS detergent removes remnant cellular material.
2. Substrate Forms

ECM isolated from the decellularization process can be utilized in several different configurations for many different applications. The intended objective determines how this material must be used.

2.1 Decellularized Tissue Constructs

One of the main uses of decellularized organs and vascular constructs is for tissue engineering. With a large disparity between donor organs and patients requiring transplantations, the goal of organ engineering is to narrow this gap by supplying lab grown tissues, preferably with patients’ own cells to avoid an immune response. To engineer complex organs, one of the primary objectives is to create a fully cellularized and patent vasculature. This task is made easier by maintaining an intact vessel basement membrane.

Several groups have successfully generated re-endothelialized and functional vascular grafts through seeding of decellularized scaffolds (41-46). Similar strides have been made at a reduced scale in the recellularization of small animal organs or small tissue samples (39, 47-49). Decellularization not only preserves the ECM composition but also the structural architecture of the tissue. This remnant microarchitecture allows cells to attach, align, and self-organize into proper functional units within both parenchymal and vascular regions.

Challenges remain in whole organ engineering. Consistency of decellularized tissue samples and the ability to control the physical and biochemical properties of the decellularized matrix remain a problem. The exact physical and biological toll various
detergents take on ECM during decellularization is not fully controllable or known. Certain key growth factors or smaller microstructures may need to be replaced or reconstructed in order to produce an optimally functional cell scaffold. Microscopic structures are easily damaged by decellularization reagents, including glomeruli of the kidney, as well as bile canaliculi and sinusoids of the liver. Future studies should focus on modifying and improving methods for preserving these delicate but important structures. Research is still needed to optimize exact concentrations of reagents, duration of individual steps, and storage methods for decellularized tissue. Studies also need to gain a better understanding of complex interactions within tissue-specific ECM, including the binding and biological activity of growth factors. New knowledge will build upon current advances and lead the field closer to achieving functional tissue products.

2.2 ECM Hydrogels

Hydrogels provide a versatile platform to isolate and test individual physical and chemical properties. The flexibility of gel substrates allows for alteration of properties such as stiffness, growth factor content, and ECM composition. These substrates can be applied to everything from drug testing to a variety of novel therapies and treatments. Supplementing these gels with organ-specific ECM improves attachment, growth, and phenotype of cells similar to levels exhibited in fully functional tissue.

One of the uses of ECM hydrogels is to reproduce conditions found in vivo in order to model certain disease or repair states. Three-dimensional cell culture in gels effectively model tumors and metastatic phenotypes for cancer research (50, 51). High stiffness gels
can simulate diseases like liver cirrhosis, while lower stiffness mimics a developmental or regenerative environment (52, 53). An upregulation of cellular production of specific growth factors and matrix proteins also occurs during disease or regeneration (54-57). Exogenous growth factors can be bound to active sites in ECM supplemented gels, or ratios of specific structural molecules can be altered. This capacity for modifications allows accurate modeling of ECM microenvironments for many tissue types and physiological scenarios. Allows us to explore effects of composition

2.3 Species Differences

For this thesis project, decellularized rat liver tissue was integrated into HA gels for preliminary primary rat hepatocyte studies and subsequently, primary human hepatocyte culture. ECM components including collagens, polysaccharides, and fibronectin are highly ubiquitous in mammalian tissue (58, 59). Molecular structures and cell binding sites of these proteins are highly conserved between rats and humans and interact with common cellular factors (60-62). GAGs and associated growth factors also contain conserved structures and functions (58, 63, 64). In our own assays, we have observed human growth factors including HGF and EGF stimulating rat cells and vice versa. Because of their structural similarities, species differences between rat and human liver ECM proved to be inconsequential in our assays.

3. Results and Advantages of ECM Use in Cell Culture

Traditional cell culture methods use synthetic materials or incorporate single protein coatings like collagen into substrates for cell attachment and growth. With the development of decellularization, a fuller representation of organ-specific ECM
components could be included. Several studies have shown the advantages of culturing different cell types on natural, tissue-specific ECM substrates compared to purely synthetic substrates. ECM from different tissue types can direct stem cells towards various cell lineages (65-70). In liver culture systems, specific ECM cues can promote the differentiation of hepatic progenitor cells into fully mature hepatocytes or cholangiocytes (44, 71-74).

To develop effective and successful treatments, long term viability and phenotype maintenance needs to be supported. Heparin and HA hydrogels are favorable substrate bases because these molecules are naturally found in the body, do not induce immune reactions upon implantation, and have the ability to bind and present growth factors (75, 76). We hypothesize addition of tissue-specific ECM would allow cells to function at higher levels for use in cell based therapies. Previous studies have shown how tissue specific ECM improves long term cell function in vitro. Both liver progenitor cells and adult hepatocytes seeded onto isolated liver ECM maintained viability and function over longer periods of time than cells grown on tissue culture plastic or collagen alone (44, 77-79). Decellularized matrix has supported growth and phenotype of cells of several different tissue types for a variety of uses (80-82). By recreating a natural microenvironment in culture, cells are able to function and interact as they would within normal, healthy tissue of the body.

With the addition of diverse species of GAGs present in natural ECM, greater control over growth factor binding and release could be achieved to aid the efficiency of cell therapies. Improvements in phenotypic maintenance were observed in cells encapsulated with a combination of several structural molecules and growth factors,
especially hepatocyte growth factor (HGF) and epidermal growth factor (EGF) with
hepatocytes (83, 84). New data on the cellular effects of various substrate compositions
and properties will enable better design of cell therapies. Combining innovative methods
and knowledge will optimize their effectiveness in combating or correcting diseases in
patients.

4. Altering Stiffness in Cell Culture Substrates

4.1 Methods for Manipulating Hydrogel Stiffness

As mentioned in the previous chapter, physical properties are as important as the
presence of specific ECM components for differentiation or maintenance of various cell
types during seeding of intact tissue. Mechanical properties are modified by a wide
variety of strategies and techniques in cell culture. Previous groups have used increased
concentrations of proteins or synthetic compounds such as acrylamide to bolster substrate
stiffness (85-87). However, these alterations can influence cells in ways beyond stiffness,
and tend to complicate assay results. To isolate the property of stiffness during
experiments, the best techniques avoid addition of biologically active molecules.

One of the simplest and most common methods for modifying stiffness is through
substrate crosslinking. Crosslinking allows precise manipulation with limited
confounding effects on attachment sites or biological activity of the substrate. Stiffness
alterations can be made by varying the crosslinker type, the structure and arm length of
the crosslinker, the crosslinker concentration, or the duration of the crosslinker treatment
(88). The choice for crosslinker type is contingent on which molecules are available in
the substrate. These reagents chemically react with several different moieties including;
thiols, amines, carboxyls, hydroxyls, and carbonyls (89). Crosslinkers can also be photoreactive and are activated by exposure to ultraviolet light (90, 91).

A crosslinker is either classified as homofunctional or heterofunctional, reacting and covalently binding to one or more classes of functional groups. Homofunctional crosslinkers act at rates and strengths dependent on the concentration of the crosslinker, as well as the density of reactive groups in the substrate’s constituents. Greater concentrations of the reagent cause a faster and higher degree of crosslinking. A larger quantity of active sites in the substrate also translates to quicker bonding and stiffer materials (92). Multiple homofunctional crosslinker types can be used in procedures to gradually stiffen materials during activities such as bioprinting (93). Unlike homofunctional reagents, a single heterofunctional linker type can be utilized in multiple step reactions to produce viable substrates. Instead of combining all substrate components at once, the crosslinker is allowed to react with one molecule before additional compounds are added. This process provides greater control over specificity of interactions and spacing of bonds for more exact material construction (93, 94).

Properties of the crosslinker are not only dependent on its chemical specificity but also vary according to the length and number of structural arms it contains. A longer distance between crosslinks creates a looser assembly of compounds and a softer substrate. Therefore, to increase stiffness, one must use a crosslinker that is shorter in length (92, 95). The typical reagent is bifunctional and holds two binding sites. However, crosslinkers have been engineered to contain as many as eight arms. Greater amount of branching leads to more reactive ends and a greater degree of binding. The geometry of
multi-armed crosslinkers is also more compact which generates tighter packed and stiffer structures (96).

For this thesis project, our group chose a polyethylene glycol (PEG) based compound for crosslinking applications during formation of liver ECM hydrogels. PEG based chemicals are nontoxic and do not influence cell function (97). They also do not initiate immune reactions upon implantation in vivo (98, 99). Because PEG is watersoluble, clumping of the compound is avoided, and crosslinking is evenly spaced within the polymer (100). PEG creates hydrophilic substrates which have been shown to promote better cell adhesion, proliferation, spreading, and function than hydrophobic materials (101, 102). The combination of these characteristics makes PEG an ideal crosslinker for use in primary hepatocyte culture and development of cell treatments.

Liver ECM gels were created by mixing solubilized liver matrix with hyaluronic acid and denatured collagen solutions. These HA gel components were specifically modified to contain reactive thiols for crosslinking (103). Acrylate is a type of molecule often attached to a PEG spacer to form a crosslinker that contains reactive double bonds which bind to sulfhydryl groups. Our assays tested several geometric variations of PEG acrylate in order to solidify the liver hydrogels at various stiffnesses.

Polyethylene glycol diacrylate (PEGDA) is one of the most commonly used crosslinkers. It has a two-arm linear structure that produces a softer gel type (104). This substrate stiffness was used as a baseline for our assays. PEG acrylate variations containing four or eight arms also exist (105). More acrylate arms allow for a greater number of reactive ends to bind thiol groups. In our own assays, PEGDA, four-arm PEG
acrylate, and eight-arm PEG acrylate were added to HA-ECM gel constituents at various concentrations. Results showed that eight-arm PEG acrylate at the highest concentration of 6% weight by volume produced the highest stiffness.

![PEG Crosslinker Structures, adapted from Zhu et al (119)](image)

Greater PEG branching or arm number creates a tighter molecular network and increases substrate stiffness.

Although most combinations of crosslinker geometry and concentration produced distinctively different stiffness levels, a saturation point of each crosslinker was also determined. Higher concentrations of crosslinker past this limit did not significantly affect stiffness, regardless of additional reaction time (Figure 3). This upper limit is reached when all thiol groups are utilized. To ensure excess crosslinker is not retained in the gel, no concentration above the observed saturation threshold was chosen for subsequent assays. Furthermore, gels were thoroughly washed with PBS to clear out excess crosslinker.
4.2 Measuring Stiffness

Several methods and instrumentation were developed to measure the property of stiffness. For centuries, the simple practice of palpation was used to diagnose patients (106). Clinicians now utilize a non-invasive method called elastography to image and quantitate stiffness of intact organs. This technique employs the use of ultrasound or magnetic resonance imaging (MRI).

Several variations of ultrasound elastography evolved over time. Early forms involved application of a manual force to the exterior of patients which was then measured internally by ultrasound to calculate organ stiffness (107). Accuracy improved through use of mechanical pulse generators and more advanced sonography systems. During transient elastography, an automated device creates shear waves that penetrate through the skin into organs of the abdominal cavity such as the liver (108, 109). The
velocity of the waves is measured by ultrasound as they pass through the tissue of interest. This data allows clinicians to calculate the stiffness of a large representative area of the organ for diagnosis (110). Instead of acting on the surface of the skin, newer adaptations employ concentrated sonography waves to generate acoustic radiation forces at the site of measurement within the tissue (111-113). These processes improve the distance pulses can be directed within the body. Acoustic radiation forces also increase the precision of deep tissue ultrasound measurements through their ability to travel across fluid build-up in the peritoneal cavity which normally impedes transient elastography (110, 112).

