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Mimicking the Arterial Microenvironment with PEG-PC to Investigate the Roles of Physicochemical Stimuli in SMC Phenotype and Behavior

William G. Herrick
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**MIMICKING THE ARTERIAL MICROENVIRONMENT WITH PEG-PC TO
INVESTIGATE THE ROLES OF PHYSICOCHEMICAL STIMULI IN SMC
PHENOTYPE AND BEHAVIOR**

A Dissertation Presented

by

WILLIAM GERARD HERRICK

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2015

Chemical Engineering

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By

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Approved as to style and content by:

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Juan Anguita, Member

John Collura – Interim Department Head
Chemical Engineering

DEDICATION

To my parents, Jane Marie Herrick and William Donald Herrick

For always supporting and believing in me

And to my brother, Corey Donald Herrick

For teaching me that life isn't always so easy, but we don't give up

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mechanic, they have always done everything in their ability to help me achieve success and happiness.

ABSTRACT

MIMICKING THE ARTERIAL MICROENVIRONMENT WITH PEG-PC TO
INVESTIGATE THE ROLES OF PHYSICOCHEMICAL STIMULI IN SMC
PHENOTYPE AND BEHAVIOR

MAY 2015

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Directed by: Assistant Professor Shelly R. Peyton

The goal of this dissertation was to parse the roles of physical, mechanical and chemical cues in the phenotype plasticity of smooth muscle cells (SMCs) in atherosclerosis. We first developed and characterized a novel synthetic hydrogel with desirable traits for studying mechanotransduction *in vitro*. This hydrogel, PEG-PC, is a copolymer of poly(ethylene glycol) and phosphorylcholine with an incredible range of Young's moduli (~1 kPa - 9 MPa) that enables reproduction of nearly any tissue stiffness, exceptional optical and anti-fouling properties, and support for covalent attachment of extracellular matrix (ECM) proteins. To our knowledge, this combination of mechanical range, low price, and ease-of-use is unmatched by any other hydrogel. We further used PEG-PC to evaluate the impact of substrate stiffness on the proliferation and adhesion properties of three cancer cell lines in 2D, from which we conclude that mechanotransduction is cell type-dependent and differences in focal adhesion-mediated signaling affect proliferation outcomes.

With PEG-PC as a substrate, we then designed a complex *in vitro* model to recapitulate characteristic changes in the surrounding microenvironment that SMCs experience during the progression of atherosclerosis. These changes include the composition of the ECM, the availability of soluble factors, and the surrounding mechanical environment. Our findings point to ECM composition as the primary regulator of SMC behaviors and characteristics, in part by modulating the effects of soluble factors. Unexpectedly, changes in substrate stiffness had a relatively modest effect. In spite of large ECM-directed differences in proliferation and motility, we did not find that these behaviors are inversely related to SMC marker expression, nor was marker expression substantially dependent on ECM composition despite being regulated by focal adhesion kinase signaling. Finally, our findings suggest that the transition from a migratory to a proliferative phenotype in atherosclerosis is mediated by the changing ECM composition, and we propose hypothetical, integrin-driven models to explain this switch. From these conclusions, we emphasize the importance of increasing the complexity of *in vitro* models to carefully match critical features of the *in vivo* microenvironments. We expect this approach to produce physiologically relevant behaviors, and in doing so we may identify novel, context-dependent therapeutic targets.

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CHAPTER 1

INTRODUCTION

1.1 The devastating impact of atherosclerosis on human health

Since at least 1900, the leading cause of non-infectious death nearly every year in the Western world has been heart disease or atherosclerosis-related [1]. In 2010 alone there were 596,577 deaths attributed to heart disease, of which 62.9% are attributed to ischemic heart disease and 5.6% are attributed to hypertensive disease, both most commonly caused by atherosclerosis. Examined further, 21% of these heart disease-related deaths were due to myocardial infarction, 42% due to ‘other forms of chronic ischemic heart disease,’ and 30.3% due to ‘other heart diseases’ such as acute endocarditis, heart failure and ‘all other types of heart disease’ [2]. Furthermore, the picture worsens if we consider cerebrovascular diseases (128,932 deaths in 2009) and aortic aneurysm (10,073 deaths), other common results of atherosclerotic plaque disruption. Altogether this represents 738,852 deaths or 29.2% of all deaths in the United States in 2011, a figure which matches the World Health Organization’s figures for atherosclerosis-related deaths worldwide in 2008 [3]. In contrast, all forms of malignant neoplasms (cancer) combined accounted for 576,691 deaths or 22.3% in the United States in 2011.

