Insights from a 3D Culture Model : Exploring the Synergistic Impact of Tissue Stiffness and Matrix Composition on Tumor Progression

by

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and that any and all revisions required by the final
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This thesis is dedicated to my beloved father

ABSTRACT

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Extracellular Matrix (ECM) is a highly versatile network that regulates essential cellular processes. The matrix contains a wide array of proteins and carbohydrates, the composition and organization of which determines the mechanical and chemical signature of the ECM. The architecture of the ECM is tightly modulated in physiological events, whereas the chemical and mechanical properties are altered aberrantly during malignancy. The elevated stiffening of lung tissues due to excessive production of the matrix components, increased crosslinking, as well as the deregulation of enzymes involved in matrix remodeling and degradation accompany tumor progression. The aberrant increase in stiffness of tumor tissues is received through mechano sensitive receptors whose activation is required for the transmission of mechanical inputs into biochemical signals. As tumor tissues stiffen, elevated activation of surface receptors leads to epithelial to mesenchymal transition of cancer cells as well as promotes their stem cell like characteristics.

Recapitulating the biochemical and biomechanical properties of the tumor microenvironment is of utmost importance in modelling cancer progression and enhancing the efficacy of therapeutic regimes. Hence, generating an *in vitro* model which enables alteration of tissue stiffening independently from ligand composition and concentration is of great value for examining the sole impact of stiffening on tumor progression. In addition, mimicking *in vivo* characteristics of tumor cells with respect to organ-specific cues in a 3D model where tissue stiffness is decoupled from other parameters enables comprehensive disease modeling.

The impact of elevating tissue stiffness on modulating the malignancy of cancer cells in different ECM compositions has not been studied in an *in vitro* 3D culture model. In this study, a fully defined double network of alginate and two different matrices, decellularized healthy bovine lung ECM and tumorigenic basement membrane, is generated. Utilizing this model, it has been revealed that EMT activation and stemness abilities of cancer cells are promoted through synergic interactions between the stiffness of the microenvironment and tumorigenic composition of the matrix. In addition, the absence of PI3K gene, a well-known mediator of mechanotransduction process, is compensated through elevated stiffness of the tumor microenvironment.

ÖZETÇE

3 Boyutlu Kültür Modelinden İçgörüler: Doku Sertliği ve Matris Bileşiminin Tümör İlerlemesi Üzerindeki Sinerjistik Etkisini Keşfetmek

Deniz ÖRNEK

Hücresel ve Moleküler Tıp, Yüksek Lisans

Temmuz 11, 2023

Hücre Dışı Matris (HDM), temel hücresel süreçleri düzenleyen çok yönlü bir ağdır. Matris, bileşimi ve organizasyonu HDM'nin mekanik ve kimyasal imzasını belirleyen geniş bir protein ve karbonhidrat dizisi içerir. HDM'nin yapısı, fizyolojik olaylarda sıkı bir şekilde modüle edilirken, kimyasal ve mekanik özellikler, malignite sırasında anormal bir şekilde değişir. Çeşitli matris bileşenlerinin aşırı üretimi, çapraz bağlanması ve bununla beraber matrisin yeniden şekillenmesinde yer alan enzimlerin düzensizliği ile meydana gelen akciğer doku sertleşmesi tümör ilerleyişine eşlik etmektedir. Tümör dokularının sertliğinin anormal artışı, mekanik girdilerin biyokimyasal sinyallere dönüştürülmesi için aktivasyonu gerekli olan mekanik olarak duyarlı reseptörler aracılığıyla algılanır. Tümör dokuları sertleştikçe, yüzey reseptörlerinin artan aktivasyonu, kanser hücrelerinin epitelden mezenkimal geçişine yol açar ve kök hücre benzeri özelliklerini destekler.

Tümör mikro ortamının biyokimyasal ve biyomekanik özelliklerini temsil etmek, kanser ilerlemesinin modellenmesinde ve terapatik rejimlerin etkinliğinin arttırılmasında son derece önemlidir. Bu nedenle, doku sertleşmesinin ligant bileşimi ve konsantrasyonundan bağımsız olarak değiştirilmesini sağlayan bir *in vitro* model oluşturmak, sertleşmenin tümör ilerlemesi üzerindeki özgün etkisini incelemek için büyük değer taşımaktadır. Ek olarak, dokuların sertleşmesinin diğer parametrelerden ayrıştırıldığı bir 3D modelde organa özgü doku bileşenlerine göre tümör hücrelerinin *in vivo* özelliklerini taklit etmek, kapsamlı hastalık modellemesine olanak tanır.

Artan doku sertleşmesinin, farklı doku bileşimleriyle etkileşimde bulunan akciğer kanser hücrelerinin malignitesini modüle etme üzerindeki etkisi, bir in vitro 3D kültür modelinde incelenmemiştir. Bu çalışmada, aljinat ve iki farklı hücre dışı matris, hücresizleştirilmiş akciğer ve tümörijenik bazal membran, kullanılarak cifte ağ yapıda hidrojel modelleri oluşturulmuştur. Bu modeller kullanılarak, kanser hücrelerinin EMT aktivasyon ve kök hücresel yeteneklerinin, mikro ortamın sertliği ile matrisin tümörijenik bileşimi arasındaki sinerjik etkileşimler yoluyla desteklendiği ortaya çıkarılmıştır. Ek olarak, mekanotransdüksiyon sürecinin iyi bilinen bir regülatorü olan PI3K geninin yokluğunun, tümör mikro ortamının sertliğinin artmasıyla telafi edilebildiği gösterilmiştir.

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ABBREVIATIONS

ECM	Extracellular Matrix
TME	Tumor Microenvironment
GAG	Glycosaminoglycan
sGAG	Sulfated Glycosaminoglycan
HS	Heparan Sulfate
HA	Hyaluronan
CS	Chondroitin Sulfate
DS	Dermatan Sulfate
PG	Proteoglycan
HSPG	Heparan Sulfate Proteoglycan
DSPG	Dermatan Sulfate Proteoglycan
CSPG	Chondroitin Sulfate Proteoglycan
FN	Fibronectin
GF	Growth Factor
RTK	Receptor Tyrosine Kinase
FGF2	Fibroblast Growth Factor 2
FGFR2	Fibroblast Growth Factor Receptor 2
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Receptor
GPCR	G protein Coupled Receptor
GPI	Glycosylphosphatidylinositol
SULF2	Extracellular Sulfatase 2
MMP	Matrix Metalloproteinase
FA	Focal Adhesion
FAK	Focal Adhesion Kinase
DDR1	Discoidin Domain Receptor 1
ERK1/2	Extracellular Signal Regulated Kinase
MAPK	Mitogen Activated Kinase
RhoA	Ras Homolog Family Member A

PTM	Post Translational Modification
EMT	Epithelial to Mesenchymal Transition
CSC	Cancer Stem Cell
LOX	Lipoxygenase
EHS	Engelbreth Holm Swarm

Chapter 1: **INTRODUCTION**

1.1 The Extracellular Matrix

The extracellular matrix (ECM) is an essential element of every tissue in the body. It is a dynamic entity in which composition and mechanical properties are constantly modulated with organ specific cues to maintain tissue homeostasis [1]. The matrix is made up of a plethora of proteins and polysaccharides. The major elements that form the ECM are proteoglycans (PGs), glycosaminoglycans (GAGs), collagens, elastin and other glycoproteins including fibronectin (FN) (*Figure1.1*) [2]. Post-translational modifications of these molecules are of great importance to produce a range of diverse proteins in different conformations [3]. Indeed, the presence of such a wide array of components and its mechanical features make the matrix highly dynamic and functional while providing the structural support to resident cells and maintaining the architecture [2, 4]. Thus, the matrix can be regarded as a hub of signaling events with a crucial role in regulating intracellular signaling pathways that stem in growth, differentiation, proliferation as well as determining stem cell fate [2, 5-9]. Although ECM has a profound impact on modulating cellular events, there is an interdependent relation between cells and matrix where the chemical composition and the physical properties of the matrix are dictated by the cells within [1].

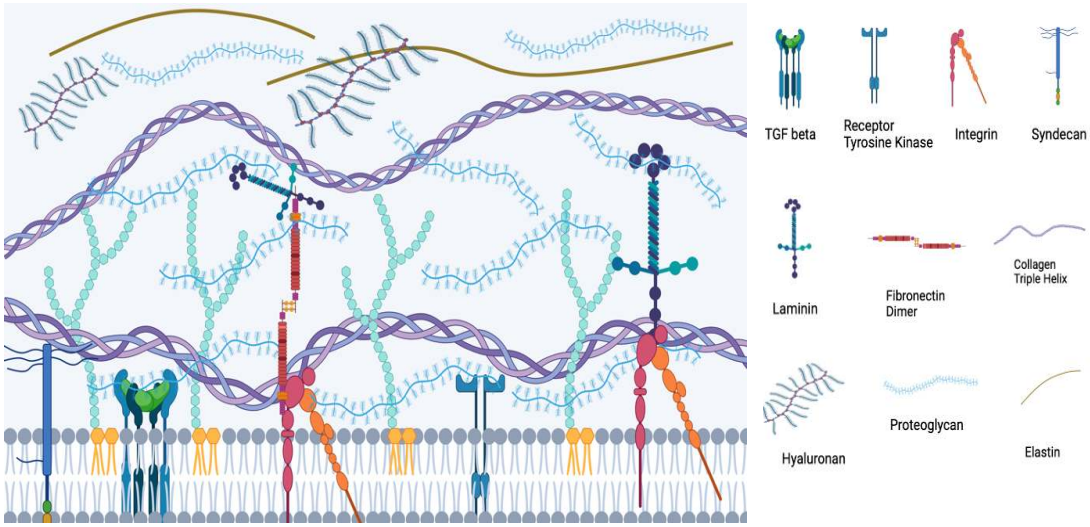


Figure 1.1 A scheme of main extracellular matrix (ECM) components and their interaction with plasma membrane receptors (Created with BioRender.com)

In healthy tissues, the synthesis, post-translational modifications and spatial organization of matrix components are tightly regulated. Hence, remodeling and degradation of these molecules are balanced with the production rate by numerous enzymes to maintain homeostasis. However, in pathological conditions such as during cancer, this balance is disrupted, thereby the chemical and physical properties of the matrix are aberrantly altered resulting in alterations in cellular events that can promote malignant progression[1].

1.1.1 Glycosaminoglycans (GAGs)

Glycosaminoglycans (GAG) are made up from recurring disaccharide units of amino sugars (N-acetylglucosamine or N-acetylgalactosamine) and uronic acid (glucuronic or iduronic) which are linked with either, α/β (1,3) or α/β (1,4) bonds [10, 11]. After being synthesized, these molecules are subjected to a range of post-translational modifications (PTMs) [12]. The presence of carboxyl units as well as the attachment of sulfate moieties by PTMs leads GAGs to become anionic structures [11, 12]. Although, many GAG molecules contain sulfate moieties (sGAG), hyaluronan (HA) is the only one that lacks sulfate groups [11]. These structures attract numerous cationic ions which creates an osmotic pressure that allows tissue matrices to retain water. This phenomenon increases the compressive strength of tissues [11].

GAGs are integral components of the ECM as they have numerous roles in regulating signaling events [11, 13, 14]. The binding of growth factors (GFs), cytokines, and chemokines to receptor tyrosine kinases (RTKs) are orchestrated by GAGs [11, 13, 14]. Among various PTMs, the level and distribution of sulfate groups have a profound impact on determining the specificity of these interactions [11, 13-15]. In addition, they facilitate the formation of ternary complexes with ligands and their receptors, hence decrease the ligand concentration for receptors to be activated [11, 13, 14].Furthemore, they can bind to other structural elements of ECM, thereby they are crucial for organizing matrix and maintaining its architecture [11, 13, 14].

1.1.2. Proteoglycans (PGs)

Sulfated GAG (sGAG) molecules are covalently attached to a protein core, giving rise to the formation of more complex structures named as proteoglycans (PGs). They are of the utmost importance in regulating both physiological and pathological cellular events [16]. These molecules have highly versatile functions since even the slightest difference in the type of sugar, the degree of sulfation and its pattern can result in major variations in their function [16]. They are very fundamental for facilitating the binding of cell surface receptors to their substrates, including GFs, cytokines, and chemokines [13, 16, 17]. They fine tune the activity of these molecules through preserving them from catabolic activities of enzymes and keeping them in a confined region to facilitate diffusion in the presence of appropriate signals. Hence, they augment the activity of bioactive molecules and decrease the threshold of receptors to be induced by acting as a physical bridge between molecules and receptors [13, 16, 17].

They are essential for establishing the architecture of the matrix as they have numerous sites where other structural units bind to [14, 16]. For example, they can covalently be attached to different GAG chains that are floating within the matrix, other PGs, and collagen molecules to facilitate the assembly of a more complex meshwork. In addition, the activation of mechano sensitive pathways upon facing a force is resulted from the ability of PGs to alter their conformation and reorganize the components of the matrix to transmit these inputs inside the cells [14]. In line with this, they are established as key molecules to mediate essential cellular events which includes growth, differentiation, adhesion, migration as well as proliferation [13].

PGs can be categorized into various subgroups based on which sGAGs are attached and where PGs can be found. PGs can either free roam in extracellular space, bind to other structural elements of the matrix and receptors, attach to the membrane or float inside the cells [16]. As an example, syndecan is one of the PGs whose core protein pass the membrane. These molecules attach to numerous structural elements, such as fibronectin, to facilitate direct binding to their receptors including RTKs and integrin [18]. Thereby, they take role in modulating the activity of receptors and downstream signaling cascade activation through reorganizing the cytoskeletal network of cells. Additionally, syndecan molecules can also interact with different GF, including Fibroblast Growth Factor 2 (FGF2), and facilitate the activation of its receptor, Fibroblast Growth Factor

Receptor 2 (FGFR2), to by establishing a complex ternary structure [19]. Yet another one of these molecules is also important in modulating Wnt pathway by activating ß-catenin signaling through activating G-Protein Coupled Receptor (GPCR) by concentrating the signal in a confined region [20]. Glypican 1-6 is an example whose core is attached to cell surface through a glycosylphosphatidylinositol (GPI) anchor that is required for the proper regulation of Wnt pathway by orchestrating the SULF2 enzyme [21, 22].

PGs that have heparan sulfate (HSs) GAG chains, known as HSPGs, can be found in various locations [16]. Syndecan and glypicans are examples of HSPGs which are located in the cell membrane. Perlecan and Agrin, however, are types of HSPGs found in basement membrane. They can attach to numerous molecules to activate their receptors, mediate communication among cells and provide linkage sites [16]. HSPGs are required for maintaining the activation of numerous RTKs. The activation of FGFR is directly mediated by the binding of FGF which is facilitated by the assembly of heparin with these molecules [13, 23]. Glypican 1 is the essential element on the cell membrane that interacts with vascular endothelial growth factor (VEGF) to ensure its efficient attachment to its corresponding RTK, vascular endothelial growth factor receptor (VEGFR) [24].

Syndecan 4 is a crucial mediator of the organization of the focal adhesion complexes through binding integrins, thereby it promotes the adhesion of cells [18]. In addition, Syndecan 1 is required for facilitating the attachment of integrins to collagen molecules, resulting in the reorganization of actin cytoskeleton in cells [25]. Therefore, these molecules are vital for the structural integrity of ECM through assisting the attachment of collagen, laminin, and fibronectin to cells as well as other matrix molecules [18]. Furthermore, these molecules merge with numerous enzymes, including metalloproteinases (MMPs) that alter their function by cleaving and scattering bioactive molecules which might induce the activation of receptors [1].

Other type of PGs are chondroitin sulfate (CS) proteoglycans (CSPGs) and dermatan sulfate (DS) proteoglycans (DSPG) where CS and DS groups are attached to their core protein respectively. Briefly, these molecules are important in terms of supporting the healthy development of neuronal networks. In addition, they are crucial for maintaining tissue architecture by facilitating the binding of one or more collagen fibers[16].

1.1.3. Collagen

Collagens, a diverse group of fibrous proteins, are one of the most important and abundant structural elements of the matrix [26]. These molecules are highly organized to give strength and support to cells to bear tensile forces. The fundamental unit that makes a collagen fiber is alpha helix polypeptide which is composed of glycine, hydroxyproline and proline amino acids. Thereafter, 3 alpha helix polypeptides are tangled to one another to produce pro-collagen molecule. Once synthesized, it is subjected to a PTM which results in N and C terminus of the polypeptide being cleaved. Next, they are crosslinked to form collagen fibers [26].