Magnetic Resonance Elastography (MRE) incorporates these same mechanisms to propagate shear waves. However, the velocity of these waves is subsequently measured by MRI. Unlike ultrasound technology, a three-dimensional image of the entire tissue is produced alongside a map of various stiffness values (114, 115). This tool provides a more complete analysis of the organ being tested. MRE has proven to be a reliable tool for detection of fibrosis in diseased liver (116). It has also successfully evaluated stiffnesses in organs such as the brain that were unattainable by ultrasound methods (117). Further refinement is needed to optimize the use of MRE in other tissue or organ types (114).

In a laboratory setting, a rheometer is one of the most popular instruments used to measure stiffness for samples prepared ex vivo. Unlike non-invasive clinical methods, Rheometry is used to analyze biopsies or excised tissue outside of a living organism. This technique is also commonly used in calculating stiffness of hydrogels, biomaterials, or synthetic substrates used in cell culture. Because samples are isolated from irrelevant
tissues, fluids, or other confounding factors, rheometry increases accuracy and precision of measurements compared to ultrasound and MRE (118). Engineers developed several variations of rheometers based upon the material being tested and the type of data required for analyses.

Rotational or shear rheometry is often used to measure stiffness in viscoelastic soft tissue, hydrogels, or polymers (119, 120). These methods were applied in our own assays to determine stiffness in liver ECM hydrogels and decellularized liver tissue. During testing with these rheometers, a sample is stabilized on a fixed platform or plate. An oscillating geometry in the form of a cone, plate, or cylinder is subsequently lowered until it comes into contact with the sample (118). From the various forces applied, an elastic modulus can be measured and calculated. The elastic modulus, or resistance to deformity, can be expressed by three types of calculations; Young’s modulus, bulk modulus and shear modulus. Young’s modulus is the ratio of tensile stress to tensile strain or the force required to compress an object (121). The bulk modulus is the amount of force needed to uniformly deform a material or the ratio of volumetric stress to volumetric strain (122). The shear modulus describes how a sample responds to opposing forces parallel to its surface and is calculated by dividing shear stress by shear strain (123).

In the stiffness tests we performed, the Discovery Series HR-2 Rheometer (TA Instruments, New Castle, DE) was used with a parallel plate geometry. Oscillation stress sweeps incrementally increased shear stress on the samples up to 10.0 Pa and were run at a constant oscillatory frequency and axial force. Both the shear storage modulus (G’) and shear loss modulus (G’”) were measured and recorded. The storage modulus describes the
elastic properties or stored energy, while the loss modulus describes the viscous characteristics or energy lost as heat (124). The storage modulus was the main stiffness value utilized for reporting and comparison of hydrogels in our assays.

Shear rheometry provides an overall representation of stiffness in materials by assessing a substantial portion of the substrate. For rheology measurements of smaller microstructures or individual cells, atomic force microscopy (AFM) is required. An atomic force microscope contains a high resolution nano-indenter or tip and detection probe that scans indented samples to evaluate microscopic areas for stiffness (125-128). Instead of measuring the combined effects of cell and ECM stiffness, AFM allows separate analysis of components. Singular structures and cells in a sample can easily be distinguished and measured to assess conditions such as cancer (129). Multiple readings of a larger homogenous substrate can also be obtained and averaged to calculate more wide-ranging stiffness (130, 131).

Despite AFM’s exceptional resolution, disadvantages also exist. AFM performed on a heterogeneous sample can give an incomplete or inaccurate representation of the material tested if too few readings are acquired or only a partial area is covered (132). Problems can also arise depending on the indenter type used. Improper tips cause artifacts to develop in samples (127, 131). Indenters can also adhere to softer materials and cause miscalculations of stiffness (133).

Every analysis of stiffness presents unique objectives and obstacles. Clinicians require a quick and noninvasive strategy, while a laboratory setting affords greater time and control over samples. By effectively evaluating the overall stiffness of cell culture
substrates, shear rheometry demonstrated the greatest ability to study altered stiffness in our liver ECM gels.

5. Conclusions and Potential Applications

In previous studies, hepatocytes cultured on different substrate stiffnesses have been reported to have generalized effects. Low stiffness is associated with a growth arrested and differentiated adult phenotype. High stiffness is associated with increased viability and cell proliferation but also dedifferentiation and possible initiation of EMT (53). However, the exact stiffness ranges defined in these studies and substrate compositions vary greatly. Stiffness levels are also often at non-physiologically achievable levels.

The assays we conducted utilized liver specific ECM isolated by decellularization to form gels so that substrate components were similar to what composes normal liver tissue. We also designed our study to focus on a narrow physiological relevant range of stiffness. The adjustable system was created to culture primary human hepatocytes to determine optimal conditions for cell function and distinguish specific mechanisms for stiffness-induced changes in cell mechanics and morphology. We hypothesized that the substrate stiffness closest to the actual stiffness of native liver tissue promotes the highest long term function.

This knowledge can enable advancement in the creation of substrates for liver cell therapies to treat disease in patients. This technology was developed to easily manipulate and test effects of mechanical properties on cells. Future studies can use these abilities to simulate the effects of disease states in a variety of organ systems and determine disease
mechanisms in order to form possible treatment strategies. Increased dimensions of complexity in architecture and cell types could also be added to the system. These ECM-containing hydrogels could eventually be used for tissue engineering applications.

Because of its semi-fluidic properties, cells encapsulated in gels can be injected into numerous sites in vivo (134-138). Gels can also be “printed” into various patterns for tissue construction. Bioprinting is a new technology that rapidly deposits substrates into a predetermined form. The process controls substrate shape, composition, and physical properties like stiffness. After gels are printed into constructs, materials can be fully cross-linked depending upon the desired tissue type. Bioprinted tissues currently in development include everything from skin patches and vessels to complex organs like the liver (80, 139-142). With the use of ECM hydrogels and new emerging technologies, the possibilities for new therapeutic products and treatments are endless.

Works Cited


CHAPTER 3

Stiffness of Hyaluronic Acid Gels Containing Liver Extracellular Matrix Supports Human Hepatocyte Function and Alters Cell Morphology

Daniel B. Deegan

This chapter includes and expands upon the results of the published manuscript, “Stiffness of Hyaluronic Acid Gels Containing Liver Extracellular Matrix Supports Human Hepatocyte Function and Alters Cell Morphology.”

Manuscript Reference:

Abstract

Tissue engineering and cell based liver therapies have utilized primary hepatocytes with limited success due to the failure of hepatocytes to maintain their phenotype in vitro. In order to overcome this challenge, hyaluronic acid (HA) cell culture substrates were formulated to closely mimic the composition and stiffness of the normal liver cellular microenvironment. The stiffness of the substrate was modulated by adjusting HA hydrogel crosslinking. Additionally, the repertoire of bioactive molecules within the HA substrate was bolstered by supplementation with normal liver extracellular matrix (ECM). Primary human hepatocyte viability and phenotype were determined over a narrow physiologically relevant range of substrate stiffnesses from 600 to 4600 Pa in both the presence and absence of liver ECM. Cell attachment, viability, and organization of the actin cytoskeleton improved with increased stiffness up to 4600 Pa. These differences were not evident in earlier time points or substrates containing only HA. However, gene expression for the hepatocyte markers hepatocyte nuclear factor 4 alpha (HNF4α) and albumin significantly decreased on the 4600 Pa stiffness at day 7 indicating that cells may not have maintained their phenotype long-term at this stiffness. Function, as measured by albumin secretion, varied with both stiffness and time in culture, peaking at day 7 at the 1200 Pa stiffness, slightly below the stiffness of normal liver ECM at 3000 Pa. Overall, gel stiffness affected primary human hepatocyte cell adhesion, functional marker expression, and morphological characteristics dependent on both the presence of liver ECM in gel substrates and time in culture.
1. Introduction

Rising incidence and cost of liver disease as well as a limited supply of transplantable organs have increased demand for new liver treatments. Experimental treatments currently being developed include liver cell transplant on biological scaffolds (1-5). However, several limitations intrinsic to primary hepatocytes have slowed development of these technologies. Challenges remain in the maintenance of viability and cell function of primary hepatocytes outside of the normal liver microenvironment (5). Therefore, developing substrates that support hepatocyte viability and function is critical to maximize the efficacy of primary hepatocytes in tissue engineering and cell therapies for liver disease.

Previous studies have identified several advantages of using tissue-specific ECM instead of generic matrix formulations such as Matrigel for supporting primary cells (6). Cells on natural liver ECM demonstrated increased ability to proliferate and maintain function over extended time periods (7). Hepatocytes cultured on intact or solubilized organ specific matrix also maintained normal cell morphologies as compared to cells grown on tissue culture plastic or collagen (3, 8, 9). The underlying mechanisms for this improved phenotypic stability are still unknown but likely relate to inclusion of several components of the natural liver microenvironment, including cell and growth factor binding sites.

For this study, decellularization of normal liver was used to isolate acellular ECM for incorporation into hyaluronic acid (HA) hydrogels. The decellularization process involved perfusion of the organ with detergents to remove native cellular material, while
preserving structural proteins and glycosaminoglycans (GAGs) (10). Removal of native cells leaves a non-immunogenic ECM scaffold that can be seeded with hepatocytes that remain viable for several weeks (11).

Many previous studies utilized ECM coated tissue culture plastic for growing primary hepatocytes. However, this method is only suitable for two-dimensional culture and is not easily translated into a three-dimensional form for use in cell based therapies (12, 13). The current study uses a hydrogel that may be formed into three-dimensional units suitable for transplantation. Preliminary findings indicated that solubilized ECM combined with hyaluronic acid gel (HA) maintained a normal epithelial morphology and strong tight junction formation with little evidence of transition to a fibroblast-like morphology, commonly seen in traditional collagen cultures. Solubilization of the ECM also prevented hepatocyte apoptosis induced by phagocytosis of cryomilled ECM powders. Other studies published by our group demonstrated that HA based hydrogels avoided an immunogenic response measured by human lymphocyte proliferation, while other gel types such as rat tail collagen 1 induced a low level immune response (11, 14). Better phenotype maintenance and biocompatibility upon transplantation signaled that HA gel was the best substrate for incorporating decellularized liver in future assays.

In the development of a liver ECM substrate, the physical characteristic of stiffness is important to cell physiology and mechanics. The general conclusions of previous publications indicate that stiff substrates promote hepatocyte spreading, proliferation, and dedifferentiation; while soft substrates promote maintenance of the functional hepatocyte phenotype (15, 16). Substrate stiffness also regulates cell aggregation, growth factor responsiveness, and cell motility with the highest cell
migration occurring on intermediate stiffness levels (17, 18). However, several of these previous studies were done on stiffness ranges well above physiological liver stiffness, which has been reported anywhere from 1.5 to 8.5 kPa (19-24).

For the current study, the crosslinker concentration was the sole variable for adjusting gel stiffness. Crosslinking eliminated potential confounding effects found in more complex systems where concentrations of structural compounds are altered. Gel stiffnesses were also limited to a narrow, physiologically relevant range of 600-4600 Pa that bracketed the stiffness of normal liver ECM at 3000 Pa. Assays were conducted to measure overall hepatocyte function as well as the mechanisms by which substrate stiffness affected cell phenotype. Specific tests performed in this study were designed to explore the mechanisms by which substrate stiffness affected substrate/cell adhesion, primary hepatocyte function, and morphology. Cell junction formation is critical for the maintenance of epithelial cell phenotype; regulating a broad range of cellular properties through cell-cell communication, adhesion, and diffusion of water and solutes (25, 26). Tight junctions act as semi-permeable barriers that establish cell polarity, which is crucial for the maintenance of bile canaliculi (27, 28). The tight junction protein, claudin-1, is required for maintenance of the selective barrier properties between adjacent hepatocytes (29). Occludin is not essential for maintaining tight junction structures, but plays a role in regulating cytoskeletal organization, cell polarity, and cell migration (30-32).