Heart disease and cancer are major public health problems that need considerable research and work to address. The scope of the problem is even greater when we consider that the rates of obesity [4] and type II diabetes [5] in the United States have risen steadily since 1990 – current estimates are that 20% of children [6] and 36% of adults [4] are obese. Both conditions are believed to be interrelated and are significant risk factors for heart disease and cancer. This is not especially surprising given the large number of similarities

between atherosclerosis and many types of cancer, including hyperproliferative cells and fibrotic tissues.

1.2 What is atherosclerosis?

1.2.1 The pathogenesis of atherosclerosis

Atherosclerosis, literally “hardening of the arteries,” is characterized by the development of atherosclerotic plaques, also known as an atheroma. The disease earns its name from the progressive stiffening or hardening of the arteries affected by plaque formation, in particular when the plaques become fibrotic and calcified. Plaques begin as small, fatty lumps or streaks on the arterial wall, but under the wrong conditions these fatty streaks coalesce into larger fatty deposits and develop into plaques which protrude into the bloodstream and occlude blood flow (Fig. 1.1) [7]. Occlusion of blood flow from plaque protrusion can lead to hypertension and eventually ischemia, which has potentially life-threatening consequences. Plaques can form anywhere in the large arteries, but are most problematic when they grow uncontrollably in the cerebral arteries (increasing risk for stroke) and coronary arteries (increasing risk for myocardial infarction) [7]. They can also develop in the aorta, increasing risk for type III aortic dissection [8], and abdominal aortic aneurysms [9]. Atherosclerosis is also a major cause of peripheral arterial disease [10], a leading cause of lower limb amputation in diabetic patients [11].

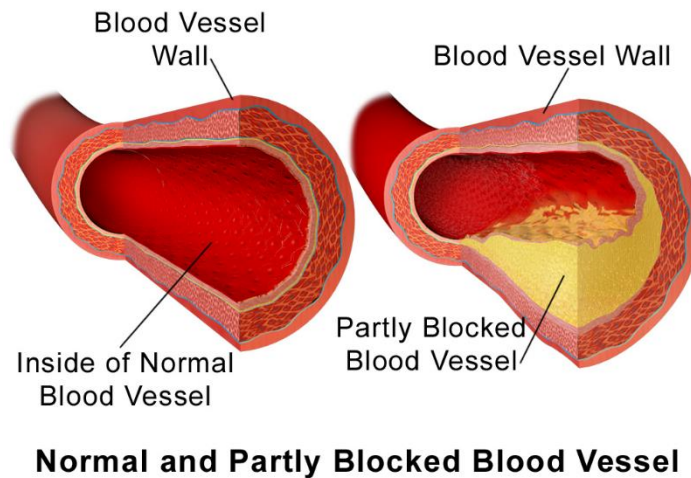


Figure 1.1: Illustration of a normal and blocked artery

"Blausen 0052 Artery NormalvPartially-BlockedVessel" by BruceBlaus - Own work. Licensed under Creative Commons Attribution 3.0 via Wikimedia Commons - http://commons.wikimedia.org/wiki/File:Blausen_0052_Artery_NormalvPartially-BlockedVessel.png#mediaviewer/File:Blausen_0052_Artery_NormalvPartially-BlockedVessel.png

1.2.2 The healthy vasculature

The medium- and large-sized elastic arteries affected by atherosclerosis are comprised of 3 distinct tissue layers (Fig. 1.2). The outermost layer, the adventitia, is largely acellular and composed primarily of connective tissues, especially collagen. Its function is to anchor arteries to the bones and organs of the body and keep them in place. The innermost layer, the intima, is in contact with flowing blood and is composed of a single layer of endothelial cells attached to a thin basement membrane of connective tissues. The middle layer, the media, is responsible for imparting the elastic stretch-recoil properties that allow the vascular wall to retain mechanical integrity under pulsatile blood flow. These properties are imparted by smooth muscle cells (SMCs) arranged in concentric layers sandwiched between connective tissues and thick sheets of elastin [7,12].

These SMCs are in a ‘contractile’ phenotype, so-called because of their robust actomyosin contractile apparatus that imparts resilience to mechanical stretch and strain.