The fact that collagen molecules exhibit numerous binding sites for cells and other structural elements of the matrix makes them an integral component of matrix organization. These molecules can interact with numerous building blocks, including FN, laminin, and HA chains [27]. They are one of the fundamental elements of the cellular mechanotransduction process due to their reorganizing of the matrix components and binding to fibronectin molecules to activate integrins [27]. The activated receptors, then modulate the cytoskeletal network of the cells through binding with kindlin and talin, which in turn reorganize actin cytoskeleton [28]. Indeed, the organization of focal adhesions (FAs) is altered in a way that it results in integrin activation leading to the phosphorylation of focal adhesion kinases (FAK) and downstream signaling cascades including PI3K-AKT, ERK1/2, RHO-ROCK [29-32]. These cascades play a crucial role in inducing the growth, adhesion, differentiation, migration as well as polarity of cells [28, 29]. In addition, the activation of discoidin domain receptor 1 (DDR1) is accomplished through the physical binding of collagen molecules, an aspect which is unique to this particular RTK [33, 34].

There are various types of collagen molecules all of which are key for determining the organization of the matrix. Type I is responsible for producing collagen fibers, however, type IX and XII are the ones which decorate these assemblies to produce a complex meshwork. Type IV is required for producing the basal lamina. In addition, type VII plays an important role in anchoring numerous molecules to these fibers [26].

1.1.4. Elastin

Tissues in the body are not rigid, they are subjected to numerous forces that might result in deformations in their structure, however, due to the presence of elastic fibers within their matrix, they can regain their shape when the deforming stress is eliminated [35]. This property is known as elasticity, and it allows cells to act counteractive against stretching forces [35, 36]. This property results from the fact that the structure of this polypeptide chain is composed of random coils, instead of a regular pattern. Moreover, the hydrophobic nature of these coils also has profound impact on demonstrating an elastic behavior [35, 36]. The polypeptide chain responsible for producing elastic fibers are mainly composed of proline and glycine. The elastic behavior of this polypeptide results from its hydrophobic nature. This fiber is also capable of forming a meshwork composed of numerous proteins which are crosslinked to each other through covalent binding to lysine groups [35, 36].

1.1.5 Other glycoproteins

ECM also contains a plethora of various glycoproteins whose role is to fine tune the organization of matrix components. These proteins play an important role in providing attachment motifs to cells and other molecules, thereby contribute to the formation of a complex meshwork. They are also important in regulating signaling mechanisms through facilitating physical interactions between receptors and their substrates. Many studies have established that the presence of RGD (Arg-Gly-Asp) motif is essential for proteins to facilitate the binding of integrin, collagen, and heparin molecules [27, 28, 37]. FN is the most studied glycoprotein, however, other proteins can also contain this domain. FN has 3 repeats all of which contain this domain [27, 28, 37]. Thereby facilitating the binding of integrin which results in the transduction of mechanical forces into chemical cues through reorganization of cytoskeletal elements. Upon a force stimulus, fibronectin molecules can attach to each other as well as integrins through this domain. This phenomenon is important in regulating mechano-sensitive pathways which trigger the growth and differentiation of cells as well as their migration [27, 28, 37].

1.1.6 Basal Lamina and Laminin

Laminins are fibrous proteins which can be found in high abundancy in basal lamina. These molecules have a unique hook-like structure in which 3 different polypeptide chains, α, β and γ, are wrapped around each other [38-41]. This structure enables the binding of collagens, FNs, HSPGs as well as integrins [40, 41]. As these molecules are crucial for forming a complex web of collagens and fibronectin molecules with other PGs, it plays a vigorous role in transducing the mechanical force applied to cells. They are important in facilitating the attachment of these components to integrins for further activating the FAK and reorganizing actin network. Thereby, the role of laminin on governing the fundamental cellular events is key for maintaining the homeostasis [40, 41].

Basal lamina is made up from laminin and collagen type IV molecules which is located at the basement of epithelial cells. This matrix is mostly synthesized by epithelia and stromal cells. As it is mostly composed of laminin molecules, it displays a thin and flat morphology. This specialized form of matrix is of utmost importance in supporting cells and dividing them from their surroundings. So that, this entity is also crucial for maintaining the cell polarity, growth, as well as differentiation [38, 42].

1.1.7 Degradation of ECM components and maintaining homeostasis

The components of the ECM are constantly being modulated by the resident cells. Cells produce these molecules and arrange their spatial organization within the matrix. However, this is a dynamic process where many of them can be altered or degraded in response to specific cues. This homeostasis should tightly be regulated as any aberrant alterations can result in the activation of signaling pathways critical for determining the behavior of cells [1]. This event is controlled by numerous enzymes including MMPs and proteases [43-45]. The mechanism of which these enzymes can degrade their substrate, including collagen, laminin, and fibronectin, differs among each other. Ca^{+2} or Zn^{+2} ions are utilized for the activation of MMPs, whereas proteases have active serine residues. As an example, in healthy cells, MMPs can degrade their substrate to release bioactive molecules that are bound to matrix components. The release of these molecules, such as GFs, activate the downstream pathways. When these enzymes are dysregulated, the rate

of degradation and the release of signaling molecules are increased, which might promote the activation of pathways responsible for controlling cellular fate [43, 45, 46].

1.2 Tumor Microenvironment

Tumor Microenvironment (TME) is of utmost importance in modulating pathological transformation. The profound impact of TME on regulating tumor initiation and further progressive events has been well established for a long time [47]. Examining the components of the TME as well as the dynamic regulation of matrix with respect to intracellular cues and forces are of great value for understanding the signaling mechanisms of cancer and therapeutic responses [1, 47].

TME is a dynamic unit composed of a plethora of cell types and surrounding matrix. The composition and physical properties of the TME is constantly altered in a way that induces or prevents tumor growth and further disease progression [1, 48]. The production rate of the ECM components as well as their removal or remodeling are tightly orchestrated in healthy tissues, whereas it is disrupted during pathological transformation of healthy cells [1]. The hallmarks of the tumorigenic environment are elevated deposition of several matrix components, changed expression of enzymes responsible for both their biosynthesis and degradation, increase in post-translational modifiers of matrix molecules, altered spatial organization of these components, augmented matrix crosslinking, stiffening and increased ECM viscoelasticity [1, 49]. Thereby, signaling mechanisms involved in numerous events are affected significantly.

Pioneering studies have established that altered expression levels of enzymes responsible for biosynthesis and post-translational modification of PG molecules during malignancy accompanies tumor progression [11, 12, 15, 16]. Numerous research has established that biosynthesis of HA is increased during malignancy which stimulates the growth of tumorigenic cells through boosting angiogenesis [50]. In addition, aberrant deposition of syndecan molecules is shown to be a key indicator of cancerous growth as they promote acquiring mesenchymal and invasive phenotypes [11, 51]. The level of sulfation and the change of related patterns are one of the hallmarks of malignant growth observed in various tissues including lung [52] breast [53] and prostate [54]. Most of the cases, the elevated expression of these molecules and sulfation degree resulting in an over-induction of RTKs. For example, the expression level of an enzyme responsible for

degrading heparin molecules known as human sulfase 1 (HSulf 1) is downregulated in various cancers triggering the FGF2-HSPG signaling which results in the induction of ERK cascades responsible for promoting the growth of tumorigenic cells [55, 56]. In addition, numerous studies have rendered that the regulation of heparanase enzyme is altered during malignant transformation of cells in a way that its over-expression results in the removal of HS from their core protein. This phenomenon leading to alterations in matrix composition through releasing an active component which further triggers tumorigenic proliferation and metastasis [57]. As it has been appreciated that GAG chains mediate the communication among GFs and their receptors, the altered expression of GAGs has a profound impact on regulating processes such as epithelial-mesenchymal transition (EMT). For instance, the elevated expression of HA leading to the activation of CD44 receptor plays a crucial role in modulating EMT in cancer cells, and sustaining their ability for self-renewal [58].

EMT process involves obtaining of a mesenchymal phenotype and disinheritance of epithelial characteristics in cells. In general, the expression of major epithelial markers such as E-cadherin are downregulated, whereas the expression of mesenchymal markers such as N-cadherin, vimentin and fibronectin are upregulated during this transition. Additionally, transcription factors including SNAIL and ZEB are a well-known mediators of this process [59]. This phenomenon is of utmost importance in facilitating the migration of cancer cells and supports metastatic spreading to secondary sites [47, 60, 61]. However, cancer cells are highly heterogenous which display distinct morphologies within the same population. Indeed, not all cancer cells are obligated to disinherit their epithelial characteristic to become invasive and gain ability to migrate. There are numerous examples of which cells preserve their epithelial characteristics by maintaining E-cadherin expression yet display metastatic behavior. Therefore, correlation of EMT, in regards to cadherin switch from E-type to N-type, and invasiveness is rather a generalized approach [62].

It has been discussed that the integrity of the basal lamina is disrupted to facilitate cancer cell migration. Thereby, altered expression of matrix degradation enzymes , such as MMPs and proteases, is tightly correlated with the EMT process. Additionally, the reorganization of the newly deposited tumor matrix is one of the crucial factors that drives tumor cell growth [63]. Indeed, elevated expression of MMPs and proteases which degrade Type 1 collagen that is found in the basement membrane, promotes the invasion

of tumorigenic cells and facilitate their metastasis through modulating macrophage activity [45]. As an example, the overproduction of collagen 1 molecules results in the formation of stiffer regions that govern the cancerous growth of pancreatic cells [64]. Moreover, the induction of TGFβ signaling promotes the crosslinking of collagen molecules which elevates the stiffness of the TME and supports the spreading of cancerous cells [65]. Furthermore, stiffened matrix is also responsible for elevating the activity of TWIST1 transcription factor which partake in EMT and its downstream mechano-sensitive signaling cascades, thereby supports their invasion [66]. As the orientation of the excessively produced collagen molecules becomes perpendicular to the boundaries of the tumorigenic cells during malignancy, invasion and further disease progression is supported [67]. In addition to collagen, excessive production and remodeling of FN results in the stimulation of mesenchymal phenotype which promotes the mobility of cancerous cells [68, 69]. Indeed, overproduction of FN and its altered spatial organization induces the activation of STAT3 signaling cascades which triggers the EMT process to promote migration of cancer cells [70].

Cancer cells with ability of self-renewal, are often stated as cancer stem cells (CSC) [71, 72]. Numerous research has established that the stemness ability of cancer cells usually accompanies tumor growth [73, 74]. The most evident indicator to mark CSCs are overexpression of CD44 and CD133 genes. They are shown to be a key surface molecule which are deregulated during malignancy. As an example, CD44 is important for mediating the attachment of cells and regulating the crosstalk with their surroundings. However, it plays a crucial role in inducing the progression of prostate cancer through interacting with CD133 and integrins [75]. Pioneering research has revealed correlation of EMT with induction of CSC phenotype with intersecting signaling pathways [76]. For instance, both canonical and non-canonical WNT pathways display a vital role in activating transcriptional factors that partake in EMT process such as SNAIL, ZEB and TWIST1 as well as JUN and FOS [71]. During this process, the impact of altered reorganization of matrix components as well as deregulation of enzymes including MMPs are essential in acquiring CSC properties [71].

1.3 Mechanotransduction

Cells are in a constant interaction with their microenvironment where the matrix, adjacent cells and fluids apply various mechanical forces including shear stress, compressive forces, tension and stretching [77]. Young's modulus, a term which designates the force needed to cause a defined deformation on a material, is used as a measure for matrix stiffness [78]. Mechanical forces are responsible for the final shape and architecture of cells and tissues [78]. In addition, these inputs are received by mechanosensitive receptors which are essential for facilitating the attachment of cells to one another as well as to ECM and transduced into chemical signals [79-86]. Thereafter, cells generate responses to these inputs through reorganizing their cytoskeletal network and regulating gene expression. This phenomenon is known as mechanotransduction and is key in modulating fundamental cellular events including growth, attachment, differentiation, and proliferation. Although there are various receptors which are evolved to receive and integrate mechanical inputs, integrins and cadherins are highly versatile receptors that drive mechanotransduction [87].

Integrin receptors are integral element of focal adhesions for accomplishing numerous functions due to the diversity of their building blocks, α and β subunits [87]. Specific combinations of these units determine their interaction partner, implying each has evolved to attract different structures to maintain homeostasis. Indeed, $\alpha_6\beta_1$ is essential for the binding of laminin, whereas $\alpha_2\beta_1$ is utilized for the attachment to collagen molecules. When cells are surrounded in a soft matrix, integrin molecules are active though they are not found clustered together [87]. The attachment between actin and integrin molecules which is facilitated by talin is flabby. Once cells are subjected to force, however, integrin molecules are assembled. As a result, talin molecules undergo a conformational change which leads to the enrollment of other proteins to the focal adhesion sites to strengthen the binding of integrin to actin network [87]. Vinculin is one such adaptor protein that is recruited after the conformational change of talin. Thereafter, a plethora of downstream signaling mechanisms are activated and determine the fate of cells [45, 88].

The phosphorylation of the FAK complexes as a result of integrin clustering is essential for initiating downstream signaling pathways which include mitogen activated protein kinase (MAPK) known as extracellular signal regulated kinase (ERK) [88]. In

addition, the activity of Rho family GTPases including RhoA is also induced by the phosphorylation of FAK, leading to the reorganization of actin cytoskeleton and changes in genomic regulation. Furthermore, YAP/TAZ are one of the most crucial TFs that translocate to nucleus when mechanical force is sensed through integrin molecules and activates FAK and its downstream pathways via RhoA [89].

As mentioned above, physiological stiffness of tissues and cells are specialized to maintain homeostasis. However, during pathological transformations, the biomechanical signature of the TME is altered which results in aberrant expression of mechanosensitive signaling cascades. It has been well-studied that the overproduction of matrix elements, elevated crosslinking and altered expression of matrix-degrading enzymes accompany elevated stiffening of TME [4, 45, 90, 91]. This leads to deregulation of signaling cascades responsible for integrating mechanical inputs, thereby, has a profound impact on modulating tumorigenic growth [92]. In addition, the aberrant reorganization of the ECM molecules, specifically collagen 1, that become perpendicular to the surface of the breast cancer cells promotes their malignant phenotype [45]. Elevated crosslinking of collagen molecules, on the other hand, enhances the stiffness of the ECM thus results in overactivation of PI3K pathway through elevated assembly of integrin which in turn promotes the spreading of premalignant epithelial cells [91]. In addition, pioneering research has revealed the dual importance of stiffness and the composition of TME on modulation of tumorigenic phenotype through aberrant activation of integrin and PI3K pathway [93]. As an example, elevated stiffening of the TME is received through integrins and cause reorganization of actin cytoskeleton which in turn promotes translocation of YAP/TAZ to nucleus and support proliferation and mobility of tumor cells [89]. Furthermore, the EMT process is activated when stiffness of the TME is increased as it causes the release of TWIST1 from its negative regulator [66]. Hence, the elevated stiffening is responsible for the loss of cellular polarity which in turn leads to uncontrolled proliferation of breast cancer cells [80].

Deregulation of matrix-degrading enzymes similarly accompanies the growth of numerous cancer types. It has been established that elevated expression of MMPs is a hallmark of numerous cancer types [43, 46]. The reason for that might arise from the ability of MMPs to destroy the basal lamina, disrupt its barrier function and stimulate the migration of tumor cells [92]. Furthermore, elevated levels of LOX, an enzyme required for crosslinking of collagens, is a well-known mediator of the aberrant stiffening of tumor

matrix, augmenting the invasive phenotype of tumor cells [91]. In line with these, numerous studies have proposed that increased fibrous collagens serve as tracks for the tumor cells during invasion [94]. Elevated stiffening of tumor matrix due to enhanced production and crosslinking of collagen molecules results in immune cells attraction into the TME [92]. This phenomenon further induces the stimulation of T cells [87, 95].

The elevated biomechanical properties of the TME has a profound impact on regulating self-renewal of cancer cells as well. Specially, TME stiffening modulates the stemness properties of cancer cells [96-98]. The increased expression of MMP enzymes including 2,3 and 9 from cancer associated fibroblasts (CAF) enhances the ability of cancer cells to invade and gain mesenchymal phenotype which in turn promotes the expression of stemness markers in cancer cells [99]. Increased expression of MMPs also results in the induction of WNT pathway and supports cells to gain stem cell like abilities [100]. Moreover, increased collagen density in the TME promotes self-renewal and stemness in lung adenocarcinoma cells [101].

1.4 Three Dimensional (3D) Tumor Models

To understand the impact of elevated stiffening of TME on regulating cancer cell behavior, previous research had been performed in two-dimensional (2D) cultures. Although these models have enlarged our knowledge of how the disease progressed and on the molecular mechanisms to target with therapies, they did not manage to fully recapitulate the hallmarks of the TME. The role of ECM in providing mechanical support, in regulating the signaling between bioactive molecules and their receptors by modulating cell-ECM interactions, and in spatiotemporal sequestration of signaling cues is entirely left out in 2D cultures. Therefore, they fail to represent the native tumor microenvironment where cells are in constant interaction with their complex surroundings.