The effects of substrate stiffness on intracellular signaling were also studied through measurement of two cytoplasmic kinases: focal adhesion kinase (FAK) and integrin-linked kinase (ILK). Cells adapt their cytoskeletal structure to specific microenvironmental conditions through interactions between focal adhesion molecules
and intracellular kinases. Appropriate expression of these kinases in hepatocytes is required for normal growth factor responsiveness, prevention of apoptosis, and normal metabolic function (33-35). Silencing or overexpression of these factors can lead to cellular dysfunction and, in the case of neoplasia, tumor progression (36). Increased expression also mediates cytoskeletal reorganization and cell protrusion formation which drives cell migration (37, 38). In other experimental systems, FAK and ILK expression have been shown to correlate with cell junction remodeling, specifically through interactions with occludin. FAK and ILK also act through downstream activation of Ras homolog family member (Rho) GTPases; cell division control protein 42 (CDC42), Ras-related C3 botulinum toxin substrate (Rac1), and Ras homolog family member A (RhoA) (39, 40). CDC42 and Rac are the two GTPases involved in actin cytoskeletal reorganization for cell motility, including formation of cells extensions called filopodia and lamellipodia. RhoA expression increases focal adhesion and stress fiber formation and is correlated with a more contractile morphology (41-44).

By studying the stiffness responsive factors that influence hepatocyte structure and function, we have gained insights into the mechanisms through which substrate stiffness affects the viability and phenotypic stability of primary hepatocytes. These findings have informed the development of a transplantable gel containing liver specific ECM at an optimal stiffness for therapeutic use in hepatocyte cell therapies.
2. Materials and Methods

2.1 Liver Decellularization

Intact rat livers were decellularized according to our group’s previously published methods (10). Ten week old male F344 rats were euthanized by administering a lethal dose of sodium pentobarbital (100 mg/kg). Livers were then cannulated and perfused by methods previously diagrammed and reported in our cell isolation protocol (45). In brief, a euthanized rat was pinned on a dissection board, and a mid-line incision was made through the peritoneum starting from the diaphragm down to the groin. Visceral organs were shifted to expose the inferior vena cava (IVC) and liver. The IVC was cannulated with a 20-gauge catheter and tied in place with sutures. The portal vein was then cut, and the superior vena cava (SVC) was clamped. Solutions were held in flasks at 37°C in a water bath (Fisher Scientific, Pittsburgh, PA) and then perfused through the catheter at a flow rate of 5 mL/minute using tubing and a Masterflex L/S peristaltic pump (Cole-Parmer Instrument Company, Vernon Hill, IL).

100 mL of PBS was first perfused to clear residual blood. The liver was then perfused with approximately 300 mL each of 1%, 2% and 3% Triton X-100 solutions in PBS. A final solution of 0.1% SDS in PBS was used to clear remaining DNA and Triton detergent. The liver was then fully excised, and overnight PBS washing was used to remove residual SDS from the matrices. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Wake Forest University.
2.2 Immunohistochemistry (IHC) on Decellularized Tissue

Following decellularization, we immunostained rat livers to assess cell clearance and retention of structural components such as collagens and laminin. As reported in our previous manuscript, decellularization completely removed cellular material from tissue sections, while collagen and laminin were preserved and represented (Figure 1) (10).

For this study, Alcian blue staining for mucopolysaccharides that comprise GAGs was performed on frozen decellularized liver sections. Tissue was frozen in OCT blocks and cut into 5 μm slices that were then fixed in acetone on slides at 4ºC for two minutes. Sections were then stained according to a previously established protocol (46). In short,
sections were incubated in 0.5% Alcian blue 8G solution in 0.5% acetic acid for 30 minutes (Sigma-Aldrich, St. Louis, MO). Sections were then washed with PBS and imaged.

2.3 GAG Function in Decellularized Tissue

To assess the ability of residual GAGs to bind growth factors following decellularization, 5 mg samples of decellularized liver were incubated at room temperature for one hour in PBS containing an array of concentrations of recombinant epidermal growth factor (EGF) and human hepatocyte growth factor (HGF) up to 1000 ng/mL (Peprotech, Rocky Hill, NJ). Following pre-treatment, tissue was washed for 1 hour at 4ºC in four separate 50 mL volumes of PBS to remove unbound growth factor. We confirmed that following the fourth wash, greater washing of up to ten washes created no differences in bound growth factor quantity. Following lyophilization, cryomilling, and solubilization of the tissue matrix in HCl- pepsin buffer solution, we quantified EGF or HGF levels in each sample by ELISA (RayBiotech Inc., Norcross, GA). Samples were normalized through measurement of total protein using the Pierce BCA Protein Assay Kit (Thermo, Rockport, IL).

2.4 Liver ECM Solubilization

Gels with added ECM were created according to previously published protocols from our group (8). In brief, decellularized liver tissue was frozen at -80ºC and lyophilized using a SuperModulyo Freeze Dryer (Thermo, Waltham, MA). Samples were maintained under vacuum at a pressure of 0.1 mbar and temperature of -80ºC for 24 hours. Tissue was then ground in liquid nitrogen using a mortar and pestle until a fine
powder was attained. Liver ECM was solubilized by mixing lyophilized decellularized tissue with 0.1 N HCl and pepsin (Porcine gastric mucosa, 3400 units of protein, Fischer Scientific, Fair Lawn, NJ). After an incubation of 48 hours at room temperature, the solution was centrifuged and filtered with a 0.2 µm syringe filter. The pH was adjusted to 7.0 and the total protein concentration to 1.0 mg/mL.

2.5 Solubilized Liver ECM Quantitative Analysis

DNA from solubilized liver ECM solutions was isolated and purified using the Qiagen DNeasy Kit. DNA amounts were quantified using a set of standards and the Quant-iT PicoGreen reagent kit (Invitrogen Corp., Carlsbad, CA) that could detect lower than 1 ng/mL of DNA in solution.

GAG levels were quantified using the Blyscan sGAG Assay kit (Biocolor, Newtownabbey, UK). 40 µL of solubilized ECM was mixed with 160 µL of 0.2 M sodium phosphate buffer containing 1 mg/mL of papain and incubated at 65°C for 3 hours. Samples were then mixed with Blyscan dye, pelleted, and washed. After dissociation reagent was added, samples and GAG standards were loaded into a 96 well plate and measured in a SpectraMax M5 Multi-Mode Microplate Reader at an absorbance of 656 nm (Molecular Devices, Sunnyvale, CA).

Collagen levels were quantified using the Sircol Soluble Collagen Assay kit (Biocolor, Newtownabbey, UK). 20 µL of solubilized ECM was mixed with 130 µL of 0.5 M acetic acid containing 0.1 mg/mL pepsin and then incubated overnight at 4°C. Samples were then mixed with Sircol dye, pelleted, and washed. Dissociation reagent
was added, and samples and standards were read in a 96 well plate at an absorbance of 555 nm.

2.6 ECM Gel Formation

Liver ECM solution was combined at a 1:1 ratio with thiol modified HA gel components (HyStem Hydrogels, ESI BIO, Alameda, CA). Components include 3,3′-Dithiobis(propanoic hydrazide) modified HA (Glycosil or HA-DTPH, $M_w$ 168 kDa, $M_n$ 79 kDa, polydispersity index 2.13), chemically modified gelatin-DTPH (Gelin-S, $M_w$ ~50 kDa), and heparin-DTPH (Heprasil, $M_w$ ~17-19 kDa). Protocols for synthesis of these proprietary compounds can be found in the literature (47, 48). HA alone does not provide sites for attachment during cell seeding. By including gelatin, or denatured collagen, and supplemented liver ECM, adequate binding sites for cells were supplied. At a sufficient stiffness, these molecules enable timely and efficient cell adhesion that resembles what is observed in seeding of tissue scaffolds or protein coated tissue culture plastic. These additions allow the hydrogels to mimic the composition and physical properties of a normal tissue microenvironment.

To test the effects of mechanical properties, three different gel stiffnesses were created. For the two lower stiffnesses, 1% and 2% weight/volume polyethylene glycol diacrylate (PEGDA/Extralink, 3.4 kDa) were added to other gel components (ESI BIO, Alameda, CA). The stiffest gel was created with 6% weight/volume 4-arm PEG acrylate (20 kDa) (Creative PEGWorks, Chapel Hill, NC). As controls, gels containing only components of the HA gel kit were created without the addition of solubilized ECM.
Gel solutions were added to wells of chamber slides or tissue culture plates and allowed to fully crosslink for two hours. Gels completely filled the growth area of the wells and polymerized at a thickness of over 1.5 mm to prevent the tissue culture plastic bottom from contributing to the stiffness of the substrate. For cell culture assays involving immunocytochemistry (ICC), 125 μL of each gel type was layered per well into 8-well Permanox Plastic Nunc Lab-Tek Chamber Slides to fill an area 80 mm² (Thermo Scientific, Waltham, MA). For stiffness quantification, 325 μL of gel was added to 24-well plates to fill an area of 200 mm². For assays measuring function, viability, and gene expression, 50 μL of each gel type was layered into 96-well plates to fill an area of 32 mm² for cell seeding (Tissue culture treated plastic, Thermo Scientific, Waltham, MA).

2.7 Stiffness Quantification

ECM-HA and HA only gels were created in 24-well plates (80 mm²) with each crosslinker concentration (1% PEGDA, 2% PEGDA, and 6% 4-arm PEG w/v). The Discovery Series HR-2 Rheometer (TA Instruments, New Castle, DE), a type of rotational or shear rheometer, was used to test stiffness following creation of gel substrates. Decellularized tissue was used as a reference control and comparison to normal liver ECM in this study. During testing with this type of rheometer, the sample was stabilized on the fixed stage of the rheometer. A geometry in the form of a 12 mm flat parallel plate was subsequently lowered until it came into contact with the sample. The rheometer performed oscillatory stress sweep tests where shear stress was incrementally increased on the samples from 0.6 Pa up to 10.0 Pa with a constant oscillatory frequency of 1 Hz and a normal axial force of 0.4 N, as has been previously described (49-52).
From the various forces applied, an elastic modulus could be measured and calculated by the instrument. The elastic modulus, or resistance to deformity, can be expressed by three types of calculations; Young’s modulus, bulk modulus and shear elastic modulus. We analyzed the shear elastic modulus, which describes how a sample responds to opposing forces parallel to its surface and is calculated by dividing shear stress by shear strain. Both the shear storage modulus (G’) and shear loss modulus (G’’) were measured. The storage modulus describes the elastic properties or stored energy, while the loss modulus describes the viscous characteristics or energy lost as heat (53). The storage modulus (G’) from oscillatory shear was the main value utilized for reporting stiffness characteristics of the hydrogels in our assays.

To calculate G’, measurements of phase lag (the phase shift between stress and strain), stress, and strain are needed. The formulas listed in Table 1 were used to calculate G’ and its different variables. Formulas were modified from the Discovery Series HR-2 Rheometer Reference Guide (TA Instruments New Castle, DE).