The extracellular matrix proteins comprising the connective tissues are crucial to the unique mechanical properties of large arteries, but they also affect cell phenotype and behavior through integrin signaling and sequestration of growth factors. In an undamaged media, SMCs are in immediate contact with a thin layer of basement membrane (or reticular lamina; the correct nomenclature is unclear) comprised largely of laminin and the non-fibrillar type IV collagen. Both proteins are major components of Matrigel™, a protein mixture purified from Engelbreth-Holm-Swarm mouse sarcoma, which is commonly used for cell culture and known to promote differentiated phenotypes *in vitro* [13,14]. Indeed, the combination of laminin and collagen IV has been shown to promote expression of contractile markers in SMCs [15,16], but evidence points to laminins having the greatest impact [16–18], especially when the cells are exposed to cyclic stretch [19]. Their effects are most likely mediated by integrin signaling, but there may be other mechanisms at work. Collagen IV has also been implicated in phenotype maintenance of smooth muscle cells, but this finding has not been confirmed in other studies [18]. Regardless, collagen IV does make up about 50% of the basement membrane and forms suprastructures with laminins [20]. It may have anti-proliferative effects, in part, by binding and sequestering growth factors including PDGF and FGF [21].

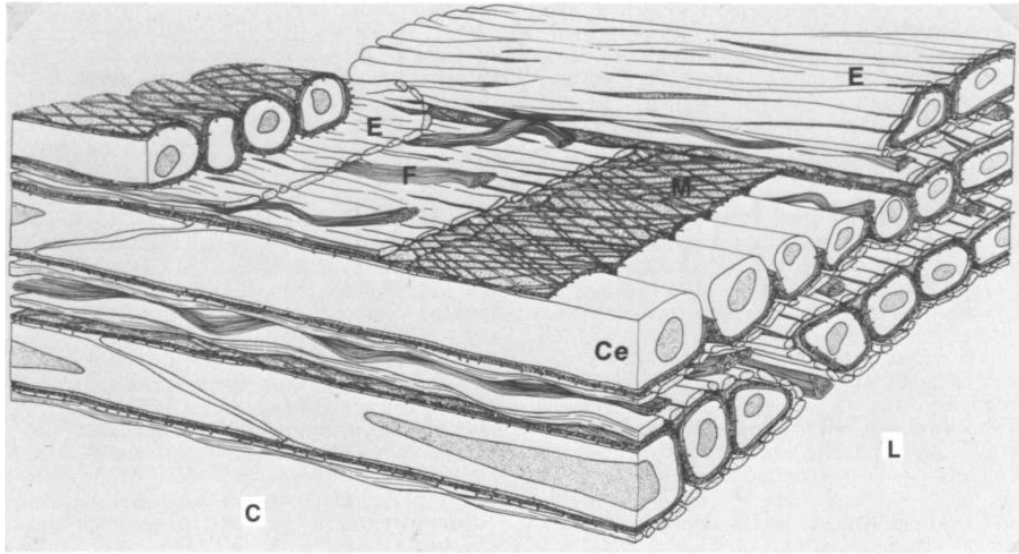


Figure 1.2: Illustration of the arrangement of SMCs and ECM proteins in the media

A detailed depiction of the healthy arterial media. E = elastin fibers, F = collagen I fibers, M = basement membrane and collagen III mesh, Ce = SMC, C = circumferential cut, L = longitudinal cut. Figure adapted with permission from [12], Copyright © 1985 Wolters Kluwer Health.

Immediately adjacent to the thin basement membrane layer resides a mesh-like network of collagen III fibrils (Fig. 1.2) [12], which is surrounded by thick sheets of elastic laminae composed of crosslinked elastin protein. Finally, thick bundles of collagen I are interspersed between the elastic laminae. These bundles of SMCs and connective tissues, collectively termed “lamellar units,” are primarily responsible for the elasticity and mechanical integrity of large arteries [22]. It is unclear if SMCs in the media are directly adhered to the collagen networks, but fibrillar forms of both types of collagen and elastin fibers have been demonstrated to promote the contractile phenotype in SMCs *in vitro* [18].