The importance of TME on modulating cancerous growth has been well appreciated after Dr. Bissell and her group had highlighted the role of matrix on regulating the tumor initiation with their pioneering approach of adding the third dimension in cancer research [48]. Further studies supported such vital role of TME on promoting angiogenesis, metastasis, tumor immunity and drug resistance [6, 102-105]. These studies established the importance of having 3D culture model which recapitulates the

hallmarks of tumorigenesis and tumor progression with respect to extracellular cues. Engineering approaches have been then implemented in cancer research for generating tissue models that mimic both healthy and diseased states [106]. Thereby, these cancer models can be used for better disease modeling and testing of therapeutics. The first model that was established was spheroids [107]. Although this culture system was more realistic in a way that supported cell-cell interactions, the absence of a tumor matrix has led to development of more complex systems. Later, to provide ECM ligands to tumor cells, materials like collagen and reconstituted basement membrane (rBM) were used to mimic and model tumor tissues [108]. Matrigel and Cultrex, commercial rBM products, are derived from Engelbreth Holm Swarm (EHS) mouse tumors [109]. Their use in cancer models has demonstrated an increase in mesenchymal phenotype, tumorigenesis and tumor-associated angiogenesis [91, 110-114]. However, these materials are mechanically weak and they fail to represent the mechanical properties of native tumor tissues. Understanding the role of stiffness in tumor biology, requires 3D tissue models where tunability of tissue mechanics can be achieved independently from parameters such as ECM ligand density and porosity [115]. To generate a model where individual parameters can be independently tuned, numerous studies have focused on engineering biological (i.e. hyaluronic acid [116], alginate [117]) or synthetic materials (i.e. polyethylene glycol (PEG) [118], poly (lactide coglycolide) [119]). An engineered double-network of alginate and rBM with independent tunability of stiffness served as a unique model where it was shown that mechanics and composition both have distinct roles in tumorigenesis of breast epithelium [93]. Other models that have been generated further established the importance of ECM stiffening, and reorganization through utilizing RGD domains to enable cell attachment [120, 121].

1.5 Alginate

Alginate is a frequently used inert biopolymer in tissue engineering to biomimic several aspects of native tissues including mechanical properties [122, 123]. It is made up from G and M monomers and has the ability of ionic crosslinking in the presence of divalent cations such as Ca^{+2}. The stiffness of the alginate matrix can be controlled by altering the Ca^{+2} concentration which makes it a suitable material for producing hydrogels to study the impact of matrix stiffening without changing the chemical composition of

the environment [93, 122, 123]. One other important aspect of alginate is that it does not have any sites for cell recognition and attachment [93, 122, 123]. Therefore, cell instructive materials such as rBM can be used in combination where alginate modulates the microenvironment stiffness without affecting cellular responses [93, 122, 123].

1.6 Scope of the Study

The scope of this study is to examine the impact of tissue stiffening on modulation of lung tumor malignancy in different matrix compositions. To do so, double network hydrogels composed of alginate and two different matrices, namely decellularized healthy lung ECM and tumorigenic basement membrane, are generated to obtain a fully defined *in vitro* model. Physical properties of these matrices are tuned through altering the concentration of the crosslinker cation, Ca^{2+}. Utilizing these models, the impact of different ECM ligands during stiffening on lung tumor cell growth, phenotype as well as induction of cellular programs including EMT and stemness were investigated in fully defined and controllable microenvironments.

Chapter 2: **MATERIALS AND METHODS**

2.1 Cell Culture

Human lung adenocarcinoma cell line A549 (#CCL-185) was obtained from American Tissue Culture Collection (ATCC). A549 cells were growth in Dulbecco's Modified Eagle's Medium (DMEM) F12 augmented with 10% Fetal Bovine Serum (FBS) and 1% PenStrep (P/S). Human embryonic kidney (HEK293T) cell line was maintained in DMEM High Glucose augmented with 10% FBS and 1% PenStrep (P/S). These cell lines were cultured in 37 °C incubator with 5% CO_2. Cells are incubated until their confluency is reached to 80%. After that point, the medium was removed, and cells were washed with PBS, then cells were trypsizined and counted for re-seeding.

2.2 Plasmid Constructs

Short Hairpin Mediated RNA inhibitor (shRNA) encoding PI3KCA (NM_0062) was purchased from Vector Builder (plasmid IDs VB9000558929, VB9000619992CNJ and VB9000558918GSY). Second generation lentiviral systems were utilized for the generation of viral particles. For that purpose, psPAX2 packaging plasmid and PCMV-VSVG envelope plasmid are used.

2.3 Lentiviral Production

Lipofectamine 3000 Transfection Reagent (Invitrogen) was used for transfecting HEK293T cells. The ratio of transfection of Lentiviral plasmid DNA, psPAX2 packaging plasmid, PCMV-VSVG envelope plasmid is given respectively 4:3:1. 6 hours after transfection the medium was discarded, and fresh medium was added. 2 days after transfection viral supernatant was collected and filtered through 0.45 μm syringe filter. Then, the supernatant was centrifuged for 10 minutes at 13000 rpm at room temperature. The viral supernatant was relocated into a new Eppendorf tube and combined with 1:3 volume of PEG8000: Viral Supernatant. The solution was mixed by vortexing and incubated for 1 day at 4 ºC. The day after, it was centrifuged at highest speed for 1 hour

at 4 °C. The supernatant was discarded, and the pellet was resuspended with DMEM F12 (0% FBS, 0% PS). The volume of PBS was 1/5 of the first volume. Viral particles were stored at -80 °C in 100uL aliquots.

2.4 Stable Cell Line Generation

The day before transduction, A549 cells were seeded at 20% confluency to 6-well plate. The day of the transduction, medium was replaced with DMEM F12 (10% FBS only) and incubated for 4 hours. Then A549 cells were transduced with viral particles with 10 µg/mL of Protamine Sulfate (Sigma) at MOI of 5. After 1 day, the efficiency of the transduction was examined by monitoring the GFP fluorescence. The viral supernatant was remained until 80% of the total population displayed GFP fluorescence. Then they were treated with Puromycin (Sigma) antibiotic at the ratio of 1.5 µg/mL. The suggested selection rate of A549 cells are 5 days. However, in this experiment, the selection process was carried on until whole population of the control group which was not transduced with the viral particles yet treated with Puromycin was killed by antibiotic treatment. After that point, the cells were trypsizined and passaged to a 10 cm dish, or cryo-preserved.

2.5 Decellularization Process of Bovine Lung Tissue

Bovine lung tissues were decellularized according to the protocol which was published in our previous study [124]. To summarize, bovine lung tissues were chopped off into small pieces and washed with dH_2O thoroughly. Then, these tissues were freeze-thawed in liquid nitrogen and subjected to 10U/mL DNase (Sigma) treatment. Next, tissues were washed with dH_2O and lyophilized to obtain a powder form of decellularized lung ECM (dLung ECM). Then, powder form of dLung ECM is treated with 1 mg/mL pepsin solution (Sigma) for 48 hours at room temperature to obtain a digest with a final concentration of 15 mg/mL (w/v). Next, this digest is neutralized and lyophilized for one last time. After lyophilization, powder form of dLung ECM is stored at -80°C until use.

2.6 Characterization of the Mechanical Properties of Double Network Hydrogels Composed of Alginate and dLung/Cultrex

Kinexus Pro Rheometer (Malvern) was used for characterizing the mechanical properties of hydrogels. The lower plate of the instrument is cooled to 4ºC prior measurements, and 20 mm parallel plate is attached to the instrument. Upon gels are prepared, they are casted to lower plate and the gap is adjusted to 1 mm. Then, the lower plate was warmed to 37ºC, the storage and loss modulus at 1% strain and 1 Hz was recorded periodically for an hour. Mineral oil (Sigma) is spread to edges of the gel to avoid drying as the instrument takes measurements. All measurements were done at least in triplicate.

2.7 Generation of the Double Network of Alginate and Cultrex/dLung and Encapsulation of A549/ shPI3K A549 cells

- Preparation of solutions:

Alginate was dissolved in DMEM F12 and vortexed thoroughly for 60 seconds, then incubated at 4ºC overnight. The powder of lyophilized decellularized lung ECM was dissolved in DMEM F12 supplemented with 2% PSA, vortexed thoroughly and incubated at 4ºC overnight. 1.2 M final concentration of $CaSO_4$ main stock was prepared and sterilized through autoclaving and diluted to the working concentration (120mM and 240mM) in DMEM F12 .

- Generation of the gels:

Alginate was sterilized by using 0.2 µm syringe filter. Cells were trypsizined and counted, then they were diluted to 250K/mL final concentration. Cultrex and dLung were placed on ice. Alginate, cell suspension and Cultrex/dLung are placed in one 1 ml Luer lock syringe in given order. Solutions were mixed and syringe was kept on ice while $CaSO_4$ and DMEM F12 were placed into another Luer lock syringe. The syringes were coupled with a lock and mixed rapidly until the mix became homogenous, and gels were casted to 24-well plates. The final solution consisted of 10 mg/mL alginate, 4 mg/mL ECM, and 6 mM $CaSO_4$ for soft hydrogels or 15 mM $CaSO_4$ for stiff hydrogels. Hydrogels were incubated to solidify at 37ºC with 5% CO_2 for 45 minutes then DMEM

F12 containing 10%FBS, and 2%PSA was added. The medium of the gels was changed every 3 days and cultured up to 21 days.

2.8 RNA Isolation from Hydrogels and Reverse Transcription of RNA to cDNA

Hydrogels were collected and washed with wash buffer composed of 150 mM NaCl and 5 mM $CaCl_2$ for 5 minutes. Gels were snap-frozen in liquid nitrogen. RNA isolation was performed with TRIzol Reagent (Invitrogen). Each gel was homogenized in 50 µL TRIzol by using a pestle, and vortexed thoroughly. Gels are mixed until no gel fragments remained. Then the samples were centrifuged for 5 minutes at 12000 rpm at 4^{o}C. The supernatant was transferred to another Eppendorf tube and 200 µL of chloroform was added. This solution was incubated on ice for 10 min and centrifuged at 12.000 rpm for 15 min. The upper layer of the solution which contains RNA was transferred to another tube and an equal volume of 70% EtOH was added. After this stage, Nucleospin RNA II (MN) kit was followed according to the manufacturers protocol. RNA concentration was determined by NanoDrop Spectrophotometer (Thermo Scientific 2000c, USA). 1 µg RNA was reverse transcribed into cDNA. This synthesis was performed according to the protocol supplied with M-MLV Reverse Transcriptase Kit (Invitrogen).

2.9 Real Time-Quantitative Polymerase Chain Reaction (RT-qPCR)

The primers were designed with IDT "RealTime qPCR Assay Entry" Design tool. The sequence that was generated from the tool was cross checked with Primer3 tool. Primers were obtained from Sentebiolab. Primers that were used for this study are given below in *Table 2.1.*

Table 2.1 Primers used for RT-qPCR

	PRIMERS (5'→3')	
Name	**Forward**	**Reverse**
Human_ GAPDH	CTGACTTCAACAGCG ACACC	GTGGTCCAGGGGTCTTA CTC

Human_ PI3KCA	CCATATCTCCAAAGT AGAAC	TAGAATGACGACTAGCA GTA
Human_SOX2	CTTTTATGAGAGAGA TCCTG	ACCGTACCACTAGAACT TT
Human_OCT3/ 4	CACTAAGGAAGGAAT TGG	GTGTGTCTATCTACTGT GTCC
Human_KLF4	TCTGTGACTGGATCTT CTAT	CTTCCTCTTCTTCTAACA TC
Human_CD133	ACCTACAGCATATTCT TCAC	CTGTTAAACTGTACACC GTA
Human_CD44	CTCACTCAAGCTCTTT AACT	GAATATCTAGAAGGAGT GGA
Human_ ZEB1	ACCCTTGAAAGTGAT CCAGC	CATTCCATTTTCTGTCTT CCGC
Human_ ZEB2	ACTCCTGTCTGTCTCG CAAA	GCTCGATAAGGTGGTGC TTG
Human_TWIS T1	GCATTCTCAAGAGGT CGTGC	TGACTATGGTTTTGCAG GCC
Human_TWIS T2	AGAGCGACGAGATGG ACAAT	ACAGACTCGAATGCATC CCA
Human_ SLUG	AGCATTTCAACGCCTC CA	GGATCTCTGGTTGTGGT ATGAC
Human_ SNAIL	GGAAGCCTAACTACA GCGAG	CAGAGTCCCAGATGAGC ATTG
Human_MMP2	CAACTGGACACATCT ACCCTG	ACGGTTCATGTCAGGTG TG
Human_CDH1	CTGCCAATCCCGATG AAATTG	TCCTTCATAGTCAAACA CGAGC
Human_ CDH2	CAGAATCAGTGGCGG AGATC	CAGCAACAGTAAGGAC AAACATC

Human_VIMENTIN	ACCAGCTAACCAACGACAAAG	AAAGATTGCAGGGTGTTTTCG
Human_ JUN	TATCTAGGTGGAGTTGAAAG	GAGGAGGAGTATAACCTGAC
Human_FOS	AGAGGAGAAACACATCTTC	GAAAGAGAAAAGAGACACAG
Human_MYC	GAAACTACAAACTCTCCTTG	GAAGACCTACTTTGAGACTG
Human_ EIF4E	ATACAAGGAAAGGTTAGGAC	CTCTTAGTAGCTGTGTCTGC
Human_CDKN1C	CCATCTAGCTTGCAGTCT	GCTCAGGAACCATTTTAAC
Human_RLF13	CCTTCGCTAGTCTCCGTATG	ACTGCCGACTGATTCCAAG

The synthesized cDNA was diluted into 1:2 with Nuclease Free water. qPCR reaction was performed by using SYBR Green PCR Master Kit (Qiagen). The reaction was set according to the manufacturers protocol. The total reaction was adjusted to 10 µL where 3 µL nuclease free water, 0.5 µL Forward Primer, 0.5 µL Reverse Primer, 5 µL 2x Master Mix and lastly 1 µL from 1:2 diluted cDNA were combined respectively into RT-qPCR reaction well plate. It was centrifuged at 1500 rpm for 1 min at room temperature. qPCR experiment was performed by LightCycler 480 Instrument II (Roche). Reaction cycles were given below in *Table 2.2*.

Table 2.2 *RT-qPCR reaction settings*

Cycle Number	Stages	Temperature (ºC)	Time
1	Initial Denaturation	95 ºC	5 min
40	Denaturation	95 ºC	30 s
	Annealing	55 ºC	30 s
	Elongation	72 ºC	30 s
1	Melting Curve	95 ºC	10 s
		65 ºC	1 min
		97 ºC	
1	Cooling	40 ºC	30 s

2.10 Immunofluorescence Staining of Hydrogels

Hydrogels were fixed with 4% PFA at room temperature for 30 minutes. Wash buffer (WB) was prepared, and hydrogels were washed three times for 5 min. After washing step was performed, hydrogels were blocked at room temperature for 1 hour with 5% Goat Serum (Gibco) containing 1% BSA and 1% Triton X-100. Staining buffer was prepared by adding 1% BSA and 0,1% Triton X-100. Then hydrogels were incubated with primary antibody in staining buffer at 4ºC overnight. Antibodies used in this project are given in below *Table 2.3*. The day after, primary antibody solution was removed, and hydrogels were washed with staining buffer at room temperature for 10 min twice. Hydrogels were then incubated with the secondary antibody solution at overnight 4ºC. Secondary antibodies were listed in the below *Table 2.3*. Next day, secondary solution was discarded, and hydrogels were washed for 2 hours at room temperature with staining buffer. To visualize nucleus, staining solution containing 1 µg/mL DAPI was prepared, and gels were incubated for 15 minutes at room temperature. DAPI solution was then removed and washed. Leica DMI/SP8 Laser Scanning Confocal Microscope was used for visualization of the samples. ImageJ software (National Institutes of Health, USA) was used for image analysis.

Table 2.3 *Antibodies used for Immunofluorescence Staining*

	Name	**Catalog Number**
Primary Antibodies	Integrin β1/ITGB1	SantaCruz # sc-13590L
Secondary Antibodies	DAPI	Sigma # MBD0015
	Phalloidin	Sigma # P5282
	Alexa Fluor 488 goat anti-mouse IgG (H+L)	Invitrogen # A11029

2.11 Statistical Analysis

Quantitative data is represented as means with standard deviation (S.D). Statistics were carried out using t-test analysis with Welch's correction in GraphPad Prism9 Software. The significant results was considered with a p value of <0.05 .