**Table 1: Storage modulus formulas**

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<td>( G' = \text{storage modulus} = \cos(\delta) \frac{\sigma}{\gamma} )</td>
<td>( \delta = \text{phase lag or shift}, \sigma = \text{oscillation stress}, \gamma = \text{strain} )</td>
</tr>
<tr>
<td>( \sigma = \text{oscillation stress} = K_\sigma * M_{(sample)} )</td>
<td>( K_\sigma = \text{stress constant}, M = \text{oscillation torque (on the sample)} )</td>
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<tr>
<td>( K_\sigma = \text{stress constant} = \frac{2}{\pi R^2} )</td>
<td>( R = \text{radius of parallel plate} )</td>
</tr>
<tr>
<td>( \gamma = \text{strain} = K_\gamma * \Theta )</td>
<td>( K_\gamma = \text{strain constant}, \Theta = \text{oscillation or angular displacement} )</td>
</tr>
<tr>
<td>( K_\gamma = \text{strain constant} = \frac{R}{H} )</td>
<td>( R = \text{radius of parallel plate}, H = \text{gap} )</td>
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2.8 GAG Function in ECM gels

GAG function was analyzed in gel substrates of the same 600 Pa stiffness in 96 well plates either containing HA only or HA supplemented with solubilized liver ECM. Gels were incubated in solutions containing 500 ng/mL of HGF in PBS overnight and then washed for an hour in four separate 250 µL volumes of PBS. A HGF dependent epithelial cell line, 4MBr-5, was seeded at 20,000 cell/well in Ham's F-12K Medium with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, and 30 ng/mL of epidermal growth factor (ATCC, Manassas, VA). DNA synthesis of seeded cells was quantified after 48 hours in culture to assess the activity of GAG-bound HGF. Values were also standardized by cells grown on non-growth factor treated gels of each substrate type.

2.9 Primary Hepatocyte Isolation and Seeding

In preliminary assays, primary rat hepatocytes were freshly isolated from rat livers perfused with collagenase and calcium chloride solutions through the inferior vena cava at 37ºC. To purify isolations, hepatocytes were passed through a 100µm nylon mesh and centrifuged at 1000RPM as described in previous studies (102, 103).

For assays involving primary human hepatocytes, cryopreserved human hepatocytes (Triangle Research Labs, Research Triangle Park, NC) were seeded onto gels in Clonetics Hepatocyte Basal Medium with UltraGlutamine-1 and Hepatocyte Culture Medium supplements (HCM; Lonza, Allendale, NJ). Supplements include ascorbic acid, bovine serum albumin-fatty acid free, hydrocortisone, human epidermal growth factor, transferrin, insulin, and gentamicin. 50,000 or 125,000 primary hepatocytes per well were seeded on pre-formed gels in 96-well plates or 8-well chamber slides respectively. Cells
were allowed to attach to gel binding sites for 5 hours before PBS washing removed dead or non-adherent cells. Hepatocytes were maintained in incubators (37°C, 5% CO₂), and media was changed following PBS washing every 2 days until conclusion of the experiment.

2 and 7 days post-seeding were used as time points in all of our cellular assays. The majority of cell death occurs in the first 24 hours post-hepatocyte seeding (54). The day 2 time point allows for initial cell attachment, establishment of cell viability, and washing away of non-adherent or dead cells. Primary hepatocytes have also been reported to take 3-6 days to stabilize metabolic functions, especially albumin production (55). The day 7 time point allows cells to complete this adaptation period on the cell culture substrates.

2.10 DNA Quantification

DNA was extracted from 4MBr-5 cells of each well by proteinase K digestion for 2 hours at 56°C and purified using the Qiagen DNeasy Kit. DNA was quantified using the Quant-iT PicoGreen reagent kit and a set of DNA standards (Invitrogen Corp., Carlsbad, CA). Total DNA amounts were standardized by DNA levels from cells grown on non-growth factor treated HA only or ECM containing gels.

Similar to the GAG-bound HGF assay, DNA was quantified in primary hepatocytes to assess cell numbers retained on gels at various time points. DNA was extracted and purified from cells of each well at the day 2 or day 7 post-seeding time points. DNA was then quantified using the Quant-iT PicoGreen reagent kit (Invitrogen
Corp., Carlsbad, CA). Total DNA amounts were compared among various stiffness groups.

2.11 Immunocytochemistry (ICC)

For ICC, cells were seeded on gels in Nunc™ Lab-Tek™ 8-well Chamber Slides. After 7 days in culture, cells were fixed overnight in 10% neutral buffer formalin (NBF) at 4°C. Cells were then stained using the Actin Cytoskeleton and Focal Adhesion Staining Kit (Millipore, Billerica, MA), or for FAK and ILK staining, the rabbit monoclonal antibodies anti-FAK (EP695Y) and anti-ILK (EPR1592) (Abcam, Cambridge, MA) were used. Cells were blocked for 30 minutes in 8% milk and then stained with a 1:500 dilution of the anti-Vinculin, 1:500 dilution of anti-FAK, or 1:250 dilution of anti-ILK primary antibody for 1 hour. After washing, anti-Vinculin cells were then stained with 1:500 diluted TRITC-conjugated Phalloidin and 1:500 Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor 488 conjugate (Life Technologies, Grand Island, NY). Fluorescent-conjugated phalloidin stained F-actin in the microfilaments of the cytoskeleton to assess cell structure. Cells incubated with Anti-FAK or Anti-ILK were stained with 1:500 Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 594 conjugate. Following further washing, all cells were incubated in 1:1000 DAPI counterstain for 5 minutes. DAPI co-staining was used to visualize individual cell nuclei for quantification.

2.12 Cell Counting

To confirm DNA quantification methods at 7 days post-seeding, hepatocytes were quantified by counting nuclei of stained cells per 0.25 mm² area. For each gel type and
stiffness, six separate 10x magnification images (1000 μm x 1000 μm) of DAPI stained hepatocytes were captured in randomized areas of six different substrate-containing wells of an 8-well Permanox Plastic Nunc Lab-Tek Chamber Slide (Thermo Scientific, Waltham, MA). Images were broken down into four quarters (500 μm x 500 μm), and the top left quarter of the image was used for counting purposes (0.25 mm² area). DAPI stained cells were hand-counted per image and averaged to assess cell adhesion for each substrate condition.

2.13 Calcein-AM Staining

Primary hepatocytes seeded on ECM-HA or HA only containing gels were immunofluorescently labeled using a Live/Dead Viability Assay Kit (Life Technologies, Grand Island, NY). Viable cells were identified by green-fluorescent Calcein-AM positive staining.

2.14 Hepatocyte Functional Analysis

Media samples were collected at various time points from wells containing primary hepatocytes seeded on gels of varying stiffness. The Human Serum Albumin ELISA kit (Alpha Diagnostic International, San Antonio, TX) was used to compare secreted albumin in experimental groups to negative controls containing medium alone. Quantification was achieved by comparing values to a standard curve generated from purified albumin.
2.15 Liver Cell Junction and Kinase Expression

Primary hepatocytes seeded on various gel types were lysed by syringe homogenization in QIAzol solution. Solutions from triplicate wells were combined to create a total volume of 700µL of homogenized QIAzol/cell mixture. A total of 140 μL of chloroform was added to this solution and vortexed for 15 seconds. Solutions were then centrifuged at 12,000 x g at 4°C for 15 minutes. The upper aqueous layers were added to miRNeasy spin columns, and processed RNA was used for real time PCR (Qiagen, Germantown, MD).

Isolated RNA was quantified using a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA). A total of 200 ng RNA was used to create cDNA using a thermocycler and the SuperScript Vilo cDNA Kit (Life Technologies, Grand Island, NY). cDNA was mixed with the SYBR Select Master Mix (Life Technologies, Grand Island, NY) and Eurofin primers custom created from sequences designed using Primer-Blast (NCBI) (Table 2) or from published literature (56, 57). An Applied Biosystems 7300 Real-Time PCR System was used to calculate samples’ Ct values for targets, which were standardized by GAPDH. Expression was compared relative to Human Liver Total RNA (Life Technologies, Waltham, MA).
Table 2: Human primers for RT-qPCR

<table>
<thead>
<tr>
<th>Targets</th>
<th>Forward Primer (5’ to 3’)</th>
<th>Reverse Primer (5’ to 3’)</th>
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<tbody>
<tr>
<td>Claudin-1</td>
<td>CCGTTGGCATGAAGTGATAG</td>
<td>CCAGTGAAGAGAGCCTGACC</td>
</tr>
<tr>
<td>E-Cadherin</td>
<td>TGAGTGTCCTCCCGTGATCTT</td>
<td>AGAAATCATAGGCGGGGTGTC</td>
</tr>
<tr>
<td>ILK</td>
<td>AAGGTGCTGAAGGTTCGAGA</td>
<td>ATACGGGATCCAGTGCTGTGA</td>
</tr>
<tr>
<td>FAK (PTK2)</td>
<td>TTATTGGCCACTGTTGGATGA</td>
<td>TACTCTTGCTGGAGGCTG</td>
</tr>
<tr>
<td>GAPDH (56)</td>
<td>CCCACTCCTCCACCTTTGAC</td>
<td>TGCTGTAGCCAAATTTCGTGT</td>
</tr>
<tr>
<td>Integrin Beta 1 (57)</td>
<td>GAAGGTTGCCCTCCAGA</td>
<td>GCTTGAGCTTCTCTGCTG</td>
</tr>
<tr>
<td>RhoA</td>
<td>GTCCACGGTCTGGTCTTCAG</td>
<td>TTTCCACAGGCTCCATCA</td>
</tr>
<tr>
<td>CDC42 variant 1/2</td>
<td>GGTGGAAGGCTGAGGAGTCAT</td>
<td>CATCGCCCAACACACACAC</td>
</tr>
<tr>
<td>Rac1/3</td>
<td>ATCACCTATCCGCCAGGCTTCT</td>
<td>CGGATCGCTTCGTCAAAC</td>
</tr>
<tr>
<td>HNF4α</td>
<td>TCAACCGAGAAACAAAAACACC</td>
<td>ACCTGCTCTACCAACACAC</td>
</tr>
<tr>
<td>Albumin</td>
<td>TTGGCACAATGAGTTGGGA</td>
<td>AAAGGCAATCAACACACAC</td>
</tr>
<tr>
<td>SNAIL</td>
<td>CCCTCAAGATGCGACATCCGAA</td>
<td>GACTCTTGTTGCTGGAGCAA</td>
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</table>

2.16 Statistics

Data was expressed as the mean +/- standard deviation. Statistical analysis was performed using Student's t test and analysis of variance (ANOVA) where noted. A p-value of less than 0.05 was considered significant.
3. Results

3.1 Liver Decellularization and Solubilization Preserved ECM Components and Their Functions

This study incorporated liver ECM into gels through the process of decellularization and solubilization. A previous manuscript published by our group demonstrated full cellular removal and retention of specific ECM components, including

![Image]

**Fig. 2** Solubilized Liver Composition and Activity - **A)** Alcian blue staining detected GAG presence in decellularized tissue. **B)** Solubilized liver ECM solutions were analyzed for collagen, GAG, and DNA content (n=3, ±SD). DNA content was non-detectable (ND) at an assay limit of <1 ng/mL. **C)** During growth factor treatments to determine GAG functionality, HGF bound to isolated ECM in a dose-dependent manner (*p<.05, Student’s t-test, n=5). **D)** The activity of growth factor treated gels was tested by seeding a HGF-dependent cell line on HA gels or HA gels supplemented with solubilized ECM after 500 ng/mL HGF pre-treatment. Cell growth was quantified by DNA (*p<.05, Student’s t-test, n=7).
collagen IV and laminin, in the decellularized liver tissue (Figure 1-Methods) (10). Alcian blue staining was performed in a new IHC assay to confirm presence of GAGs in the tissue (Figure 2A). GAG-containing decellularized matrix also demonstrated the ability to bind HGF in a concentration dependent manner (Figure 2C). In addition, the GAG binding growth factor HGF abound in greater quantities compared to EGF, which only has non-specific interactions with collagens of the matrix (Supplementary Figure 1).

Following liver ECM solubilization, key molecular constituents were quantified. DNA levels were insignificant and below detection levels (<1 ng/mL). However, significant levels of GAGs and collagen were detected (Figure 2B). An assay was performed to measure the ability of GAGs in solubilized liver ECM gels to bind and present growth factor. The assay showed increased growth of a HGF-dependent epithelial cell line on HGF pre-treated HA gels supplemented with solubilized ECM compared to pre-treated gels only containing HA (Figure 2D).