1.2.3 Physicochemical factors in atherosclerosis

While it is not known with certainty how atherosclerosis starts, it is widely believed that vascular inflammation is the most likely initiator of plaque formation. The inflammatory response leading to atherosclerosis is initiated, in part, by low density lipoproteins (LDLs) and very low density lipoproteins (VLDLs) embedding in the vascular subendothelial space [7]. Atherosclerosis results when vascular inflammation is chronic, and the persistent inflammation seems likely to be the result of a combination of factors. Implicated pro-inflammatory factors include those mentioned above, plus oxidized low density lipoproteins (oxLDLs), advanced glycation end-products (AGEs) [23], and inflammatory factors such as interleukins and interferons [24].

Regardless of the initial cause, fatty streaks form from the embedding of LDL and VLDL particles and their uptake by invading monocytes [7]. In the subendothelial space, monocytes differentiate into macrophages and impact plaque progression in multiple ways: they secrete growth factors and cytokines that ‘activate’ endothelial cells, which, in turn, also produce growth factors and cytokines; they express scavenger receptor A to increase the uptake of LDL particles [25]; they express matrix metalloproteinases (MMPs) and migrate towards the intima, digesting the connective tissues in their path; they increase uptake of LDL particles and can eventually turn into fat-laden ‘foam cells’ which apoptose at a higher rate and fill the subendothelial space with extracellular fat deposits [7]; and they may also secrete disordered fragments of elastin and collagen [26,27].

All of these factors serve to irreversibly damage the arterial wall and contribute to phenotype switching of SMCs in the media. SMCs respond to the soluble growth factors and cytokines – which have either diffused there or are secreted by invading macrophages

– by expressing MMPs [28], digesting their surrounding matrix and migrating towards the developing plaque [7]. These atherosclerosis-associated SMCs have dedifferentiated into the so-called ‘synthetic’ phenotype, which is similar to that of myofibroblasts. In some plaques, SMCs proliferate rapidly and secrete excess collagen, fibronectin, and elastin fragments that contribute to plaque size and form a fibrotic cap, which is the type plaque from which atherosclerosis derives its name [7]. However, they may also apoptose and/or take up LDL particles and become foam cells, much like macrophages, which may contribute to a softer plaque that is believed to be more prone to rupture [29]. Of course, the degradation of the medial connective tissues during their migration to the plaque results in the loss of mechanical integrity, which along with occlusion of blood flow will contribute to hypertension [30].

The atherosclerotic plaque is a heterogeneous environment with dozens of soluble factors identified *in vitro* as having potential roles in disease progression. These soluble chemicals are believed to, on the whole, increase the rates proliferation of resident cells, as well as their uptake of LDL particles and synthesis of extracellular matrix proteins and lipids [31,32]. The majority of these soluble factors are classified as cytokines with roles in modulating the immune response at sites of vascular inflammation. Interleukins, secreted peptide cytokines, are one such class of immunomodulators produced by macrophages and endothelial cells. Interleukin-1s (IL-1), actually a family of 4 proteins, are believed to prolong the inflammatory response by inducing resident endothelial cells to produce more cytokines (IL-6, IL-8, monocyte chemoattractant protein, tumor necrosis factor), by upregulating MMP expression, and by promoting transendothelial migration of leukocytes and monocytes by upregulating ICAM-1 and VCAM-1 [33]. Interleukin-8 has similar

positive feedback effects on inflammation and can simultaneously activate several signaling pathways associated with cytoskeleton dynamics, protein synthesis and enhancement of growth factor signaling [34]. Interleukin-6 also has pro-inflammatory effects and is produced by immune cells and SMCs in plaques [35]. Tumor necrosis factor alpha (TNF α) is another cytokine produced by macrophages and endothelial cells implicated in proliferation, cell survival and production of pro-inflammatory eicosanoids and interleukins [36].

Growth factors are another major promoter of atherosclerotic lesion formation through their effects on cell proliferation, survival and migration. The most potent growth factor to SMCs *in vitro*, platelet derived growth factor BB (PDGF-BB), is released in lesions by endothelial cells, macrophages and SMCs. It has potent proliferative and migratory effects on synthetic phenotype SMCs, but little effect on contractile SMCs [37]. Proliferation is induced by promoting splicing of histone deacetylase 7 (HDAC7) [38] and migration by promoting phosphatidylinositol turnover and calcium signaling [37,39].

Prostanoids, a subclass of eicosanoids, are another class of soluble factors with roles in atherosclerosis. These include thromboxane, a molecule involved in thrombosis by promoting platelet aggregation, and a variety of prostaglandins with effects on inflammation, proliferation and platelet aggregation (i.e. prostacyclin I₂, prostaglandin E₂) [40]. However, whether prostaglandin E₂ is atheroprotective or promotes atherosclerosis is unclear, and its effects are concentration-dependent [41].