Chapter 3:**RESULTS**

3.1 Generation of Stiff and Soft Double Networks Composed of Alginate and dLung/Cultrex

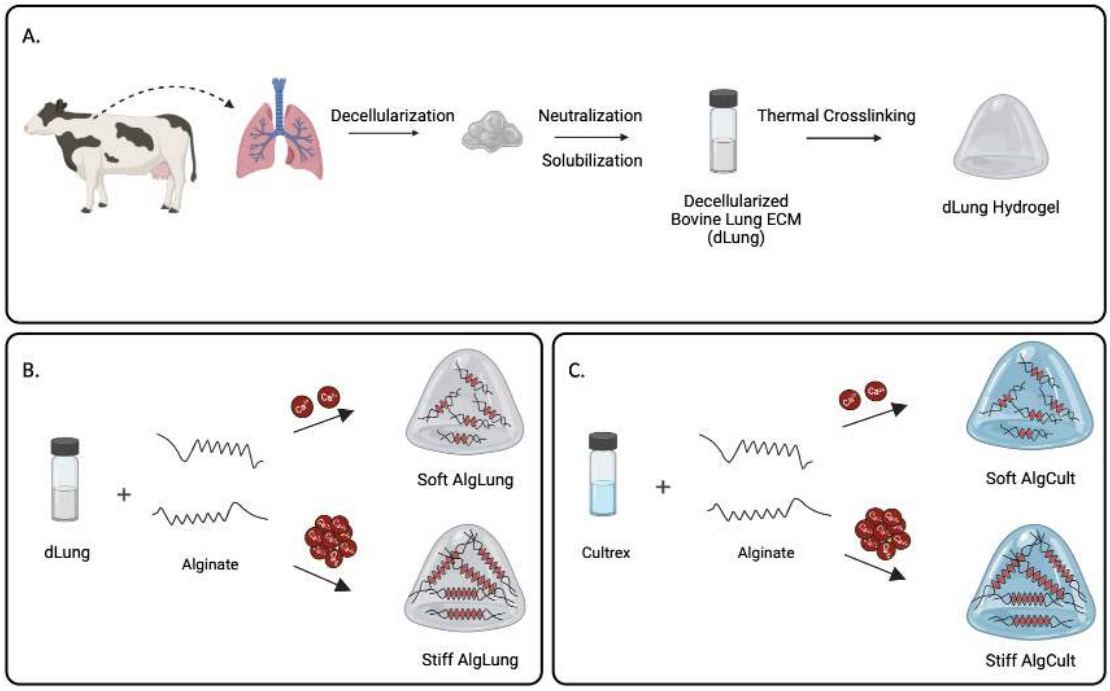


Figure 3.1 Double Network Formation Steps. a) Schematic representation of generation of decellularized bovine lung ECM (dLung) b) Alginate and dLung are crosslinked with altering Ca^{2+} concentrations to produce AlgLung with different stiffness values c) Alginate and Cultrex are crosslinked with altering Ca^{2+} concentrations to produce AlgCult with different stiffness values (Created with BioRender.com)

To examine the importance of stiffness on modulation of tumorigenic growth, a double network composed of alginate (Alg) and decellularized bovine lung ECM (dLung) or Cultrex rBM (Cult) gels are generated with different stiffness values. Alg is a well-known engineered polysaccharide that can be used as a bio-scaffold for 3D in vitro models. The ability of Alg to adjust the stiffness of the gels without altering biochemical composition of the ECM demonstrates the importance of its use in engineering fully defined 3D models [123]. Lung decellularized bovine ECM (dLung) has been revealed

as an ideal tool for recapitulating the native like properties of a healthy lung tissue with respect to organ-related cues [124]. It has been established that this matrix can be used to culture A549 cells up to 4 weeks as it provides the mechanical support and ligand composition required for cells to maintain functions. Thereafter, the gels made up from Alg and dLung is termed as AlgLung. This hydrogel is a model in which the composition and mechanical properties of a healthy lung ECM is mimicked to recapitulate the native-like properties of lung tissue. On the other hand, Cult is a commercial matrix that is used for tissue modeling of cancer progression where it provides tumorigenic cues that is obtained from EHS sarcoma. The gels generated from Alg and Cult is termed as AlgCult. This hydrogel recapitulates the properties of what tumorigenic ECM has in terms of ligand density, composition as well as mechanical properties.

The stiffness of healthy lung tissue is estimated between 1-2 kPa, whereas the fibrotic tissues are higher than 5 kPa [125]. So that, the stiffness range of the gels are determined according to these ranges reported in literature. The stiffness of AlgLung and AlgCult gels are tuned by changing the Ca^{2+} concentration through using different concentrations of $CaSO_4$. In the meanwhile, the ligand concentration is kept constant by adjusting the final concentration of Cult and dLung to 4 mg/mL. That means the ligand concentration are equal, however the composition differs significantly. Thereby, the importance of altering the stiffness of the environment in healthy and tumorigenic ECM composition can be studied with these models(*Figure 3.1*).

The assessment of mechanical signature of the gels are performed by oscillatory rheology. The storage moduli of the gels which represent their stiffness values are given in *Table 3.1* with their standard deviations.

Table 3.1 Storage moduli of hydrogels

	Storage Moduli (G') of Hydrogels (Pa)
AlgCult Soft	632.7 ± 252.7
AlgCult Stiff	4073 ± 202.1
AlgLung Soft	442.2 ± 181.3

AlgLung Stiff	4843 ± 717.3

Considering that the Young's modulus range of healthy lung tissues is between 1 to 5 kPa, our soft AlgCult as well as AlgLung hydrogels can be used to recapitulate the physiological stiffness values of lung (*Table 3.1*) [125]. As it is seen in *Figure 3.2,* difference of the stiffness values among these two models is neglectable. Meaning that, although the composition of their ECM is differed significantly, their stiffness values can be tuned without the composition and concentration of the matrix. Thereby, these models would be an ideal tool for studying the impact of ECM composition on cancerous growth under healthy stiffness conditions.

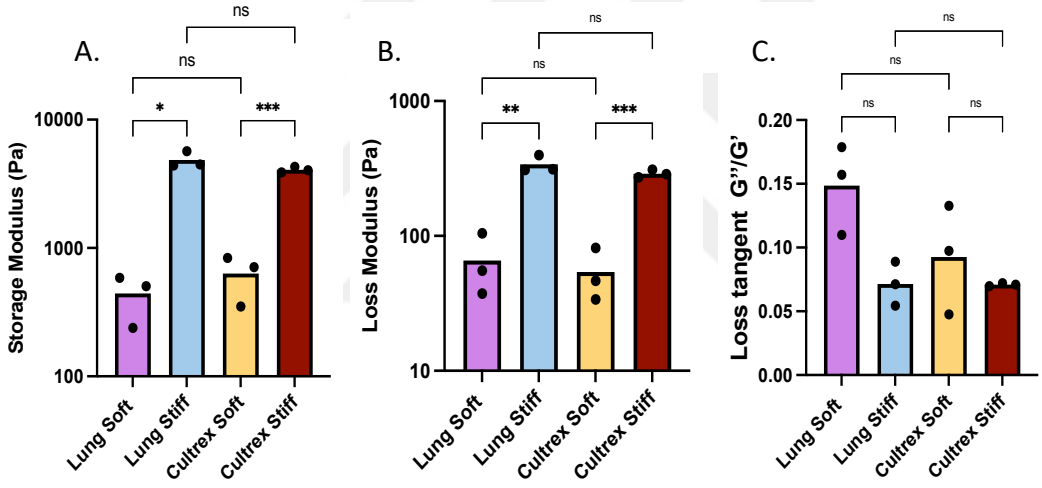


Figure 3.2 Mechanical Assessments of Double Network of Alginate and dLung/Cultrex by Rheometry. a) Storage moduli (G') of AlgLung Soft/Stiff and AlgCult Soft/Stiff gels b) Loss moduli (G'') of AlgLung Soft/Stiff and AlgCult Soft/Stiff gels. c) Loss tangent values (G''/G') of AlgLung Soft/Stiff and AlgCult Soft/Stiff gels

On the other hand, tissue stiffening accompanies lung tumor formation. As given in *Table 3.1*, the stiffness ranges of AlgCult Stiff and AlgLung Stiff are appropriate to be used in a model that recapitulates lung fibrosis. Moreover, comparable stiffness values of these two hydrogel systems were achieved, indicating that, while the stiffness of the environment is increased, ligand concentration remains constant whereas composition is altered (*Figure 3.2*). Thereby, the ability of decoupling tissue stiffness from other parameters renders the impact of stiffness and composition individually.

3.2 Growth of Lung Tumor Cells in AlgLung and AlgCult gels with differing stiffness

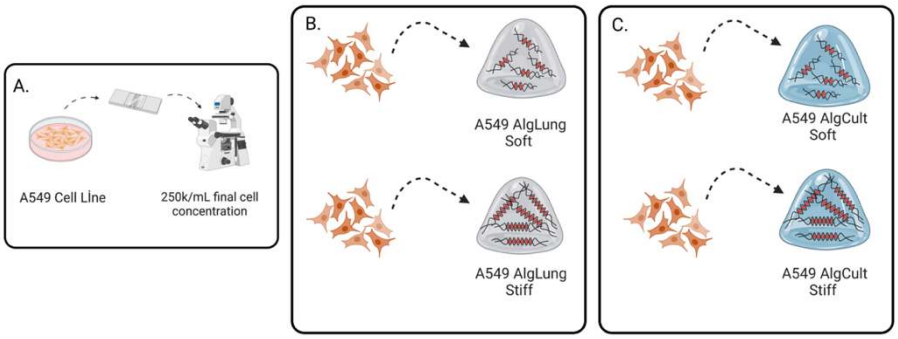


Figure 3.3 Schematic representation of A549 cell encapsulation to hydrogels. a) A549 cells were maintained 2D tissue culture plate. For encapsulation, cells were trypsizined and counted as 250k/mL final concentration. b) A549 cells encapsulated to soft and stiff AlgLung hydrogels. c) A549 cells encapsulated to soft and stiff AlgCult hydrogels (Created with BioRender.com).

A549 cells are encapsulated in 4 different hydrogel systems (AlgLung Soft, AlgLung Stiff, AlgCult Soft, and AlgCult Stiff) and cultured for 3 weeks (*Figure 3.3*)

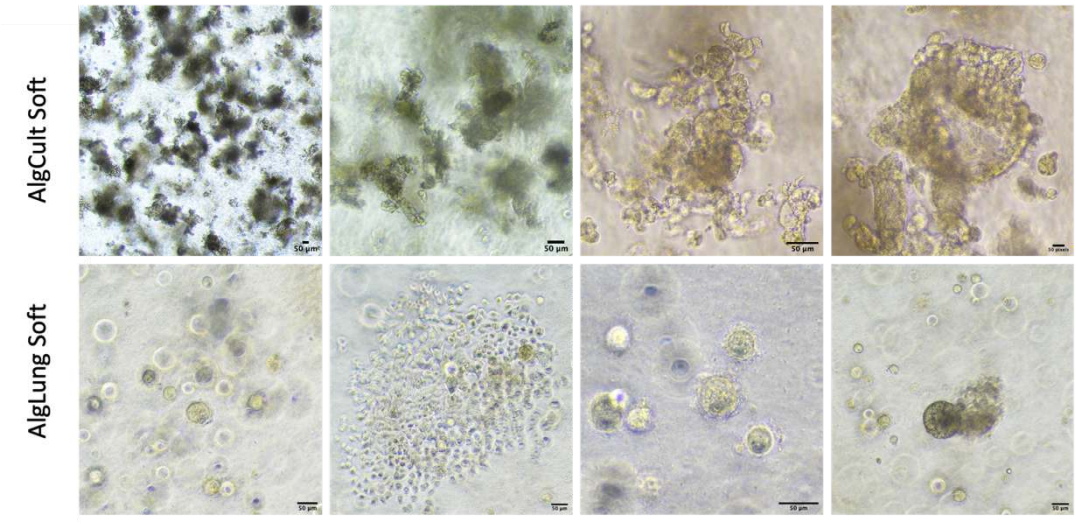


Figure 3.4 Bright-field images of A549 cells grown in AlgCult Soft and AlgLung Soft hydrogels. The top line represents the end point bright field images of A549 cells embedded in AlgCult Soft gels. The bottom line represents the end point bright field images of A549 cells grown in AlgLung Soft gels. (Scale bar: 50 µm).

The end-point images of lung tumor cells that are grown in soft hydrogels are given above in *Figure 3.4*. The proliferation of A549 cells that are encapsulated in AlgCult Soft was significantly higher than A549 cells grown in AlgLung Soft hydrogels at the end of the culturing period. That means, tumorigenic composition and biomechanical properties promote the growth of cancer cell lines in soft microenvironment.

The morphology of A549 cells encapsulated in AlgCult Soft hydrogels differs from those of AlgLung Soft in terms of the invasive phenotypes observed in AlgCult Soft gels. A549 cells embedded in AlgCult Soft were spread within the gels and displayed invasive phenotype. Cells in AlgLung Soft displayed circular clump formation, however, in different regions of the gels a monolayer sheet like cell morphology was also observed. Tumorigenic ECM composition at a healthy, soft range promoted the spreading of cancer cells in the gels, and cells were more prone to producing invasive structures.

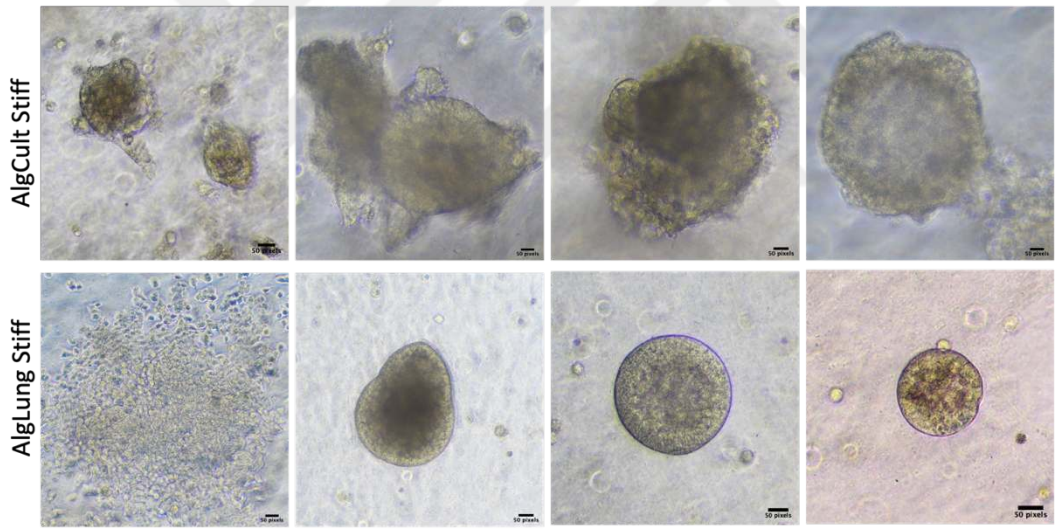


Figure 3.5 Bright-field images of A549 cells grown in AlgCult Stiff and AlgLung Stiff hydrogels. The top line represents the end point bright-field images of A549 cells embedded in AlgCult Stiff gels. The bottom line represents the end point bright field images of A549 cells grown in AlgLung Stiff gels. (Scale bar: 50 µm).

After 3 weeks of culture period, the proliferation of A549 cells that were encapsulated in AlgCult Stiff hydrogels was similar to those who were encapsulated in AlgLung Stiff hydrogels (*Figure 3.5*). However, their morphology differed drastically. The cells in AlgLung Stiff environment displayed various growth patterns. These cells are mostly grown as large circular clumps with well-defined borders and epithelial polarity, whereas, in some regions of hydrogels, the cells also displayed a sheet-like growth pattern. On the other hand, cells that were grown in AlgCult Stiff hydrogels displayed a uniform growth morphology within the gels. Tumor cells in AlgCult Stiff hydrogels demonstrated a significantly more invasive phenotype compared to AlgLung Stiff hydrogels with loss of circularity and epithelial polarity. The cell clumps observed in AlgCult Stiff hydrogels were notably larger as well.