In cell culture experiments with rat hepatocyte, substrates with solubilized ECM improved viability compared to gels containing cryomilled tissue (Figure 3). Incorporation of decellularized liver tissue into HA cell culture substrates also showed benefits over single protein coatings or unmodified gel substrates. Preliminary studies showed greater hepatocyte aggregation on HA gels containing liver ECM versus basic collagen I gels (Figure 4). Compared to cells grown on gels containing solely HA, primary hepatocytes seeded on HA gels supplemented with solubilized ECM attached in greater numbers and showed greater E-cadherin expression (Figure 4E and 4F).
Fig. 3) Primary rat hepatocytes were seeded for 7 days on gel coatings with A+C) powdered or B+D) solubilized liver ECM. Ethidium bromide levels stained dead cells red, while calcein-AM stained live cells green. Cell viability was A) low on gels with powdered ECM and B) high on solubilized ECM gels. Phase contrast images showed C) vacuolization and development of lamellar bodies in hepatocytes grown on powdered ECM. D) Hepatocytes on solubilized ECM gels maintained normal cell morphologies.
Fig. 4) Primary rat hepatocytes were seeded for 7 days on either A+C) pure collagen I gels or B+D) HA gels containing solubilized liver ECM. Phase contrast imaging and cytochrome P450 staining with a DAPI counterstain show greater cell aggregation on liver ECM gels. ECM gels also initiated greater E) cell attachment and F) E-cadherin expression (*p<.05, **p<.01, Student’s t test, DNA n=10, E-cadherin n=3).
3.2 Crosslinker Concentration Altered ECM Gel Stiffness

1% PEGDA, 2% PEGDA, and 6% 4-arm PEG (w/v) crosslinker formed three different ECM gel stiffness groups for stiffness testing. Oscillatory stress rheometry quantified the storage modulus (G’) to compare each crosslinked gel’s stiffness to the stiffness of normal liver ECM. Gels formed by 6% 4-arm PEG had a mean G’ of 4600 Pa. This value was higher than the mean stiffness of normal liver ECM, which fluctuated around 3000 Pa, but fell within the standard deviation of the data set. The stiffnesses of gel groups formed by 1% and 2% PEGDA at 600 and 1200 Pa respectively were significantly lower than the storage moduli of both the 6% 4arm PEG crosslinked gels and normal liver ECM. The 600 and 1200 Pa gel groups were also significantly different from each other (Figure 5).

Fig.5) HA-ECM Gel Stiffnesses Formed by Varying Crosslinker Concentrations-
HA-ECM gels were formed with 1% PEGDA, 2% PEGDA, or 6% 4-arm PEG (20kDa). All gel stiffness values were significantly different from each other. Decellularized liver tissue had a significantly higher stiffness than 1% and 2% PEGDA, however, it was not significantly different compared to the 6% 4-arm PEG stiffness. (*p<.05, Student’s t test, n=4,)
3.3 ECM Supplementation and Stiffness Affected Viable Hepatocyte Attachment and Morphology

Calcein-AM, which is only metabolized in the cytoplasm of live cells, was used to stain hepatocytes to assess the role of ECM gel stiffness in cell viability and morphology. An increase in viable human hepatocytes was observed on ECM containing gels with increased stiffness at day 7 post-seeding. Hepatocytes also survived on ECM gels in greater numbers than cells grown on gels containing HA alone (Figure 6).

Cell morphology varied among substrate groups. No obvious differences were observed between cells seeded on varying stiffnesses of gels containing only HA (Figure 6A, 6C, and 6E). However, cells on ECM supplemented gels of 4600 Pa developed organized, polyhedral morphologies compared to irregularly-shaped cells on the lower stiffnesses of 600 and 1200 Pa. Increased cytoplasmic protrusions or filopodia formation at day 7 post-seeding was demonstrated with Calcein-AM staining on 600 and 1200 Pa ECM gels (Figure 6B and 6D).
Cell Viability and Filopodia Formation - Green-fluorescent Calcein-AM staining was used to visualize primary hepatocyte viability and morphology at Day 7 post-seeding at 4x magnification. Mean gel stiffnesses were; A+B) 600 Pa, C+D) 1200 Pa, or E+F) 4600 Pa. Gels were composed of only HA (A,C,E) or were supplemented with liver ECM (D,B,F). Filopodia were highlighted with white arrows.
Fig. 7) Cytoskeletal Staining - Phalloidin and DAPI staining was used to visualize F-actin filaments and nuclei of primary hepatocytes at day 7 post-seeding at 10x and 20x magnification. Mean gel stiffnesses were; A+B) 600 Pa, C+D) 1200 Pa, or E+F) 4600 Pa. Gels were composed of only HA (A,C,E) or were supplemented with liver ECM (D,B,F). Actin stress fibers were highlighted by white arrows.
More detailed immunocytochemistry was then used to analyze hepatocyte attachment and cytoskeletal structure of the hepatocytes. Clear differences were observed in phalloidin staining of the hepatocytes’ actin cytoskeletons on varying ECM gel stiffnesses. Increased gel stiffness correlated with increased attachment and cytoskeletal organization, evidenced by greater actin microfilament presence and stress fiber formation (Figure 7F). ECM supplemented gels of 600 and 1200 Pa stiffnesses generated lower cell densities and less actin staining (Figure 7B and 7D). Differences in cytoskeletal structure were less obvious in cells seeded on HA only gels but in general, cells on the 4600 Pa gel stiffness experienced greater stress fiber formation than cells on the 600 and 1200 Pa stiffnesses (Figure 7E).

From the DAPI staining, cells were counted by number of nuclei per mm² of gel. Results showed a significant increase in cell attachment with increased ECM gel stiffness. Stiffness did not significantly influence cell number on gels composed of only HA. The data also indicated significant differences in attachment between ECM-containing and HA-only gels at 600 Pa and 4600 Pa. Human hepatocytes attached with greater efficiency on HA gels at 600 Pa. At 4600 Pa, a more physiologically normal stiffness, hepatocytes attached best to gels containing ECM (Figure 8A).

An additional assay was used to validate these findings. In this assay, DNA was isolated and quantified from different stiffness groups at two different time points. ECM gels at day 2 did not show significant differences. However, by day 7, 4600 Pa ECM gels showed significantly greater DNA levels (Figure 8B).
3.4 ECM Gel Stiffness Altered Human Hepatocyte Marker Expression and Functionality over Time

Expression levels of the hepatocyte markers HNF4α and albumin on gels of varying stiffnesses were measured and normalized to native human liver expression. There were no significant differences in HNF4α expression at the day 2 time point (Supplementary Figure 2A). However, at day 7 post-seeding, cells expressed both HNF4α and albumin at significantly lower levels on the 4600 Pa gel stiffness, while cells on the 1200 Pa stiffness demonstrated increased expression of these genes (Figure 9). SNAIL expression was also quantified to determine if any stiffness-induced epithelial-mesenchymal transition was
occurring. No significant differences were seen at either time point (Supplementary Figure 2B and 2D).

ELISA for albumin production was used as a measure of human hepatocyte function, and to determine if functional output matched hepatocyte marker expression. A significant increase in albumin production was seen with increasing gel stiffness at day 2 post-seeding. This increase remained significant when albumin was normalized to total DNA (Figure 10A). By day 7, albumin production improved in cells seeded on all gel stiffnesses, but cells on the 4600 Pa gel stiffness no longer produced albumin at a significantly higher rate. Cells on the 1200 Pa stiffness created albumin at a significantly higher rate than the lowest stiffness and appeared to overtake the functional rate of cells on the 4600 Pa stiffness (Figure 10B).
To further characterize underlying mechanisms of primary hepatocyte organization and morphology on various ECM gel stiffnesses, cell junction maintenance was determined by RT-qPCR. Data was again normalized to expression in normal human liver. Claudin-1 is an important protein in tight junction formation and maintenance in epithelial cells. Primary hepatocytes grown on the 4600 Pa stiffness for 7 days expressed the highest levels of claudin-1. Hepatocytes on all stiffnesses expressed claudin-1 at levels greater than those measured in normal liver (Figure 11A).

Expression of occludin, another constituent of tight junctions, also proved to be dependent on substrate stiffness, but in an inverse manner. Expression of occludin decreased relative to stiffness, although all values were at or above levels of expression seen in normal liver (Figure 11B). Taken together, the expression patterns of occludin

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**Fig.10) Hepatocyte Functional Output**- ELISA measured albumin secretion of primary human hepatocytes per well on ECM containing HA gels of varying stiffnesses at A) day 2 and B) day 7 post-seeding. Quantities were standardized for cell number by DNA quantity per well (*p<.05, **p<.01, Student's t test, n=12).
and claudin-1 have alternative roles in adapting primary hepatocyte phenotype and structure to substrate stiffness.

To determine the relationship between cell junction gene expression and integrin sensing, RT-qPCR was used to analyze cytoplasmic kinase activation on varying substrate stiffnesses. At early time points, cells expressed FAK and ILK similarly regardless of substrate stiffness (data not shown). However, at 7 days post-seeding, the highest expression levels of FAK and ILK were measured on the 600 Pa substrates.

**Fig. 11** Expression of Cell Junction Molecules and Focal Adhesion Regulators—RT-qPCR measured expression of A) Claudin-1, B) Occludin, C) FAK, and D) ILK in primary hepatocytes grown on ECM containing HA gels of varying stiffnesses at 7 days post-seeding. Expression was standardized relative to normal liver expression (*p<.05, Student’s t test, n=3).
(Figure 11C-D). Hepatocytes grown on the 4600 Pa gels expressed FAK and ILK at levels similar to and greater than physiological liver levels, respectively. On 600 and 1200 Pa stiffnesses, both kinases were expressed at higher than normal liver levels.

**Fig.12** A,C,E) FAK and (D,B,F) ILK staining was performed on primary hepatocytes at day 7 post-seeding at 20x magnification. Mean ECM gel stiffnesses were; A+B) 600 Pa, C+D) 1200 Pa, or E+F) 4600 Pa. FAK and ILK stained in red, along with autofluorescent nuclei of dead cells. DAPI counterstaining stained all nuclei in blue.
To correlate gene expression with protein expression, ICC for FAK and ILK was performed on cells seeded in the same conditions and ECM gel stiffnesses. FAK staining was most prominent at the leading edge of cells at the 600 Pa gel stiffness (Figure 12A). Staining was also seen on the 1200 Pa stiffness (Figure 12B). Cell staining for ILK was less specific, but showed increases on the 600 and 1200 Pa stiffness gels (Figure 12B and Figure 12D).

3.6 Rho GTPase Expression Correlates with Cytoplasmic Kinase Overexpression and Hepatocyte Morphology

Next, we quantified expression of vital downstream targets of FAK and ILK that are responsible for initiating actin polarization and polymerization to alter cell morphology. RT-qPCR was performed on ECM gels of varying stiffness to measure the Rho GTPases; Rho, Rac, and CDC42. Similar to FAK and ILK, CDC42 expression was highest in hepatocytes seeded on the 600 Pa gel stiffness (Figure 13A). In contrast, cells on the 4600 Pa substrates expressed Rho at the highest levels (Figure 13B). Rac expression was not altered with stiffness (Figure 13C).
4. Discussion

The challenge of maintaining primary hepatocyte phenotype in vitro has led to the development of more advanced culture systems. Traditional cell culture methods utilizing collagen coated tissue culture plastic experienced loss of primary hepatocyte viability and function by 7 days post-seeding (16). Cells grown on substrates supplemented with natural liver ECM maintain viability and function for extended time periods as compared to these traditional substrates that contain a limited number of matrix proteins (6).