The downregulation of certain atheroprotective compounds, for instance, nitric oxide (NO), is also implicated in atherogenesis. NO is normally produced by the endothelial cells of the intima, which upregulate expression of endothelial nitric oxide

synthase (eNOS) under laminar flow conditions *in vitro* [42]. This latter observation may explain why atherosclerotic plaques are more commonly found near bends, turns and bifurcations in the arterial tree [42], where laminar flow is disturbed. NO is known to inhibit migration and proliferation of vascular smooth muscle cells *in vitro*, possibly implicating defective NO production in their aberrant behavior during atherosclerosis [43,44].

Transforming growth factor beta (TGF β) is another factor believed to protect against atherosclerosis, possibly through its role in reversing the wound repair process [45]. Patients with advanced atherosclerosis have been observed to have reduced serum levels of TGF β [46], but concentrations in plaques are not necessarily altered. Instead, SMCs in atherosclerotic lesions in humans are found to have reduced levels of the type II TGF β receptor [45], expression of which is required for functionality of the type I receptor. This alteration in the ratio between the type I and II TGF β receptors with disease and aging may explain, in part, the different responses to TGF β by young and old SMCs and other differential, context-dependent effects [47–52].

Besides these soluble factors, it is becoming increasingly clear that the extracellular matrix components in contact with cells can have an impact on disease progression. SMCs involved in atherosclerosis must degrade and migrate through basement membrane proteins and fibrous collagens and elastin, and it is highly likely that the changing environment affects SMC phenotype and behavior. While the basement membrane proteins are observed to have protective effects *in vitro*, the effects of the collagens and other proteins are definitely context-dependent.

Although the effects of collagen III on SMC phenotype have not been extensively studied, it has been reported that fragmentation of collagen III with UV irradiation increases proliferation and expression of ICAM-1 and VCAM-1, while simultaneously downregulating β_1 integrins and the focal adhesion-associated proteins vinculin, talin, and the intermediate filament vimentin [53]. The synthesis of collagen III by synthetic phenotype SMCs increases when treated with TGF β , IL-1 or PDGF, however, interferon-gamma reduces synthesis, even with co-treatment with TGF β [54].

Similar to collagen III, monomeric collagen I has been shown to promote proliferation and suppress synthesis of extracellular matrix proteins, actin-binding proteins and signaling molecules [55,56]. The effects of collagen I on SMCs *in vitro* is rather similar to the effects of collagen III [57], and it is found in abundance in atherosclerotic lesions, especially within the SMC-laden fibrous cap [58]. SMCs have been shown to increase collagen synthesis in response to many factors commonly found in lesions, especially PDGF-BB, endothelin-1, IL-1, homocysteine, TGF β and mechanical stretch [58]. However, despite the typical association of collagen I and III with the synthetic phenotype, other studies have found that polymerized or fibrilized collagens can help induce the contractile phenotype, in some instances [55,56]. If this effect is relevant *in vivo*, it is presumably reversed when the SMCs are induced to degrade their environment and invade a plaque.

A major extracellular matrix protein found in plaques, fibronectin, is initially laid-down with fibrin as a provisional matrix during the wound repair response [59]. However, since the wound repair process is disrupted in atherosclerosis, the fibronectin deposits are not necessarily cleared out and replaced with appropriately-structured collagen fibers. The

abundant fibronectin in plaques is believed to help promote the proliferative, synthetic phenotype in SMCs, which is supported by *in vitro* studies [60].

The soluble and physical factors discussed above are just a small subset of the dozens or hundreds of chemicals and proteins that may have significant effects in the onset and progression of atherosclerosis. Not discussed in detail but still of importance are molecules such as various proteoglycans (especially chondroitin sulfate and heparan sulfates), which have roles in sequestration of growth factors and as co-receptors [61], as well as LDL and oxidized LDL particles [7], advanced glycation end products which may additionally stiffen plaques [23], and insulin and insulin-like growth factors (but the role of IGF-I is debated) [31] that are frequently elevated in patients with atherosclerosis. All of these factors and likely many more are important to understanding atherosclerosis, but the heterogeneity of plaques suggest that they may not all be important in every instance.