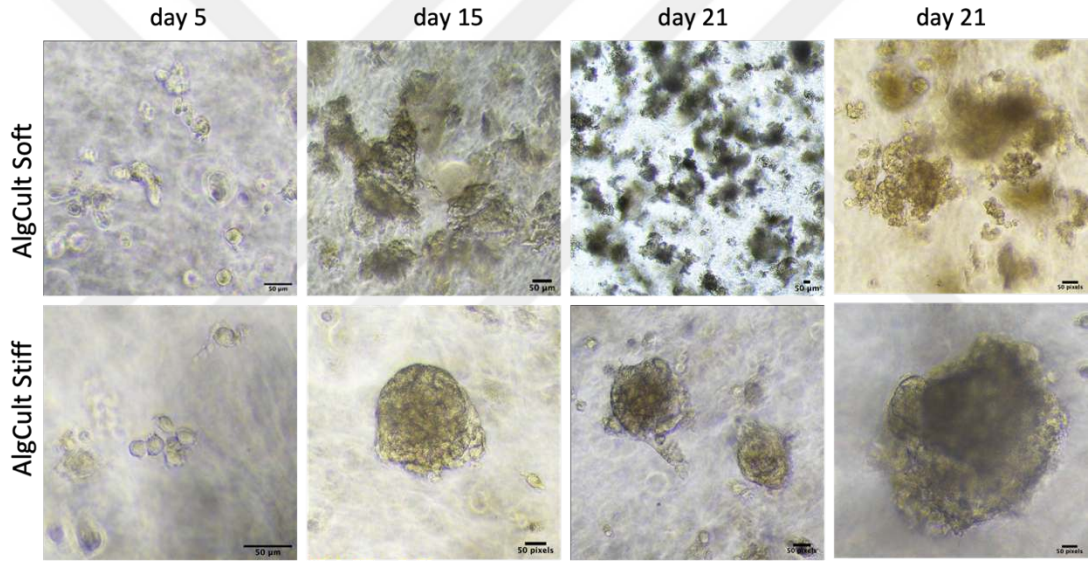


Figure 3.6 Bright-field Images of A549 cells grown in AlgCult Soft and AlgCult Stiff hydrogels at day 5, day 15 and day 21 respectively. The top line represents the images of A549 cells embedded in AlgCult Soft gels taken at different time points. The bottom line represents the images of A549 cells embedded in AlgCult Stiff gels taken at different time points. (Scale bar: 50 µm).

The change in cell growth and morphology upon stiffening was also compared in hydrogels with the same tumorigenic ECM composition. A549 cells embedded in both AlgCult Soft and AlgCult Stiff hydrogels displayed similar growth in their first week (*Figure 3.6*). Within two weeks, the growth rate of cells in AlgCult Soft gels were higher than the ones embedded in AlgCult Stiff gels. Although, the background of AlgCult Soft

gels is more crowded, the size of the clusters were much smaller than in stiff hydrogels and scattered in the matrix.

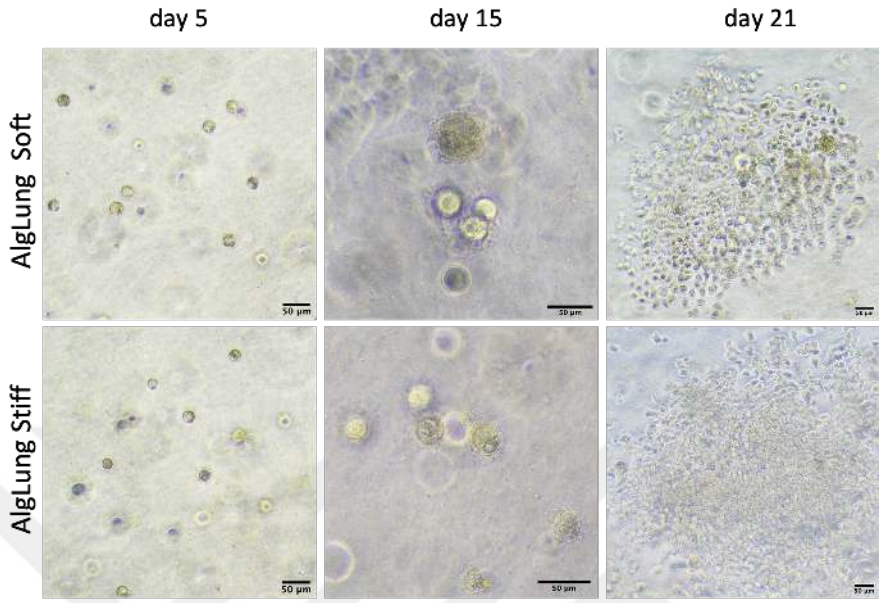


Figure 3.7 Bright-field Images of A549 cells grown in AlgLung Soft and AlgLung Stiff hydrogels at day 5, day 15 and day 21 respectively. The top line represents the images of A549 cells embedded in AlgLung Soft gels taken at different time points. The bottom line represents the images of A549 cells embedded in AlgLung Stiff gels taken at different time points. (Scale bar: 50 µm).

A549 cells encapsulated in AlgLung Soft and AlgLung Stiff hydrogels showed a striking trend during the culture period where their growth rates and proliferation capacities are very similar (*Figure 3.7*). The morphology of cells grown in AlgLung Soft and AlgLung Stiff gels also showed resemblance with the formation of small, circular clusters and in certain regions of the hydrogels, cells had the tendency to grow in sheet-like, monolayer structures. Therefore, increased stiffness of the microenvironment did not alter cellular growth and morphology when the ECM component represented a native-like, healthy composition as opposed to tumorigenic ECM.

3.3 Morphological Staining of A549 Cells Encapsulated in AlgCult and AlgLung hydrogels with differing stiffness

To examine the impact of ligand composition and stiffness on the growth and phenotype of A549 cells, actin cytoskeleton of cells were visualized by using Phalloidin staining and nuclear staining was performed with DAPI (Figure 9).

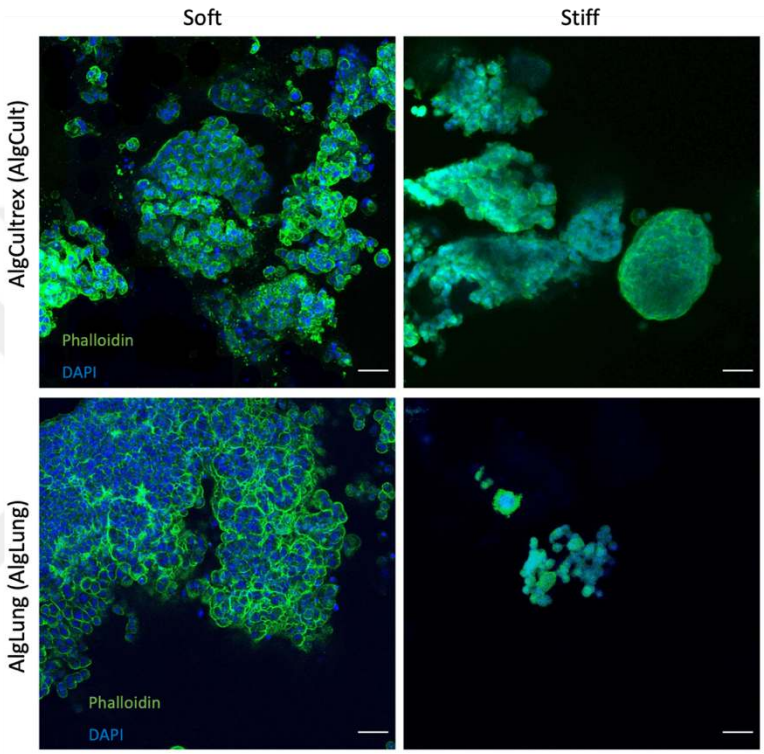


Figure 3.8 Staining of A549 cells encapsulated in AlgCultrex and AlgLung with soft and stiff microenvironment, at day 21. Green: Phalloidin; blue: DAPI; scale bar represents 50 μm.

Morphology of A549 cells were significantly altered based on the composition and the stiffness of the TME. As it is appreciated from the Phalloidin staining of cells in *Figure 3.8*, cells exhibited more dispersed behavior when encapsulated in tumorigenic composition at a lower stiffness. They tended to spread within the gel by forming loose structures. However, when the stiffness of the TME is increased, cells became more compact and formed distinct and confined clusters. The majority of the clumps though had lost circularity and adapted an invasive phenotype.

On the other hand, A549 cells encapsulated in healthy ECM composition, regardless of its stiffness, exhibited different phenotypes than that observed in tumorigenic ECM. As the stiffness of AlgLung hydrogels mimicked the healthy physiological levels at soft range, A459 cells tended to growth in sheet-like, epithelial morphology. However, when the stiffness of the ECM is elevated, the proliferation rate of the cells was observed to decreased compared to the soft ECM, as opposed to similar growth and morphology assessed with bright-field microscopy.

3.4 Knock-down of PI3K gene expression in A549 cells and cell line generation

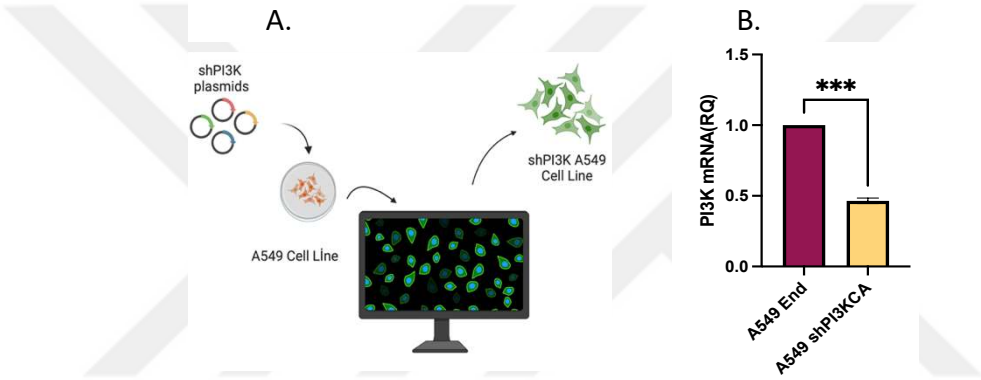


Figure 3.9 Generation of PI3K knock-down A549 cells. a) Schematic representation. b) Relative mRNA transcription expression levels of PI3K gene of control A549 and A549 shPI3K cells grown in 2D culture. (Created with BioRender.com).

PI3K gene expression in A549 cells is knocked down via short-hairpin RNA interference (shRNA) technology. The shRNA itself has an exogenous GFP tag which means that cells having GFP fluorescence are transduced with shRNA viral particle. This is also a good indicator of the transduction efficiency and show the homogeneity of the population . Cells demonstrated a significant decrease in the expression of PIK3CA gene following the knock-down procedure (*Figure 3.9*).

3.5 Growth of shPI3K A549 cells in AlgCult and AlgLung gels with differing stiffness

After generating the shRNA-mediated PI3K knock-down A549 cells, they were encapsulated in AlgCult Soft/Stiff and AlgLung Soft/Stiff gels and cultured for 3 weeks. During that time, growth and morphology of cells were monitored with bright-field microscopy.

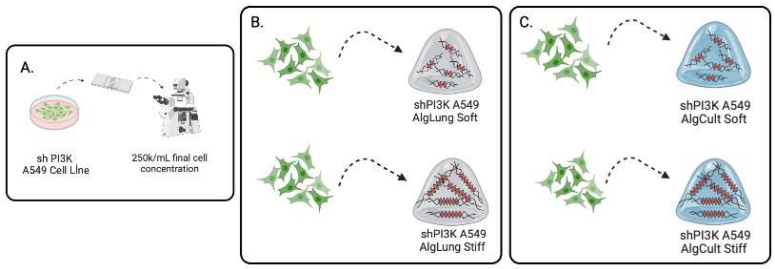


Figure 3.11 Schematic representation of shPI3K A549 cell encapsulation to hydrogels. a) shPI3K A549 cells were maintained 2D tissue culture plate. For encapsulation, cells were trypsizined and counted as 250k/mL final concentration. b) shPI3K A549 cells encapsulated to soft and stiff AlgLung hydrogels. c) shPI3K A549 cells encapsulated to soft and stiff AlgCult hydrogels (Created with BioRender.com).

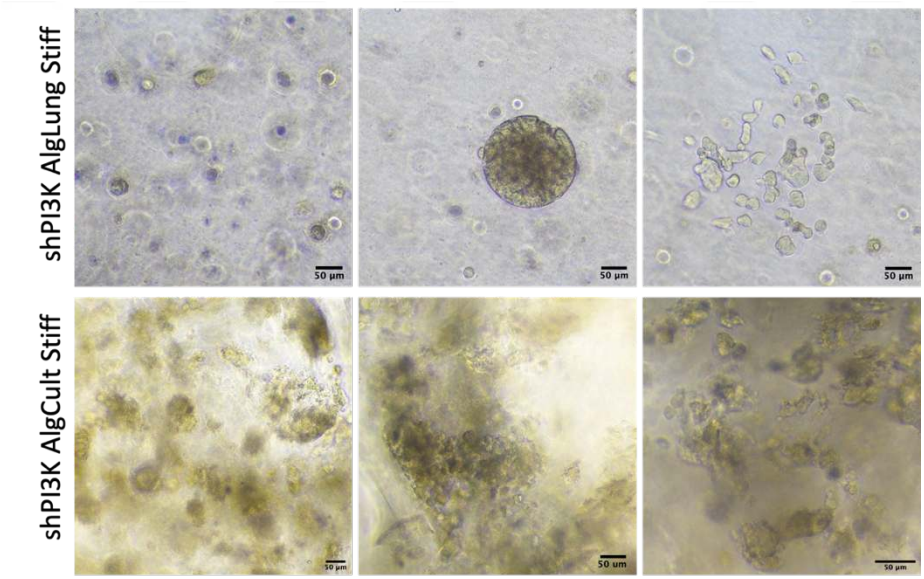


Figure 3.10 The bright-field images of shPI3K A549 cells encapsulated in AlgLung Stiff and AlgCult Stiff hydrogels. The top line represents the end-point bright-field images of shPI3K A549 cells embedded in AlgLung Stiff gels. The bottom line represents the end-point bright-field images of shPI3K A549 cells embedded in AlgCult Stiff hydrogels. (Scale bar: 50 µm).

After 3 weeks of culture period, the growth of shPI3K A549 cells that were encapsulated in AlgCult Stiff hydrogels was higher than the ones encapsulated in AlgLung Stiff gels (*Figure 3.11*). Distinct cell clump formation that was observed with wild type cells was inhibited with the knock-down of PI3K in stiff hydrogels with tumorigenic composition where cells grew in a more dispersed manner. Their growth resembled to that of wild-type cells in soft and tumorigenic hydrogels, indicating that silencing PI3K abrogated the effect of microenvironment of stiffness in these cells. On the other hand, under healthy ECM conditions with AlgLung Stiff hydrogels, knock-down of PI3K did not have a big impact on cell growth and circular clump formation was observed comparable to wild-type cells.

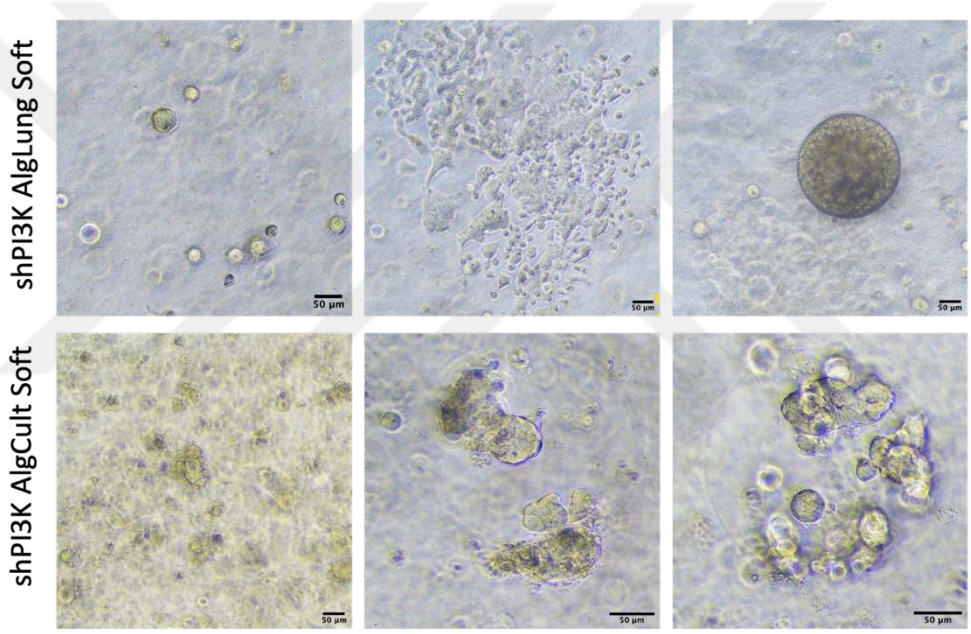


Figure 3.12 The bright-field images of shPI3K A549 cells encapsulated in AlgLung Soft and AlgCult Soft. The top line represents the end-point bright-field images of shPI3K A549 cells embedded in AlgLung Soft gels. The bottom line represents the end-point bright-field images of shPI3K A549 cells embedded in AlgCult Soft hydrogels. (Scale bar: 50 µm).

shPI3K A549 cells embedded in AlgCult Soft gels displayed higher growth rate than the ones in AlgLung Soft gels. A549 cells encapsulated in AlgLung Soft gels displayed non-uniform growth in both clump and sheet-like form . However, tumorigenic ECM microenvironment in AlgCult hydrogels, which recapitulates the ligand composition of the native tumors, scattered growth and smaller clumps (*Figure 3.12*).