However, studies have yet to completely optimize methods for incorporating ECM into culture substrates and the mechanical properties of these substrates. Development of liver specific gels supplemented with ECM has been slowed by the lack of a reliable method

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**Fig. 13** Expression of the Cytoskeleton-Regulating Rho GTPases- RT-qPCR measured expression of the Rho GTPases; A) CDC42, B) RhoA, and C) Rac1 in primary hepatocytes grown on ECM containing HA gels of varying stiffnesses at 7 days post-seeding. Expression was standardized relative to normal liver expression (*p<.05, Student’s t test, n=3).
for purifying ECM that retained all of the bioregulatory properties of the native matrix. Perfusion decellularization solved this problem by allowing selective removal of all of the cellular components of a tissue, while leaving the remaining acellular material relatively unperturbed and functional as characterized in Figure 1 and Figure 2 (10).

Initial attempts to incorporate cryomilled ECM into substrates yielded suboptimal results. Powders accumulated in endosomes and compromised the health of the cells (Figure 3). This build-up of phagocytosed matrix likely caused congestion of intracellular lysosomes, vacuolization, and induction of apoptosis in highly endocytic hepatocytes (58-60). Solubilization of the ECM allowed for easy integration into HA hydrogel substrates and circumvented the negative effect that particulate ECM had on primary hepatocytes (Figure 3). HA-based gels have also proven to be non-immunogenic following implantation into experimental animals (11, 14), opening up the possibility to use these substrates in future cell based therapies.

Preliminary studies with rat hepatocytes also showed greater viability, cell aggregation, and expression of E-cadherin on ECM supplemented HA gels compared to standard gels containing only collagen I gels or HA (Figure 4). These features are especially important in primary hepatocytes, which rely on cell contacts and junction formation for functional maintenance and cell polarity (61-63). E-cadherin (E-Cad) is a calcium-dependent glycoprotein vital in epithelial intercellular adhesion, liver tissue architecture, and hepatocyte functional maintenance (64, 65). This glycoprotein has also been correlated with reduction in anoikis, or detachment related apoptosis, during culture of primary hepatocytes (66). Increased E-cadherin expression on our ECM gels indicates the abilities of these substrates to support proper hepatocyte phenotype.
In our chief study with human cells, ECM supplementation of HA gels was shown to positively affect primary hepatocyte attachment and viability. Inclusion of solubilized ECM increased cell coverage of the gels at two of the three stiffnesses tested (Figure 8). ECM contains several different molecules that promote cell adhesion. Many of these cell attachment proteins interact with cell membrane integrins through Arg-Gly-Asp (RGD) motifs. RGD-containing molecules include laminin, fibronectin, vitronectin, and several of the collagen sub-types. (67). RGD peptides support survival of hepatocytes via the beta1-integrin-ILK-pAkt pathway (68). The results of the current study indicate that a strong correlation exists between supplementation of HA substrates with ECM and integrin beta-1 expression, which may explain why liver ECM mediates efficient hepatocyte attachment (Supplementary Figure 3) (68). Several other ligands or cell adhesion molecules contribute to cell-ECM interactions, but promotion of integrin expression clearly represents a key factor in forming and maintaining cell/substrate attachment.

The mechanical properties of gel substrates also play a significant role in the preservation of hepatocyte cell viability and function. Previous studies on hepatocellular carcinoma cell lines showed increased proliferation on stiff polyacrylamide gels (defined as 12 kPa) as compared to soft gels (1kPa) (24). A study on primary rat hepatocytes compared growth on what was defined as soft (11 kPa), medium (27 kPa), and stiff (116 kPa) heparin gels (69). In that study, the softest gel was well above the stiffness of normal liver, which has been reported by various groups as ranging anywhere from 1.5 to 8 kPa (19-24). The study concluded that “soft” gels promoted greater cell attachment, better morphology, higher albumin output, and more robust cell aggregation (69).
lack of consensus on normal liver stiffness likely arises from variations in tissue preparation, instrumentation, and how the measurement was obtained. Similar discrepancies exist in the reported stiffness of fibrotic livers, which have varied from 8 kPa to 75 kPa (20, 70-73). The variance in definitions of soft and stiff gel substrates complicate the direct comparison of results among various studies. An internal control of normal liver ECM was used in the current study, allowing for a point of reference that takes into account the equipment and methods used.

The gels employed in the current study use different crosslinker formulations to adjust gel stiffness, rather than varying the matrix concentration. This minimizes potential confounding effects such as the number of bioactive sites, diffusion rates, and gel swelling properties. The study’s goal was to determine an optimal stiffness range for maintaining the viability and function of primary hepatocytes in the presence of normal liver ECM. The two lower crosslinker concentrations produced sub-normal stiffnesses at 600 and 1200 Pa, which would be representative of matrix undergoing remodeling in scenarios such as recovery from injury or during development. The highest gel stiffness at 4600 Pa was marginally higher than what was measured as the average stiffness of decellularized liver ECM (3000 Pa), but not significantly different. Intact liver tissue had a significantly higher stiffness, which can be attributed to the presence of cells organized within the structure. The decellularization process could potentially soften the isolated liver ECM causing lower values than non-processed ECM (74). However, histological analysis confirmed the presence of all major structural molecules, suggesting that any difference in stiffness should be minimal (10). A more precise measurement of the contribution of ECM to the total stiffness of specific tissues would be beneficial to organ
bioengineering strategies that use decellularized natural scaffolds. The three stiffnesses used in the current study provide a good indication of interactions of primary human hepatocytes cultured within a narrow, physiologically relevant stiffness range.

In the ECM supplemented gels, primary hepatocyte attachment and growth was maximized at the 4600 Pa gel stiffness level. Attachment not only correlated with increased presence of ligand sites, but also the stiffness of the substrate. Stiffer substrates, in general, provide more stable attachment sites for cells (15). They also provide good traction for cell motility, but hamper cell detachment. Soft substrates allow cells to detach more easily, although they provide lower traction for cell migration (75, 76). Cells sense stiffness through mechanosensing of strain on their cytoskeletons by “outside in signaling” mechanisms (40, 77). These forces exerted on their cell structure trigger changes in morphology and phenotype (78-80). Human hepatocytes on the two softer ECM stiffnesses of 600 and 1200 Pa developed long protrusions and a disorganized structure. Hepatocytes seeded on 4600 Pa gels exhibited an organized microstructure and cuboidal morphology. These cells also developed actin stress fibers by day 7, which are required for cell adhesion, cell junctions, and mechanotransduction (Figure 7). However, a significant accumulation of stress fibers can eventually lead to phenotypic changes (79, 81). Cells seeded on HA gels without ECM did not display these same stiffness dependent variations. It is possible that components of the ECM could affect the activation state of cells and responsiveness to physical cues. In a time dependent manner, results indicate that ECM supplementation and substrate stiffness both affect primary hepatocyte attachment and morphology; the latter of which is determined by cytoskeletal actin organization.
Increases in gel stiffness led to early increases in hepatocyte function on the ECM gels. Greater stiffness improved total and normalized albumin secretion at day 2. Normalized function of hepatocytes increased across all stiffness groups by day 7 post-seeding. However, secretion on the 4600 Pa stiffness now matched albumin levels measured on 1200 Pa gels. This correlated with a decrease on the 4600 Pa stiffness and increase on 600 and 1200 Pa stiffnesses in gene expression of the hepatocyte markers albumin and HNF4α. Cells may require time to establish themselves on a substrate before modifying their gene expression levels and functional output. These data support the notion that the highest metabolic function occurs on 4600 Pa gels, as shown by hepatocyte plasma protein secretion at the earliest time point. However, over time a slightly higher than physiological stiffness can lead to loss of phenotypic marker expression, and function is surpassed by cells on a more intermediate stiffness. 600 and 1200 Pa stiffnesses could also promote active remodeling of the matrix to better suit phenotypic maintenance after a certain period of time on the ECM substrates. These alterations in cell function directly correlated with previously observed changes in morphology. This association informed the design of experiments to study the mechanisms of mechanosensing-induced cell signaling and the expression of cell junction components.

As discussed previously, cell junction maintenance is vital for primary hepatocytes function and polarity. Tight junctions are types of cell junctions that act as chemical and electrical barriers, transduce cell/cell signals, and control permeability across cell layers (82, 83). Claudin-1 is an integral component of epithelial tight junctions. It has been shown to be critical to the maintenance of biliary function and the
prevention of epithelial-to-mesenchymal transition (EMT) (28, 82, 84, 85). Disruption of claudin-1 expression can cause diseases such as neonatal sclerosing cholangitis, while overexpression can restore tight junctions in fibrotic cells, resulting in restoration of cell function (29, 86-88). The 4600 Pa gel stiffness promoted the greatest claudin-1 expression at the 7-day timepoint, correlating with more stable hepatocyte morphology.

Another component of tight junctions, occludin, responded to substrate stiffness in a different manner. Cells expressed increased levels of occludin relative to normal liver at all stiffness studied. However, in contrast to claudin-1 expression, occludin levels decreased with increased gel stiffness. A threshold occludin level is required for maintenance of cell polarity and morphology (89). Increased expression of occludin promotes focal reorganization of tight junctions that leads to the formation of cell protrusions which are critical for directional migration (30). The observed cell elongation and increased occludin expression profiles at the 600 and 1200 Pa gel stiffnesses suggest adoption of a pro-migratory phenotype observed previously at low substrate stiffnesses.

Two cytoplasmic kinases linked to cell junction organization and integrin-mediated signaling, FAK and ILK, were also studied (90). FAK and ILK regulate cell cytokine responses, cell growth, and cell viability (36, 91-93). They also modulate focal adhesion turnover and formation of cell protrusion, which affect cell motility and migration, by regulating the expression of the Rho GTPases: CDC42, Rac1, and RhoA (30, 40, 77, 94, 95). Both ILK and FAK demonstrated increased gene expression at all stiffness levels as compared to normal liver, while expression showed an inverse correlation to gel stiffness. Expression levels were sufficient to allow survival at all stiffness levels, but ICC showed marked increases in FAK expression and localization at
the leading edge of hepatocytes on the 600 Pa gel stiffness (Figure 12A). This result reinforces previous studies which suggested FAK is required for assembly and direction of the leading edge of motile cells (96). Greater expression of FAK, ILK, and occludin all suggest adoption of a migratory phenotype at the lower stiffnesses of 600 and 1200 Pa. This notion is further supported by measured upregulation of filopodia-initiating CDC42. Decreases in FAK and increases in RhoA on 4600 Pa gels suggest favorable signaling for establishment of focal adhesions, stress fiber formation, and cytoskeletal contraction (97). Taken together, these results indicate stronger attachment and greater cytoskeleton organization occur on gels at the 4600 Pa stiffness, while greater ability to detach and move occurs on the softer gels of 600 and 1200 Pa. It is likely that the 1200 Pa gel stiffness would allow for efficient hepatocyte migration, as both traction and adhesion/release are in good balance. Understanding how cell morphology, attachment, and migration are influenced by substrate stiffness and gel formulation are integral to the design of cell substrates for cell based therapies and tissue engineering. Some applications would require a static cell component, while others would benefit from increased migratory potential for promoting cell organization and microarchitecture remodeling.

Hepatocyte function was maximized at a specific stiffness depending on time in culture. Future experiments could determine how longer time points or additional liver cell types impact hepatocyte function in vitro at these particular ECM gel stiffnesses. Additionally, regional variance in stiffness within a single organ should be taken into account depending on the requirements of specific applications. There are also implications for stiffness in models for aging and diseases, as these organs demonstrate
an accumulation of certain matrix components. Studies conducted by our group including data in Figure 2 show that natural liver matrix has the potential to bind beneficial growth factors such as hepatocyte growth factor (HGF) which can compensate for deficiencies induced by inappropriate substrate stiffness and regulate function. A preliminary study using isolated primary rat hepatocytes showed increased viability and cell junction expression on 600 Pa ECM gels pretreated with EGF and HGF (Supplementary Figure 4).