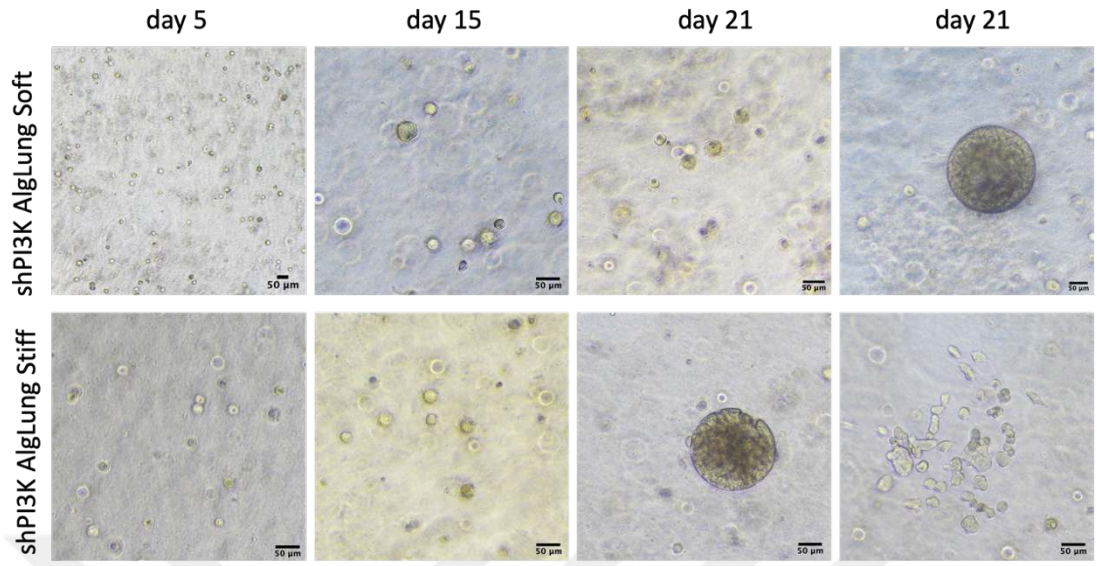


Figure 3.13 Bright-field images of shPI3K A549 cells grown in AlgLung Soft and AlgLung Stiff hydrogels at day 5, day 15 and day 21. The top line represents the images of shPI3K A549 cells embedded in AlgLung Soft gels taken at different time points. The bottom line represents the images of shPI3K A549 cells embedded in AlgLung Stiff gels taken at different time points. (Scale bar: 50 μm)

The growth rate of shPI3K A549 cells encapsulated in AlgLung Soft and AlgLung Stiff was similar throughout the culture period. There were not significant differences in the growth and proliferation rate of shPI3K cells in soft and stiff microenvironments. The morphology of cells in two different stiffness values was similar as well. Cells were more prone to grow as single clumps in both AlgLung Soft and AlgLung Stiff gels. Additionally, in various regions within the hydrogels, these cells displayed sheet-like growth.

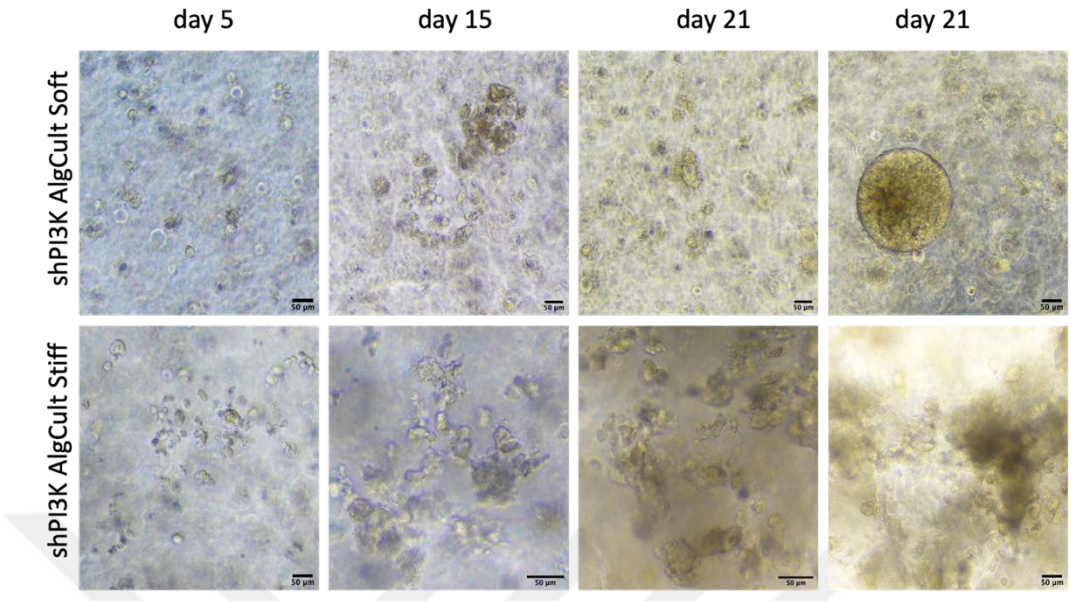


Figure 3.14 Bright-field images of shPI3K A549 cells grown in AlgCult Soft and AlgCult Stiff hydrogels at day 5, day 15 and day 21. The top line represents the images of shPI3K A549 cells embedded in AlgCult Soft gels taken at different time points. The bottom line represents the images of shPI3K A549 cells embedded in AlgCult Stiff gels taken at different time points. (Scale bar: 50 μm).

The tumorigenic ECM environment promoted the growth of shPI3K A549 cells regardless of hydrogel stiffness. However, shPI3K A549 cells that were encapsulated in AlgCult hydrogels displayed higher growth rate during culture period. Morphology of cells which were grown in AlgCult Stiff hydrogel were more invasive and they were spread throughout the hydrogel. However, cells in AlgCult Soft gels displayed at least some circular cluster formation with epithelial polarity.

3.6 Oncogenic gene expression of A549 cells in AlgCult Soft/Stiff and AlgLung Soft/Stiff

After observing that cells encapsulated in AlgCult and AlgLung gels with different stiffness values has demonstrated distinct morphology during their culture period, EMT and stemness properties of these cells were assessed with gene expression in *Figure 3.15*.

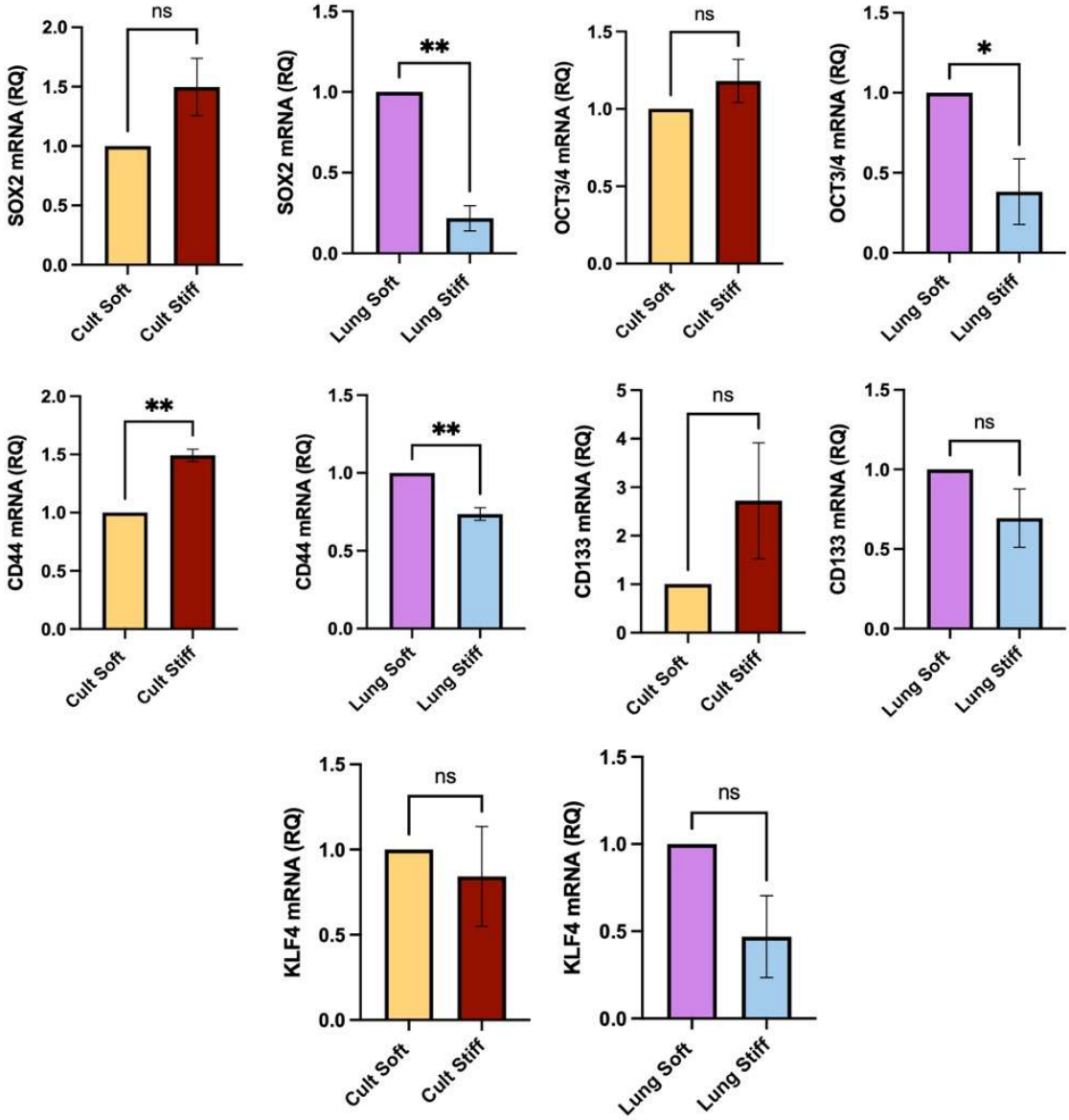


Figure 3.15 mRNA expression levels of stemness markers of A549 cells encapsulated in AlgCult Soft, AlgCult Stiff, AlgLung Soft and AlgLung Stiff hydrogels.

The mRNA expression of CD44 gene is upregulated when the stiffness of the tumorigenic matrix is increased. Cells encapsulated in AlgCult Stiff hydrogels displayed higher expression of CD44 than those who are encapsulated in AlgCult Soft hydrogels. Other stemness markers including SOX2, KLF4, OCT3/4, and CD133 are not significantly changed when the stiffness of tumorigenic environment is increased. On the other hand, the expression of stemness markers SOX2, CD44, and OCT3/4 are significantly downregulated with the increase in stiffness within AlgLung hydrogels with healthy ECM composition. Cells encapsulated in AlgLung Soft environment showed

elevated expression of SOX2, CD44 and OCT3/4 genes compared to AlgLung Stiff hydrogels(*Figure 3.15*).

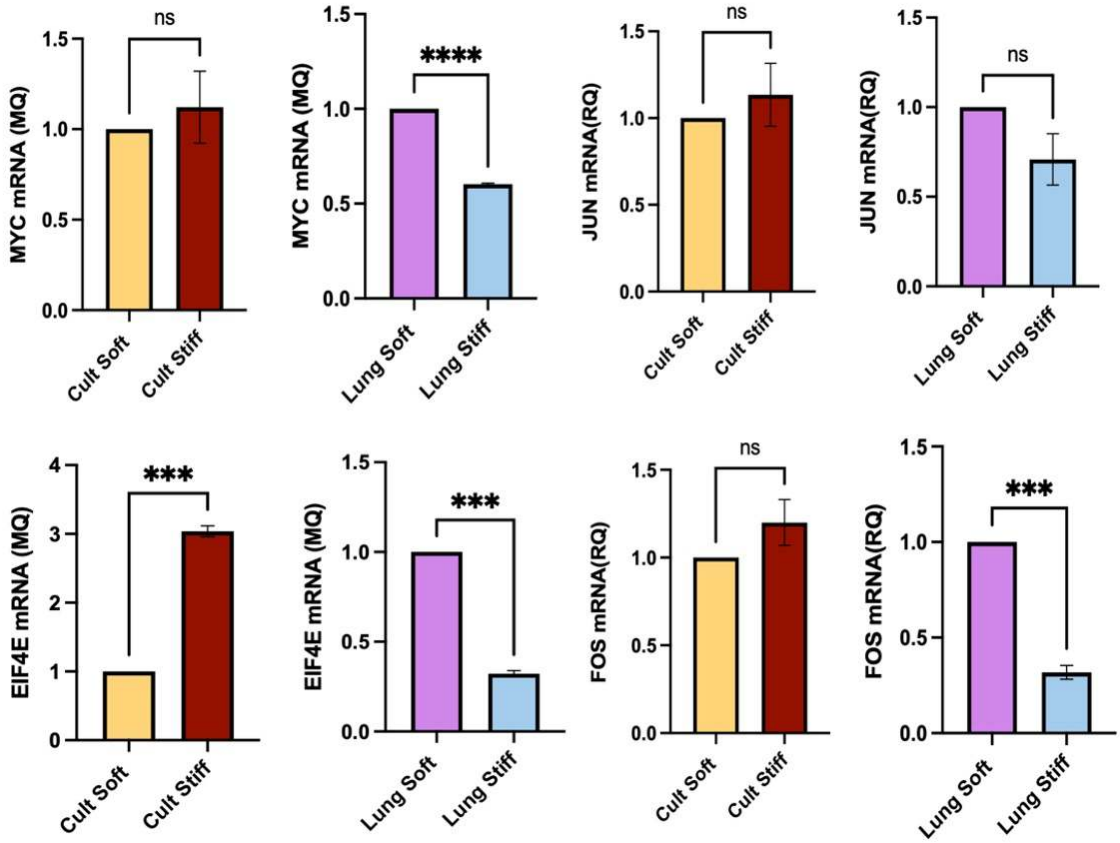
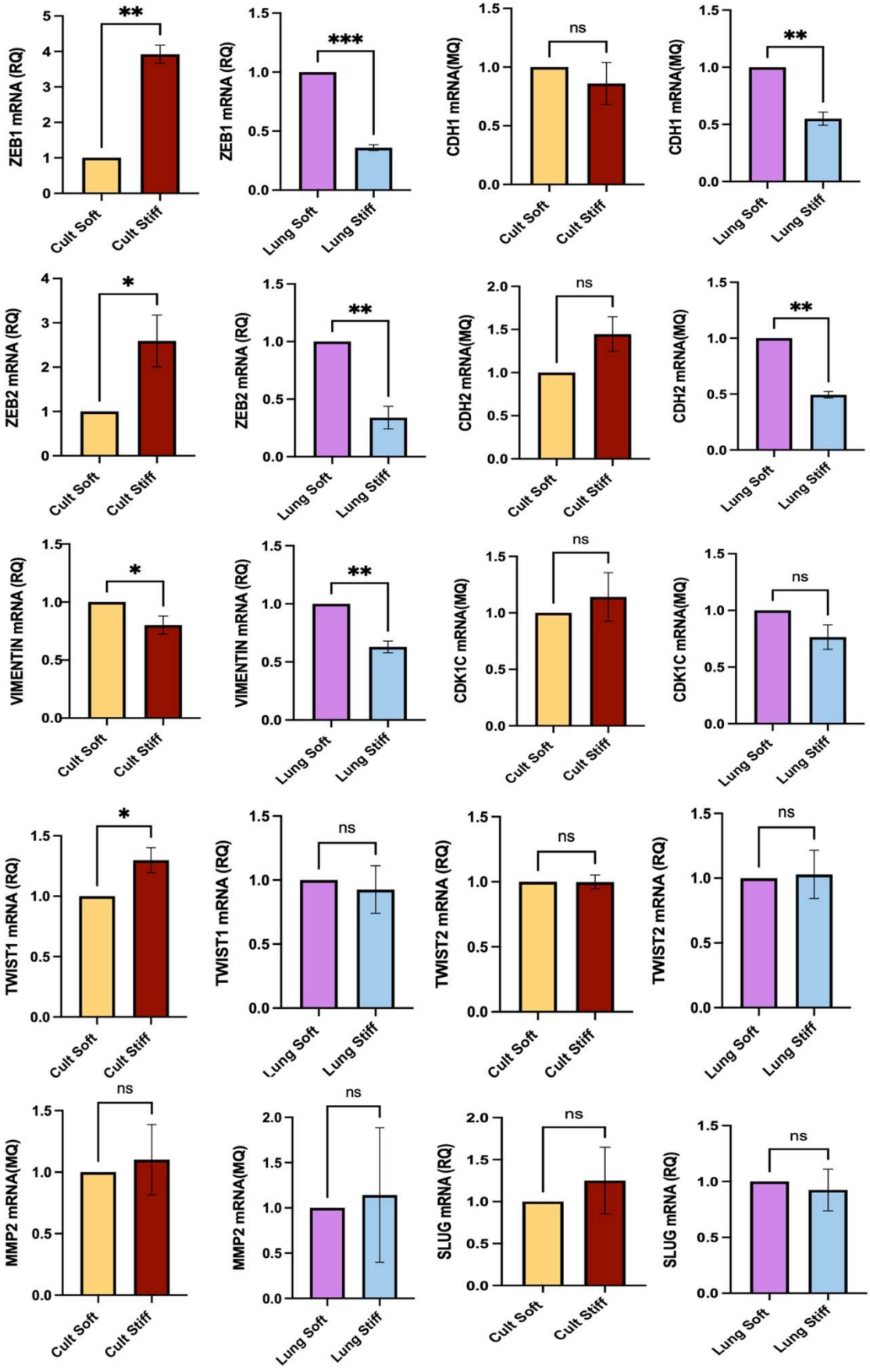


Figure 3.16 mRNA expression levels of oncogenic markers of A549 cells encapsulated in AlgLung Soft, AlgLung Stiff, AlgCult Soft and AlgCult Stiff hydrogels

The expression of oncogenes including JUN, FOS, MYC, and CDKN1C did not show a significant upregulation when the stiffness of tumorigenic matrix is increased. On the other hand, the expression of EIF4E gene is upregulated when the stiffness of the tumorigenic environment is increased in AlgCult hydrogels. The expression level of FOS, MYC and EIF4E genes are downregulated upon increase in stiffness of the healthy-mimetic matrix in AlgLung hydrogels, however, the expression of JUN and CDKN1C was not changed significantly(*Figure 3.16*).


ZEB1 mRNA (RQ)
**
Cult Soft
Cult Stiff
ZEB1 mRNA (RQ)
***
Lung Soft
Lung Stiff
CDH1 mRNA(MQ)
ns
Cult Soft
Cult Stiff
CDH1 mRNA(MQ)
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Lung Soft
Lung Stiff
ZEB2 mRNA (RQ)
*
Cult Soft
Cult Stiff
ZEB2 mRNA (RQ)
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Lung Soft
Lung Stiff
CDH2 mRNA(MQ)
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Cult Soft
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CDH2 mRNA(MQ)
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VIMENTIN mRNA (RQ)
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Figure 3.17 mRNA expression levels of EMT markers of A549 cells encapsulated in AlgCult Soft, AlgCult Stiff, AlgLung Soft and AlgLung Stiff hydrogels.

The expression levels of EMT markers, including ZEB1, ZEB2, and TWIST1 are significantly elevated when the stiffness of the tumorigenic ECM is increased. Indeed, the expression of these markers are upregulated in cells encapsulated in AlgCult Stiff compared to AlgCult Soft gels. The expression of TWIST2, SLUG, E CAD, N CAD and MMP2 genes are not altered as the stiffness of the tumorigenic matrix is increased. However, the expression of ZEB1, ZEB2, E CAD, N CAD and Vimentin are significantly downregulated when the stiffness of the healthy mimetic ECM is increased. Indeed, the expression of these markers were higher in AlgLung Soft than AlgLung Stiff hydrogels(Figure 3.17).

As PI3K is well-known mediator of EMT, we sought to determine the expression level of PI3K in AlgLung Soft and AlgCult Stiff gels where the EMT is induced. The findings have elevated that that the expression of PI3K gene is upregulated in tumorigenic ECM with elevated stiffness whereas it is downregulated in healthy-mimetic ECM as tissues stiffen (*Figure 3.18*).

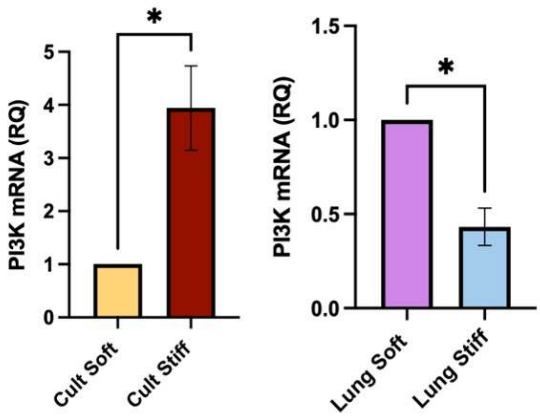
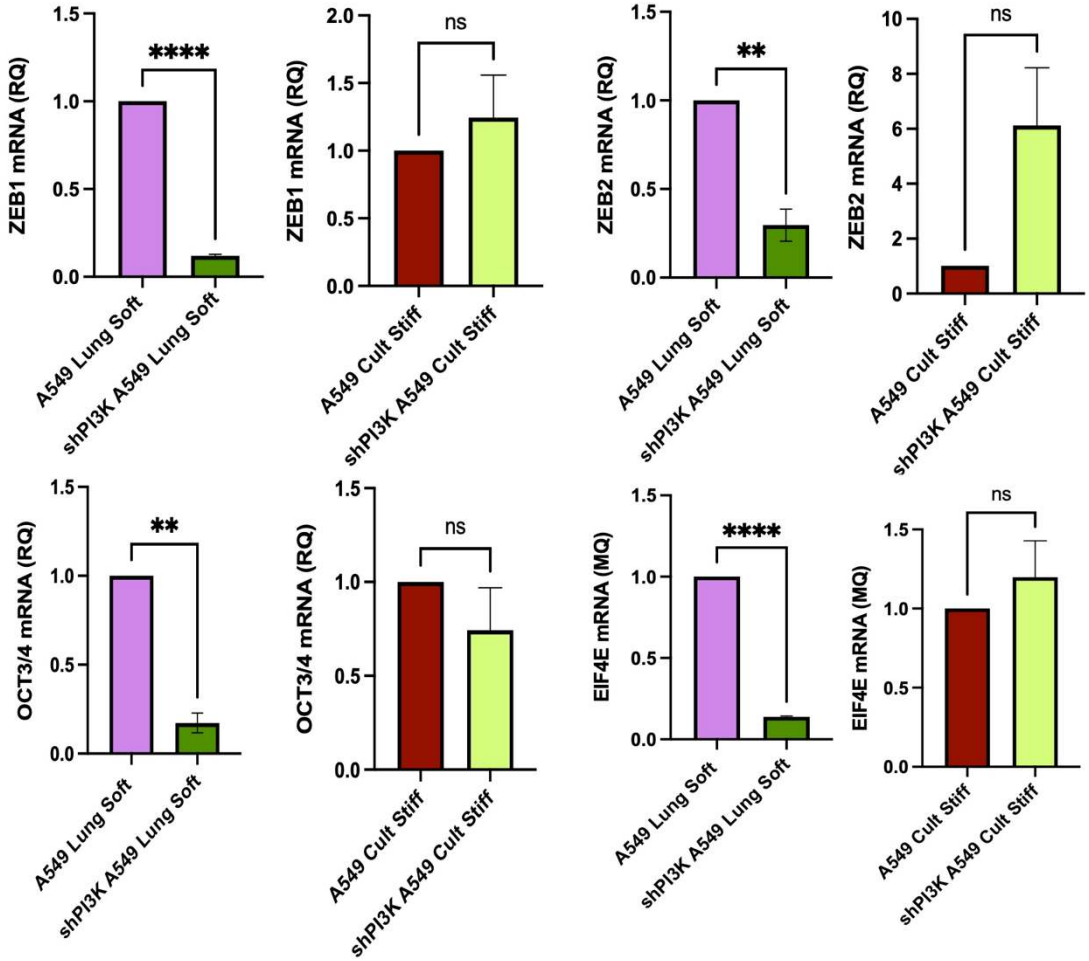


Figure 3.18 mRNA expression levels of PI3K gene of A549 cells encapsulated in AlgCult Soft, AlgCult Stiff, AlgLung Soft and AlgLung Stiff hydrogels.

3.7 Oncogenic gene expression of shPI3K A549 cells in AlgCult Soft/Stiff and AlgLung Soft/Stiff hydrogels

As the expression of PI3K gene is upregulated concomitantly with EMT and stemness markers in AlgCult with elevated stiffness, and is downregulated when the stiffness of the healthy ECM is increased, the expression of these markers are quantified in shPI3K A549 cells and compared (*Figure 3.19*).



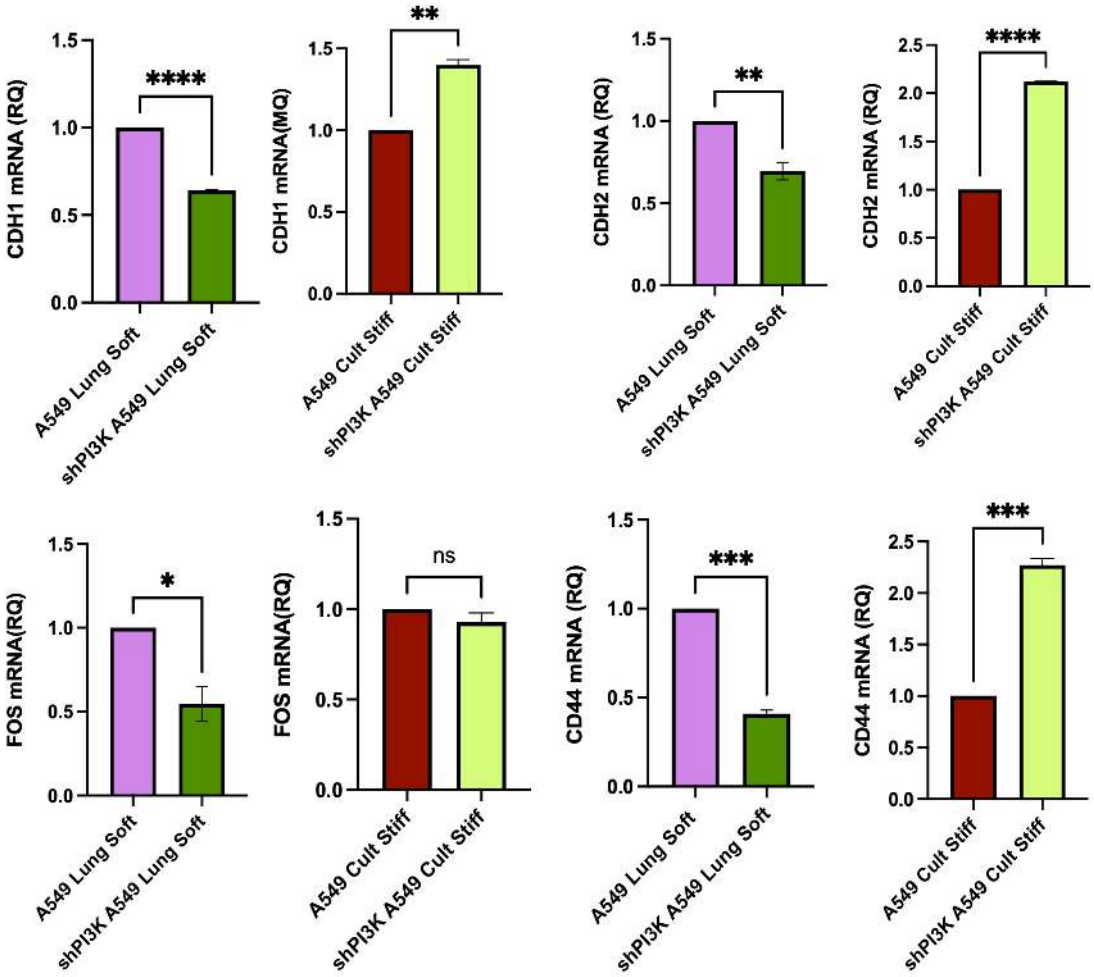


Figure 3.19 mRNA expression levels of EMT markers of shPI3K A549 and A549 cells encapsulated in AlgCult Stiff, and AlgLung Soft hydrogels.

The expression levels of ZEB1, ZEB2, OCT3/4, EIF4E, CDH1, CDH2, FOS and CD44 is downregulated in shPI3K A459 cells encapsulated in AlgLung Soft hydrogels compared to control. In addition to that, the expression levels of ZEB1, ZEB2 , OCT3/4, EIF4E and FOS were very similar between A549 cells and shPI3K A549 cells that are embedded in AlgCult Stiff hydrogels. However, the expression of CDH1, CDH2 and CD44 are upregulated in shPI3K A549 cells encapsulated in AlgCult Stiff hydrogels (*Figure 3.19*).

3.8 Integrin-β1 staining of Cells in AlgCult Soft/Stiff and AlgLung Soft/Stiff Hydrogels

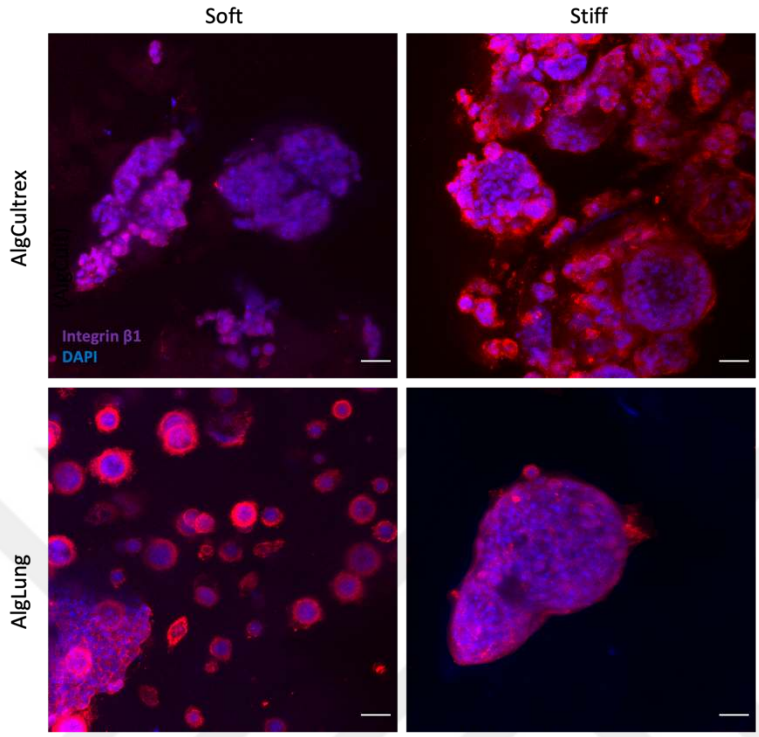


Figure 3.20 Immunofluorescence staining of A549 cells encapsulated in AlgCult and AlgLung hydrogels with differing stiffness at day 21. Magenta: Integrin β1; blue: DAPI, scale bar represents 50 μm.

To assess whether elevated expression of EMT and stemness genes on A549 cells grown in AlgCult Stiff and AlgLung Soft is correlated with increased protein expression of Integrin β1, cells were immunostained and visualized with confocal microscopy. Although, cells are positive for Integrin β1 expression in each gel conditions, the activation is increased in tumorigenic ECM with elevated stiffening. Moreover, cells AlgLung Soft gels expressed elevated levels of Integrin β1 compared to AlgLung Stiff in line with the gene expression of EMT markers (*Figure 3.20*).

Chapter 4: **DISCUSSION**

Biomimicking the TME composition and mechanical properties is of utmost importance for developing a model which recapitulates the hallmarks of tumorigenesis and further disease progression [2]. Examining the molecular cues that drive malignancy is a great value for developing novel treatments that inhibit cancer spread. Although, traditional culture systems where 2D cell culture plates are utilized have enhanced our understanding of how intrinsic mutations lead to uncontrolled growth of cells and further progression, extracellular signals which are important in determining the behavior of cells are absent in these systems [126, 127]. The generation of an organotypic tumor model has a great value for providing in-depth knowledge of how mechanotransduction modulates tumor phenotype by recapitulating the alterations in the composition of TME, and elevated stiffness. The reciprocal interaction between cancer cells and the aberrant stiffening of TME should be mimicked with respect to inputs obtained from organ-specific TME. Thereby, a model that allows to study both cellular and extracellular drivers of cancerous growth based on organ-specific cues is crucial for improving the knowledge of how cancer develops and responds to therapeutics.