Cell junction protein expression and localization vary according to cell type, substrates stiffness, and growth factor microenvironment. Cadherins, integrins, and growth factor receptors are transmembrane structures that form an interdependent network of adaptor proteins, effector molecules, and cytoskeletal elements to regulate signal transduction (98). Mechanical forces sensed by integrins of focal adhesion sites lead to assembly or disassembly of cytoskeletal structures. Tension on the cytoskeleton initiates conformational changes in cell-cell adhesions and growth factor receptors to either promote or block cellular responses (99).

Many downstream cytoplasmic elements are also conserved between the various transmembrane structures. In the case of HGF signaling, β-catenin complexes with both the HGF receptor c-Met and E-cadherin. Results have varied on the effects of HGF binding on E-cadherin expression. HGF induced E-cadherin loss and EMT in liver cells during development and hepatocellular carcinomas leading to cell scattering or increased tumor invasiveness (100-102). Studies which cultured primary hepatocytes showed positive influences of HGF on E-cadherin expression and fibrosis prevention (103-105). It has been theorized that polarized cells behave differently than other cell types because of variances in the utilization and localization of β-catenin following HGF binding (106,
HGF-induced dissociation of β-catenin from c-Met could actually bolster the formation of independent E-cadherin-β-catenin complexes and cell junctions in hepatocytes seeded on certain substrate compositions and stiffnesses, which our data possibly indicated (108).

Separate preliminary assays using human hepatic stellate cells demonstrated the ability of stiffness to affect growth factor secretion and GAG production. Greater stiffness led to increased GAG production by HSCs, while cells on lower stiffness levels released higher quantities of HGF at day 2 post-seeding (Supplementary Figure 5). This suggests that further refinement of both stiffness and substrate biochemical formulation may be used to fine tune control of cell phenotype for specific therapeutic applications and tissue engineering. Other factors such as substrate hydration kinetics and solute/ion diffusion properties might also be modulated to control cell phenotype. A greater understanding of how substrates and scaffolds influence cell phenotype would maximize the efficacy of cell based therapies and would provide greater control of cells in tissue engineering strategies.

5. Conclusions

HA substrates supplemented with solubilized liver ECM supported improved growth factor binding capabilities, increased hepatocyte attachment and viability, and increased phenotypic maintenance. These gels served as a solid foundation for studies of liver interactions and cell function. Recreating the natural liver microenvironment in HA hydrogels through supplementation with liver ECM revealed that hepatocyte function and morphology changed over time based on substrate stiffness. Hepatocyte morphology and
expression profiles on sub-physiological stiffnesses of 600 and 1200 Pa were consistent with cytoskeletal reorganization and filopodia formation. Gels at a higher stiffness of 4600 Pa had high metabolic and functional rates at early time points, but later developed stress fibers and demonstrated a loss of hepatocyte gene expression. The optimal stiffness for maintenance of long term primary hepatocyte function, in vitro, and in gel substrates intended for the delivery of therapeutic cells is an intermediate stiffness between 1200 and 4600 Pa at the lower end of liver physiological limits.

6. Supplementary Figures

Supplementary Fig. 1) Growth factor binding comparison- Decellularized tissue was incubated with EGF and HGF at a concentration of 500 ng/mL, and ELISAs quantified growth factor binding. Residual growth factor amounts measured in non-treated samples were subtracted. Sample measurements were standardized by DNA amount (**p>.01, Student's t test, n=6)
Supplementary Fig. 2) RT-qPCR measured expression of A) HNF4α, B) SNAIL, and C) Claudin-1 in primary hepatocytes grown on ECM containing HA gels of varying stiffnesses at 2 days post-seeding. No significant differences were found. D) SNAIL expression was also measured at day 7 post-seeding. Expression decreased from day 2, but no differences were found between stiffnesses. Expression was standardized relative to normal liver expression (p > .05, Student’s t test, n=3).
**Supplementary Fig. 3** A) RT-qPCR measured expression of integrin beta-1 in primary hepatocytes grown on ECM containing HA gels of varying stiffnesses at 2 days post-seeding (n=3, Student’s t test, *p<.05). B) Expression was also measured at day 7 post-seeding, averaged between all stiffnesses, and compared between gels containing only HA and gels supplemented with ECM (n=9, Student’s t test, *p<.05). All expression values were standardized relative to normal liver expression.
Primary rat hepatocytes were seeded for 2 days on 600 Pa HA gels supplemented with liver ECM that were untreated or pretreated with combined solutions of EGF and HGF. Calcein AM stained live cells green, or ethidium homodimer stained dead cells red. Viability staining on A) untreated or B) pretreated ECM gels showed increased cell viability with growth factor treatment. C) Cell attachment and D) E-cadherin expression also increased on growth factor pretreated ECM gels (*p<.05, **p<.001, DNA n=5, RT qPCR n=3)
Supplementary Fig. 5) Human HSCs were seeded for 2 days on HA gels supplemented with liver ECM at mean stiffnesses of 600, 1200, and 4600 Pa. A) HGF levels per well were measured in the media by ELISA. B) Total GAG production per well was measured by a colorimetric assay (*p<.05, **p<.01, ***p<.001, HGF n=3, GAG n=5)
Works Cited


103. Jones CN, Tuleuova N, Lee JY, Ramanculov E, Reddi AH, Zern MA, Revzin A. Cultivating hepatocytes on printed arrays of HGF and BMP7 to characterize protective effects of these...


Conclusions and Future Work

Daniel B. Deegan
The field of regenerative medicine uses a variety of tools, technologies, and advanced models to gain knowledge of disease mechanisms and to develop new therapies and treatments. Each approach has a set of advantages and disadvantages depending on the field of research. Three-dimensional liver spheroids, or organoids, recreate entire organ systems on a miniature scale (1-5). Spheroids can be maintained in a variety of substrates like hydrogels. In microfluidic devices, organoids are arranged in a circuit and interact in a way that mimics how organ systems work together (6, 7). Consequently, they are a great platform for toxicology and drug testing. Because of their longevity and long term maintenance of function, testing can be carried out in vitro without the need for complex in vivo models. However, organoids are not always suitable for ECM based studies. These cell structures form tight junctions which prevent manipulation of ECM composition and mechanical properties at the core of the organoids. Since cells only sense ECM composition and mechanical forces in adjacent areas, these cell centers would be relatively unaffected by substrate alterations.

Decellularized tissue is an excellent biomaterial for tissue engineering. Optimized decellularization methods preserve ECM components in their native architecture and maintain physiological proportions in the tissue. This scaffold material enables cells to home to specific sites of the matrix and either sustain function or, in the cases of stem cells, differentiate into appropriate cell phenotypes (8-10). However, challenges remain in producing consistent decellularized products. The success of cell removal or the remaining composition of the decellularized tissue can vary because of differences between animals and differences in the size or vascularity of the organ. When designing studies on small tissue segments, it can be hard to control for structural variances in the
tissue that may exist even within the same organ. Matrix samples used for cellular assays that are excised from different regions of the organ, certain areas that are not fully decellularized, or sections damaged by the perfusion process can create error or deviation in the results. It is also difficult to precisely manipulate physical and chemical properties of the intact matrix. Without a complete analysis and mapping of the tissue, methods to initiate these changes could be unpredictable.

Hydrogel substrates are viable alternatives to intact decellularized tissue constructs. Although no longer possessing the native microarchitecture, gels enable better control and manipulation of individual substrate properties. The concentration of specific molecules in the hydrogels can be controlled and altered. In the case of this thesis study, ECM from decellularized tissue was solubilized and incorporated into HA gel substrates to replicate a natural liver microenvironment. Since the matrix of the entire organs was solubilized in a homogenous solution, there was little variability within the material. All regions of the ECM and multiple liver samples were combined, stored, and utilized across multiple assays, allowing for consistent composition of the substrates. ECM molecules were also quantified to ensure that all assays used the same total concentration of solubilized material, and there were similar ratios of structural and bioregulatory molecules. This ability standardized substrate production and resulted in more consistent results and less experimental error.

This thesis work demonstrates that HA gels supplemented with ECM provided some of the same functional advantages for seeded cells as fully intact matrix scaffolds. Use of ECM containing gels in past studies were shown to greatly improve long term viability and function of culture of cells, as compared to traditional in vitro methods (11,
12). Our human hepatocyte assays showed increases in viability, adhesion, and cell junction formation. To determine the mechanism for some of the improvements seen in primary hepatocyte attachment and viability, expression of integrin β-1 was analyzed in cells seeded on ECM supplemented gels versus cells on HA only gels. At day 7, hepatocyte expression increased on gel substrates with the addition of liver matrix.

Although only one integrin class was measured, integrin β-1 may be the most critical type for maintenance of cell function. Integrin β-1 is the most highly expressed beta-integrin, dimerizing with more than 10 alpha-integrin subunits (13). These integrin complexes directly interact with the actin cytoskeleton (14). Integrin β-1 expression likely correlates with overall integrin expression in the presence of liver ECM and explains the increases in hepatocyte attachment and viability relative to cells seeded on simpler substrates. To establish this conclusion, expression of a greater variety of integrin types could be analyzed. Varying concentrations of solubilized liver matrix could also be used in substrate formation to determine if concentration dependent changes were observed. As controls, certain integrins could also be blocked with increasing concentrations of integrin-specific antibodies or RGD peptides in order to measure reduction in binding or survival of the hepatocytes. Manipulations of the cells and the adaptable gel substrate could help determine the most likely mechanisms for differences observed.

Greater control of the composition of the substrate also correlates with better control of the chemistry and physical properties of the material. These characteristics can be adjusted by adding chemically-modified compounds to the substrate preparation. Thiol-modified HA, heparin, and collagen were used for gel formation in our study. PEG-
acrylate crosslinker reacted with these moieties to solidify hydrogels and alter stiffness within a narrow range. Uniform composition of our ECM supplemented gels allowed creation of sample replicates at equivalent stiffness levels. Different concentrations and configurations of the crosslinker also generated adjustable degrees of stiffness to establish separate experimental groups. Crosslinking can vary the property of stiffness without modifying the concentration of functional elements in the ECM substrates. These advantages make ECM supplemented gels optimal for use in cell culture assays to identify the effects of substrate stiffness variations.

Following the development and creation of liver ECM supplemented gels, this thesis evaluated the effects of substrate stiffness on primary hepatocyte function. Results revealed hepatocyte gene marker expression and the functional output of albumin varied with stiffness and time in culture. In early time points, higher gel stiffness stimulated greater metabolic activity and albumin output. However, after metabolic functions stabilized for 7 days, hepatocytes grown on an intermediate stiffness of 1200 Pa produced albumin at the greatest rate. This observation was correlated to a significant drop off in gene expression of hepatic markers by cells on the highest stiffness of 4600 Pa. To test the hypothesis that the highest 4600 Pa stiffness causes a loss in long term phenotype, experiments could utilize time points past day 7. These assays would indicate if observed variances in function and gene expression widen between cells seeded on the different stiffnesses in the long term. It would also be relevant to examine if markers for EMT like SNAIL were upregulated by cells on the highest stiffness group.