In this study, to investigate the impact of stiffening and altered composition of the matrix on modulation of tumorigenesis, a double network composed of alginate and Cultrex or dLung is generated. It is a fully defined 3D culture model which is an ideal tool for examining the effect of matrix mechanics without changing the ligand concentration, as its components are crosslinked in the presence of divalent Ca^{2+} . In our model, the proliferation rate of A549 lung adenocarcinoma cells is increased when they are cultured in tumorigenic environment. It has been well established that the altered composition of the ECM, where the production of its components, crosslinking, removal, and remodeling rates are altered, tumorigenesis is promoted [28, 128, 129]. In this regard, our results are consistent with previously reported literature. It has been well-studied that the proliferation rate of tumors is increased as a result of increased stiffening of the tumorigenic matrix [45, 91, 129, 130]. However, numerous studies have emphasized the importance of high stiffness in modulating tumorigenic proliferation by creating a model that allows the stiffness of the medium to be increased by adding collagen I [86, 131]. This is not an appropriate representation that can be used to simulate tissue stiffening as

the addition of ECM components to increase stiffness results in activation of intracellular cascades that are not due to these mechanical inputs [7, 132, 133]. Yet another model has established the necessity of the activation of oncogenic genes responsible for spread of cancer cells by elevating collagen crosslinking to generate stiff hydrogels without changing the ligand concentration [91]. Though, in this model, the increased crosslinking results in the formation of bundle-like structures which limits the presentation of bioactive compounds. To address these issues, pioneering studies have come up with a model where independent regulation of tissue stiffness is achieved through using various polymers [134, 135]. However, they are lacking cell adhesive components that are important in supporting the physiological growth and proliferation of the cells. To tackle this issue, the generation of double networks composing of a scaffold polymer and cell adhesive ligand is an optimal tool for studying the molecular mechanisms of the cancer progression based on the reciprocal crosstalk between extracellular inputs and cells [136, 137]. Indeed, the fact that these systems are capable of tuning tissue stiffness without altering ligand concentration, made them an ideal tool for studying mechanobiology of cancer cells.

In line with this, our hydrogel system is a great example of those models where the stiffness of the environment is decoupled from the matrix composition and concentration. Our results are consistent with previous research where an interpenetrating network of alginate and Matrigel is generated to understand how increased ECM stiffness modulates the malignant phenotype of mammary epithelium cells [93]. In that work, where the mechanism of which drives the malignancy is examined in a fully defined 3D model has revealed that the elevated stiffening of the tumorigenic ECM induces the growth and invasive characteristics of MCF10A cells [93]. Accordingly, our results have highlighted that A549 cells encapsulated in a soft and tumorigenic matrix form smaller clusters, however, as the stiffness of the matrix is increased, the clusters get significantly larger.

Whereas cancerous growth of A549 cells in healthy matrix are not as induced as the ones surrounded with a tumorigenic matrix. This result is also in line with pioneering research where the healthy composition of the matrix abrogated the effect of being treated with a mutagen which results in overactivation of H-Ras, one of the essential modulators of the tumorigenic growth [128]. In addition to that, the proliferation rate of healthy breast cells are induced when they are co-cultured with fibroblasts that are isolated from

tumorigenic breast tissue, whereas, their proliferation rate is not changed as they are cultured with cells obtained from healthy breast tissue [138, 139]. Furthermore, similar outcomes are observed when heathy prostate cells are co-cultured with fibroblasts isolated from carcinoma and exhibit tumorigenic phenotypes [140]. To better recapitulate the growth patterns of cancer cells in a healthy ECM, organotypic models are generated. Though they have revealed that the abnormal expression levels of signaling mechanisms can be constrained when cells are cultured in a healthy ECM [1, 86, 141], these models do not fully capture native-like characteristic of cancer cells, as they do not allow the study of the formation of structures that can only be observed in 3D systems [93]. On the other hand, our double network of alginate and dLung ECM is an ideal tool for recapitulating the native-like biochemical and biomechanical properties of the lung tissue [124]. Indeed, it contains organ-specific cues that is derived from healthy lung ECM, therefore it is an organotypic model which is appropriate for examining the physiological response of cells to their environment.

As mentioned above, AlgLung hydrogel is an ideal organotypic model for studying the impact of healthy ECM composition on modulating cancerous growth. The fact that the morphology of cancer cells and their proliferation can be normalized as they are cultured in a healthy microenvironment, renders the importance of our model considering previous research. To add on, our model allow us to study the sole impact of stiffening in healthy lung tissue on modulating the cancerous growth without altering the composition of the ECM. The elevated stiffening of the healthy tissues does not elicit the phenotype of cancerous cells resulting from stiffening of tumorigenic ECM. That means, irregular boundaries of the tumor clumps as well as the increased proliferation rate are normalized and suppressed with the healthy ECM composition regarding stiffness.

The expression of ZEB1, ZEB2, TWIST1, CD44 and EIF4E is upregulated as the stiffness of the tumorigenic matrix is elevated in AlgCult hydrogels. This finding could have been the result of excessive production of collagen molecules in stiff tumor microenvironment. As it has been well studied that the overproduction of collagen molecules, increased crosslinking between them and their reorganization are accompanied by tumor stiffening which promotes the expression of mesenchymal markers, the upregulation of ZEB1, ZEB2, and TWIST1 EMT TFs is predicted [66, 130, 131, 142, 143]. Additionally, the elevated expression of PGs which is widely observed in stiff tumor microenvironment might have affected the upregulation of these genes as well.

As an example, the upregulation of the CD44 receptor might have occurred through aberrant production of HA upon tumor tissue stiffening [58]. In addition, as tumor tissue stiffness increases, aberrant GF signaling modulate the mesenchymal phenotype of cells [1]. For instance, the elevated stiffness of the tumor microenvironment promotes EMT through augmenting TFG-β1 signaling in various cells including hepatocellular carcinoma and kidney epithelium [144, 145]. That means, the increased expression of EMT markers in AlgCult Stiff gels might have rooted from the overactivation of GF signaling due to elevated stiffening of TME. Hence, this outcome might also have resulted from the elevated activation of MMP and protease activation when stiffness of the TME is increased [43]. As this process results in the release of bioactive materials that are once sequestered, it activates the surface receptors upon inappropriate cues [43]. Hence, deregulation of these enzymes due to tissue stiffening might have determined the mesenchymal phenotype of cancer cells, and elevated the activation of TFs responsible for EMT such as ZEB1, ZEB2 and TWIST1.

Interestingly, the expression of genes involved in EMT and stemness pathways are downregulated as the stiffness of the healthy ECM is increased in AlgLung hydrogels. The impact of increased stiffening of TME on elevating ZEB1 and ZEB2 activation is abrogated in healthy tissues where the soft environment is more suitable for activating ZEB1 and ZEB2. That means, composition of the matrix has a profound impact on regulating the activation of EMT TFs. In line with this, the expression of SOX2, OCT3/4, and CD44 are also downregulated as the stiffness of the healthy microenvironment is increased. In addition, integrins might have been more active in AlgCult Stiff and AlgLung Soft environment (*Figure 3.20*) as they display more clustered phenotype. As it has been well established that integrin molecules orchestrate EMT and stem cell like characteristics of cells, our findings might be consistent with previous research [28, 82, 88, 91, 146]. The downregulation of EMT and stemness markers as the stiffness of the healthy lung microenvironment is increased might be manifested from the decreased activation of integrin molecules (*Figure 3.20*). This phenomenon might have also been the result of organization and spatial distribution of the healthy matrix components. The healthy based ECM used in this experiment is mostly composed of laminin molecules [124]. As it has been well appreciated that laminins are one of the most important ligands of integrin receptors the spatial organization of laminins and the topography of soft microenvironment might favor the binding of integrins and activate EMT pathways [41].

As the stiffness of the hydrogels are modulated through altering the concentration of the crosslinking cation, the reorganization of the laminin molecules might not be favorable for facilitating the binding of integrin, thereby EMT and stemness phenotype of cancer cells are decreased. Therefore, the reorganization and architecture of the healthy lung ECM as a result of the tissue stiffening might manifest the distribution of integrins thereby modulate downstream pathways accordingly [91].

Together with these results, the impact of elevated stiffness is shown to be dependent on the composition of the matrix. It has been revealed that, cells having intrinsic mutations do not exhibit an abnormal morphology when they are not in synergistic interaction with their microenvironment. Elevated tissue stiffness does not always correlate with an increase EMT phenotypes, oncogenic and stemness expression of cancer cells. When these cells are grown in a healthy like composition, the impact of increased tissue stiffening on promoting the expression of certain genes responsible for EMT, stemness as well as the oncogenic properties are abrogated, and normalized. However, it should be kept in mind that EMT is a rather complex phenomenon [62]. Indeed, not all cancer cells have to lose their epithelial properties to acquire an invasive phenotype. Numerous research has highlighted the fact cells can preserve their epithelial characteristic yet display metastatic behavior [62].

It has been well appreciated that the PI3K gene is an integral mediator of the mechano-sensitive pathways [91]. The mechanical inputs that result from the excessive deposition of matrix components and increased crosslinking is transmitted to cells by integrins and RTKs, thus activate downstream pathways such as PI3K-AKT [30, 68, 91, 145]. This cascade is has key importance in modulating the transition of epithelial cells to mesenchymal phenotype, and regulating their self-renewal ability [28, 87, 147]. Hence, examining the impact of matrix composition on regulating the behavior of cancer cells with PI3K knock-down in a fully defined *in vitro* model would be interesting for developing future therapeutics regimes.

The proliferation of PI3K knock-down lung adenocarcinoma cells is promoted as the stiffness of the tumor microenvironment increase. In addition, the invasive characteristics of the A549 cells lacking PI3K gene is induced when tumorigenic matrix stiffens. On the other hand, encapsulation of these cells in healthy mimetic lung microenvironment, the proliferation rate as well as their invasive phenotypes are not altered as the stiffness of the environment is increased. The morphology of these cells

differs from the endogenous A549 cells, where bigger clumps with irregular boundaries are formed as the stiffness of the TME is increased. Whereas, when PI3K knock-down cancer cells are grown in stiff TME, their morphology is normalized. Although increased stiffness promotes the invasive phenotype of A549 cells, this phenotype could not be observed when cells lacked PI3K.

PI3K pathway is a hub where it plays a crucial role in modulating EMT mechanisms as well as stemness properties of tumorigenic cells [68, 91]. As it can be seen in our results, the soft environment with healthy composition induces mesenchymal phenotype and oncogenic profile of cancer cells as well their stemness properties. However, this effect is abrogated as the stiffness of the tumorigenic ECM results in the induction of EMT, and stemness pathways. Producing a cancer cell line whose PI3K gene activation is knocked down allows to study whether stiff matrix with tumorigenic composition can promote the EMT and stemness pathways through compensating for the absence of PI3K gene. SImilarly, the impact of having healthy ECM composition on regulating the invasive phenotype in the absence of PI3K can be studied in different stiffness degrees. The absence of PI3K gene cannot been compensated by cancer cells grown in soft matrix with healthy composition as the expression levels of EMT markers, ZEB1, ZEB2, CDH1, and CDH2, oncogenic markers EIF4E and FOS as well as stemness markers OCT3/4, FOS and CD44 are downregulated. On the other hand, the absence of PI3K gene is compensated when cells are surrounded with a tumorigenic ECM. Although PI3K gene is knocked down, the expression of CDH1 and CDH2 genes, as well as CD44 are upregulated, meaning that tumorigenic ECM can induce further progression of tumorigenic phenotype even when cells lack key mediators of these pathways.

Our findings might have been manifested from versatility of integrin molecules to activate numerous signaling cascades as cells faced with mechanical force. As it is discussed above, when the stiffness of the TME is increased, integrin clustering is activated which in turn promotes EMT and stemness characteristics of the cancer cells. On the other hand, when the stiffness of the healthy lung ECM is elevated, the expression of these markers are upregulated which might lead to downregulation of integrin molecules. The EMT and stemness properties of the cancer cells are decreased as a result. As cells lacking the functional pair of PI3K gene in stiff TME, the expression of these markers is elevated suggesting that there might be another pathway which compensate the absence of PI3K through activation of integrins. As clustering of integrins are

promoted when the stiffness of the TME is increased, it is possible that integrins could have activated RAS or MAPK pathways to bypass the absence of PI3K gene [148].

The clustering of integrins and further activation seemed to be promoted in a soft and healthy environment compared to stiff one. The expression of EMT and stemness pathways are downregulated as with the absence of PI3K gene in healthy ECM composition. This might have stemmed from the fact that the integrin molecules could not activate other pathways to bypass the absence of PI3K gene when the stiffness of the healthy matrix is increased. As the activation of integrins seemed to be downregulated in the stiff matrices due to matrix content, cells might not cope with the downregulation of integrin receptors in the absence of PI3K gene. Thereby, the synergistic impact of the downregulation of both integrins and PI3K results in the downregulation of EMT and stemness properties of cancer cells in healthy environment. These results reveal that stiffness alone does not trigger the activation of EMT and stemness pathways, rather, the matrix composition is of vital importance in orchestrating the behavior of cancer cells.

Chapter 5: **CONCLUSION AND OUTLOOK**

Examining the native-like behavior of cells in a fully defined 3D in vitro model system where the biochemical and biomechanical properties of the native ECM are well recapitulated key in understanding how cancer initiates and progresses. Establishing a model which allow independent modulation of physical properties from chemical signature is a great value in dissecting the role of individual parameters on regulating cellular behavior. Keeping this in mind, in this study, a double network composing of alginate and decellularized healthy lung ECM or Cultrex, a commercial tumorigenic matrix that is isolated from EHS, is generated through crosslinking via divalent cation, Ca^{2+}. The stiffness of the microenvironment is tuned by altering the Ca^{2+} concentration whereas the matrix composition and concentration remains the same. Utilizing alginate and decellularized lung ECM, an *in vitro* model which recapitulates the composition and biomechanical properties of the healthy lung is established to examine *in vivo* like characteristic of the cells. In addition, the model which is made up from the crosslinking of alginate and Cultrex matrix represents the hallmarks of tumorigenic growth in a fully defined 3D model. The fact that, the stiffness of these models is decoupled from their composition made them an ideal tool for studying the impact of stiffness on regulating cellular behavior.

Mechanotransduction is an important phenomenon which regulates a plethora of cellular events. One of the most crucial mediators of the mechanotransduction is integrin receptors found in the focal adhesion sites. Integrins are evolved to received mechanical cues and transmit them into chemical signals. During malignancy, the biochemical signature of the ECM is altered which manifest stiffening of tumor tissues. Thereby, the mechanotransduction pathways are significantly altered in a way that promotes tumorigenesis which, in most cases, induces the EMT and stem-like abilities of the cancer cells. However, the role of elevated tissue stiffening on modulating the behavior of cancer cells which are surrounded with either healthy or tumorigenic ECM compositions have not been studied to our knowledge. Thereby, the impact of being surrounded with a healthy or tumorigenic ECM composition with same stiffness values on regulating tumorigenesis should be investigated to better model disease growth.

According to our findings, elevated stiffening of the tumor microenvironment promotes the proliferation, EMT and stemness properties of the cancer cells. Whereas the stiffening of the healthy ECM abrogates this phenomenon as EMT and stemness pathways are upregulated in soft environment. In line with this, our findings demonstrate that, increase stiffening of the tumor microenvironment promotes the clustering of integrins, whereas it decreases the activation of integrin molecules in healthy ECM. This is consistent with our hypothesis that the elevated stiffness alone is not sufficient for promoting the tumorigenic phenotype, rather, the composition should be tumorigenic as well.

As the activation of integrins correlates with the upregulation of EMT and stemness factors in both healthy and tumorigenic matrix, PI3K gene, an integral mediator of integrin dependent mechanotransduction, is knocked down to examine whether tumorigenic ECM could compensate its absence. Based on our finding, elevated stiffening of the tumorigenic ECM bypass the absence of functional ECM. Whereas, in healthy lung where the activation of EMT and stemness properties are promoted in soft microenvironment, the absence of PI3K gene could not be compensated with ECM composition. This result might be orchestrated by the composition of the tumorigenic matrix which dictates the activation of integrin dependent pathways as tissues stiffen.

According to our findings, matrix composition is of utmost importance in mediating malignancy of tumors. The impact of stiffening depends on the composition of matrix. Tumorigenic ECM promotes acquiring mesenchymal properties and preserves stem like characteristics, whereas these effects are abrogated in healthy ECM when stiffness is increased in a fully defined model. In addition, the clustering of integrins and its downstream pathways are promoted as the stiffness of tumorigenic matrix is increased, on the other hand, they are downregulated in healthy composition. In addition to that, tumorigenic ECM composition can compensate the absence of PI3K which is considered as one of the most integral players of integrin dependent mechanotransduction pathways, however, healthy matrix composition is not capable of such compensating. This phenomenon might be orchestrated by other cascades that act synergistically with integrin in the absence of PI3K in tumorigenic matrix. According to these findings, stiffening of the tissues do not provoke cancer progression when they are surrounded with a healthy ECM.

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