The balance between EMT and MET is vital in liver development and could possibly have an integral role in liver repair and regeneration. It is theorized that
following injury, epithelial cells undergo EMT and travel to the mesenchyme to proliferate and produce ECM molecules. These cells then differentiate into hepatocytes or cholangiocytes to repopulate and restore the liver parenchyma (15). Imbalances in this process can lead to increases in fibroblasts and a progression of disease (16). During liver disease, many experts theorize that the stiffening ECM microenvironment promotes additional EMT of hepatocytes and contributes to worsening fibrosis and eventual organ failure (16). Our work has helped delineate a proper substrate stiffness for long term maintenance of hepatocyte phenotype as well as a stiffness range where EMT could be initiated. Future experiments could be designed using the liver ECM gels to further replicate events of liver repair or liver disease in culture and study the delicate balance between EMT and MET.

While studying the effects of stiffness on hepatocyte phenotype, assays were designed to study the mechanisms behind observed changes in attachment, viability, and morphology. Experiments monitored gene and protein expression of FAK and ILK, two integrin-localized kinases with vital roles in mechanotransduction and cytoskeletal remodeling. As previously discussed, hepatocytes on all gel stiffness expressed the FAK and ILK genes at levels higher than cells of normal liver tissue. However, at day 7 post-seeding, FAK and ILK gene and protein expression levels were highest in the lower stiffness levels, with FAK concentrated at the leading edge of epithelial cell layers. Although FAK and ILK have been shown to have effects on viability, trends we observed seemed to correlate most closely with morphological changes in the hepatocytes, specifically with formation of cellular projections.
Previous studies have shown FAK and ILK expression stimulates pathways controlling cytoskeletal structures. In addition, overexpression of these kinases has been observed in migrating cells. With this knowledge, expression of the downstream targets and actin regulating Rho GTPases were measured to determine if differences in cytoskeleton activation and regulation were occurring in human hepatocytes seeded on ECM gels of varying stiffnesses. Cells expressed RhoA, which controls stress fiber formation and contractility, on stiffer ECM gels and exhibited greater attachment and assembly of actin fibers. CDC42, which initiates polarization and filopodia formation, decreased with substrate stiffness. This result demonstrated how FAK, ILK, and CDC42 overexpression correlates with lower substrate stiffness, morphological changes, and most likely, cell migration.

Further experimentation could help confirm detected morphological and migratory trends and help strengthen our hypotheses. Determining exact mechanisms and sites of FAK and ILK phosphorylation induced by substrate stiffness could help explain the numerous functions of these kinases. Previous studies have shown blocking FAK and ILK in cell culture assays may not be ideal because deficiencies in FAK and ILK lead to reductions in viability. However, plasmid transfections could be used to upregulate FAK, ILK, or CDC42 in primary hepatocytes, as has been successful in previous studies (17). Site specific mutations could also be used to pinpoint the importance of specific phosphorylation sites. RhoA could also be upregulated to determine if increased expression initiates development of a more anchored phenotype with greater actin expression. Induction of morphological effects similar to what was observed on the varying gel stiffnesses would support our hypotheses and further implicate FAK, ILK,
and the Rho GTPases in the changes we observed. Hepatocyte culture experiments and imaging specifically designed to track and map cell motility and migration throughout time points past 7 days would also more definitively validate our theories.

In addition to analyzing the expression and localization of FAK, ILK, and the Rho GTPases, additional targets related to mechanotransduction could be included. Src tyrosine kinase binds to and phosphorylates various FAK sites. Several adaptor proteins including PI3K, CAS, GRB7, and N-WASP associate with FAK and act as intermediates between FAK and the Rho GTPases. Similar to FAK, ILK also stimulates the PI3K pathway. Unique adaptor proteins of ILK include PINCH1 and ELMO2 which aid in cellular polarization. Other focal adhesion components important to mechanosensing, integrin attachment, and actin modification include talin, vinculin, and paxillin. Assays testing expression and localization of all these proteins would provide a clearer understanding of the interactions between ECM gel stiffness, substrate sensing, and the regulation of the hepatocyte cytoskeleton and focal adhesion formation.

ECM composition and substrate stiffness play important roles in hepatocyte phenotype; however, growth factor presence also represents a factor in determining the fates of cells. Thesis studies have shown the ability of ECM gel substrates to bind active growth factor for cellular use (Chapter 3- Figure 2). Future assays like phospho-labeling will be required to pinpoint exact binding and interaction sites in the gels. Nevertheless, the presented studies demonstrate the ability to manipulate the growth factor concentration in the liver cell substrates. With these ECM supplemented gels, future assays could be designed to measure how stiffness intensifies or reduces growth factor effects on liver cells.
Preliminary studies exhibited the ability of HGF to bind to our liver hydrogels and improve cell junction formation and hepatocyte function on low stiffness gels (Chapter 3-Supplementary Figure 4). A previous publication showed similar results with HGF and EGF-treated hepatocytes seeded on Matrigel of varying stiffnesses. Growth factor co-stimulation improved aggregation and function on the lower stiffness, while the converse occurred at the higher stiffness level (18). Additional experiments on HGF and EGF stimulated epithelial cells implicated stiffness in cell polarization and migratory responses (19-22). Other research has also shown the direct effects of substrate stiffness on transforming growth factor beta (TGF-β)-induced dedifferentiation of hepatocytes and possible EMT (23). Studies could be conducted to determine if these same changes occur on our liver ECM gels. Further data could define the exact stiffness ranges that induce these responses and the molecular mechanisms behind these behaviors.

Opportunities exist to improve both the complexity and overall function of the hepatocyte cell culture system we have developed. Other cell types could be individually seeded on the ECM supplemented substrates to test effects of stiffness on different cells of the liver, or co-culture of multiple cell types could determine how they interact and function in varied mechanical environments. Hepatic stellate cells (HSCs) are one of most important liver cell types involved in ECM production and maintenance. HSCs exist in either a quiescent or myofibroblast form. In vivo these cells are vital to both hepatocyte and stem cell mediated regeneration and repair. They secrete and assemble ECM molecules including fibronectin, collagens, and GAGs (24, 25). During a fibrotic disease state, hepatic stellate cells deposit excess amounts of collagen, a process worsened by myofibroblastic activation and proliferation on stiffening ECM. Several fields from
cancer biology to regenerative medicine also explored their ability to secrete growth factors. Assays revealed HSCs produce HGF, EGF, TGFα, and multiple insulin-like growth factor binding proteins within the liver (26-28).

Previous studies have shown the abilities of stiff substrates to stimulate myofibroblastic differentiation of HSCs (29, 30). Less is known about the function of quiescent cells in the normal liver state and the exact stiffness range where myofibroblastic activation occurs. Results on the liver ECM gels determined that hepatic stellate cell production of GAGs and HGF were time and stiffness dependent. HGF production was highest in the lowest gel stiffness, while ECM production was greatest on the highest gel stiffness (Chapter 3- Supplementary Figure 5). More detailed studies would reveal if these cells could substantially modify the composition or physical properties of the substrate microenvironment. Co-culture with primary hepatocytes should improve long term viability of the cells and provide insight into the relationships between cell types and certain substrate properties. In addition, modeling a fibrotic stiffness and determining methods of controlling excess ECM production and maintenance of hepatocyte function could help in the understanding and treatment of liver diseases like cirrhosis.

Besides increasing diversity of cell types grown on the substrate, increasing the three dimensionality of this system could improve capabilities to model disease states and enable advances in tissue engineering. Hepatocytes are arranged in epithelial sheets in the liver. 2D systems replicate individual cell layers, but not the complex interactions of the entire tissue. New 3D bioprinting technology could provide a method of creating these 3D structures. This tool enables careful layering of cells and gel substrate. Using
knowledge gained from this thesis, a bioprinter could alternate printing cell sheets and ECM gel layers at a set stiffness level to produce a tissue-like system. In this way, *in vitro* modeling could better mimic an *in vivo* environment, while still providing an adjustable platform to study specific ECM properties and mechanisms.

Despite the advantages of a fully 3D gel system, new challenges and complications are created that are non-existent in 2D culture. Gel swelling, pore size, and the flow of cell nutrients through the substrate all become important. Crosslinking affects stiffness on a gel layer, but could have confounding effects in 3D. Substrate fabrication and multiple gel properties would need to be monitored, tested, and studied to prevent the introduction of unwanted variables. Although several areas still need to be addressed, the abilities and flexibility of ECM gel substrates provide a promising future.

Overall, liver ECM gels improve long term cell function and allow isolation and testing of single substrate-related properties. This thesis work elucidated specific mechanisms of mechanotransduction and cytoskeletal regulation and determined stiffness ranges where hepatocyte function was optimized *in vitro*. Future advances and development of this substrate system could further clarify molecular interactions and produce more accurate disease models. Liver ECM gels could also be utilized in cell encapsulation technologies or cell therapies to repair liver injuries or disorders. The thesis established methods to integrate a natural liver ECM microenvironment in gel substrates at a physiological stiffness range. This new knowledge and innovation has facilitated numerous possibilities to pursue and can ultimately lead to future advances and practical therapies or treatments.
Works Cited


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Education

Wake Forest University Graduate School of Arts and Sciences
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Research Experience

Wake Forest Institute of Regenerative Medicine - PhD Thesis Lab
Research Advisor: Dr. Thomas Shupe (2010-Present)
- Researched the recellularization of decellularized kidneys and livers for transplant
- Involved in protein analysis of the ECM and cell culture on ECM gels
- Analyzed influences of ECM bound growth factors on primary hepatocyte culture
- Analyzed effects of altered substrate stiffness on cell culture
- Experience in submitting grants, poster presentations, and seminar lecturing
- Author on publications

Wake Forest University- Molecular Medicine and Translational Science - 1st Year Rotations
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- Translational research performed on topics including diabetes, molecular pathways of osteoarthritis
- Experience shadowing clinicians related to fields of research
- Abstract for American Society of Nephrology

Virginia Tech - Plant Pathology Lab, Honors Undergraduate Research
Research Advisor: Dr. John McDowell (2007 – 2009)
- Assisted graduate student and professor with research on plant disease in Arabidopsis
- Focused on GUS and trypan blue staining of plants to see when auxin responses and cell death are triggered after infection of *Hyaloperonospora parasitica*
- Pictures of microscope slides used for publication
Rutgers University - Research Experience for Undergraduates Program, Camden, NJ
Research Advisor: Dr. Heike Bücking (Summer 2008)
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- Explored uptake of nutrients by mycorrhizal fungi under sterile conditions using radioactive isotopes
- Lab skills and techniques were also presented and taught through several seminars

Laboratory and Computer Skills
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Graduate School Positions
Student Representative of the Molecular Medicine and Translational Sciences Executive Committee
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Honors and Awards
Outstanding Poster at North Carolina Tissue Engineering and Regenerative Medicine Annual Conference and Innovation Summit, 2013
Member of Phi Sigma Biology Honors Society
Graduated summa cum laude with degree in biological sciences
Honors Scholar at Virginia Tech
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Member of the University Honors Program at Virginia Tech (Freshman through Senior years)
Virginia Tech Dean’s List (every semester as undergraduate)
Member of the Biological Life Sciences Community at Virginia Tech
- Freshman year program where Biology majors lived together in student housing on campus, interacted socially and academically, participated in seminars, and completed a community service project
- Service project included Stroubles Creek watershed clean-up
Publications:

Manuscripts:


Book Chapter:


Presentations:


Deegan D., "Effects of SOD1 and MKK7 Expression on IGF Induced AKT and MAPK Signaling." Molecular Medicine Seminar. Wake Forest University, Winston-Salem. 7 May 2010.


Lectures:

Posters:
Deegan D., Zimmerman C., Shupe T., Effects of Liver Specific Extracellular Matrix Hyaluronic Acid Gels of Varying Stiffnesses on Primary Hepatocyte Function. Wake Forest Institute for Regenerative Medicine, 2015

Deegan D., Zimmerman C., Shupe T., Effects of Increased Hydrogel Crosslinking and ECM Bound Growth Factors on Primary Hepatocyte Culture. Wake Forest Institute for Regenerative Medicine Retreat, 2013