1.2.4 The influence of mechanics in the progression of atherosclerosis

Given their role in mechanical integrity of the arterial wall, the effects of various mechanical stimuli on SMCs is an extensively studied area. As mentioned previously, cyclic stretch can promote the contractile SMC phenotype *in vitro* [19], but it could also be implicated in promoting atherosclerosis by inducing production of elastin and collagen I [62]. Another effect of blood flow is reflected in how certain portions of the arterial tree are prone to plaque development due to low fluid shear stress, which has been found to induce downregulation of eNOS in endothelial cells of the intima and thereby reduce diffusion of atheroprotective NO to medial SMCs [42]. Disrupted blood flow mechanics may have other effects, for instance from transmural interstitial flow due to a damaged,

leaky endothelium, which may potentially contribute to MMP-1 expression and SMC migration [63].

In vitro studies have revealed that differences in static mechanics, typically in the form of hydrogels with varying stiffnesses, may also impact SMC phenotype and behaviors. On 2D substrates, SMC proliferation tends to increase with substrate modulus, but so does expression of contractile phenotype markers, and migration is also modulus-dependent [64,65]. In 3D cell cultures, migration is reported to be strongly dependent on mesh size [66], and once again proliferation and contractile phenotype markers increase with modulus [67]. Some of these findings are unintuitive given how atherosclerotic plaques tend to stiffen, but other studies find that mesh size is more important than modulus [68], and it is very possible that SMC phenotype in atherosclerosis is not as well understood as believed.

1.2.5 Late stage complications in atherosclerosis and prevention

Further complications arise in the late stages of atherosclerosis, especially fibrosis and calcification. As discussed above, atherosclerotic plaque composition can vary greatly, and in some cases SMCs arranged near the luminal surface of the plaque produce excessive amounts of extracellular matrix proteins forming a fibrous cap [7]. Cells in other plaques can differentiate into osteoblast-like cells that secrete bone-related proteins including osteopontin [69] and calcium salts in the form of hydroxyapatite [70]. Fibrosis and/or calcification of plaques can further harden the artery wall, but a stiffer wall may actually stabilize the plaque and prevent a deadly rupture event [71,72].

Due to the potentially life-threatening consequences of rupture, much effort has been spent on therapies to improve stability [73,74] and on ways of non-invasively evaluating stability in at-risk patients [75]. Plaque rupture is a serious complication of atherosclerosis that can cause death or brain damage. When a rupture occurs, the underlying collagenous tissues are exposed to the blood stream and the coagulation cascade is rapidly induced to attempt to patch the rupture with the formation of a clot (thrombosis) [7]. However, if the clot embolizes from the site of rupture, the effects are often severe. The most common life-threatening consequence of embolism occurs with atherosclerosis of the coronary arteries (myocardial infarction), with atherosclerosis of the cerebral arteries being the second most common occurrence (stroke).

Current efforts to treat atherosclerosis focus on prevention through dietary intervention and exercise, with an emphasis on low fat foods and ‘heart healthy’ grains. However, this view is being increasingly questioned [76–79] in light of considerable evidence that low fat diets may actually be more harmful than helpful. In fact, our current obesity epidemic began at about the same time the US Senate Select Committee on Nutrition and Human Needs issued low fat dietary guidelines in 1977 (Fig. 1.3), and obesity is a significant risk factor for atherosclerosis, diabetes, cancer and related diseases.

Since there has been limited success from dietary interventions, statins have become the first line of defense in prevention for patients with pre-existing heart disease, or at high risk of developing it. Statins, including the blockbuster drug Lipitor [80], are a class of drugs approved for inhibition of 3-hydroxy-3-methyl-glutaryl-Coenzyme A reductase (HMG-CoA reductase), a rate-controlling enzyme in the cholesterol synthesis pathway [81]; However, statins have since been shown to have other effects that can

explain their efficacy, including inhibition of both Rho kinase [82,83] and the uptake of oxidized LDL particle [84]. There is also some evidence that statins may be contributing to the incidence of dementia in elderly patients [85], but the connection is considered tenuous. Statins have been proven effective in reducing the rate of myocardial infarction in patients with pre-existing heart disease, but their efficacy at primary prevention was less certain until the last couple years [86–89]. There are also surgical options for secondary prevention, including stents which mechanically widen an occluded artery [90]. Unfortunately, while stent implantation may be life-saving in many cases, the stent itself damages the artery wall and induces inflammation and restenosis.

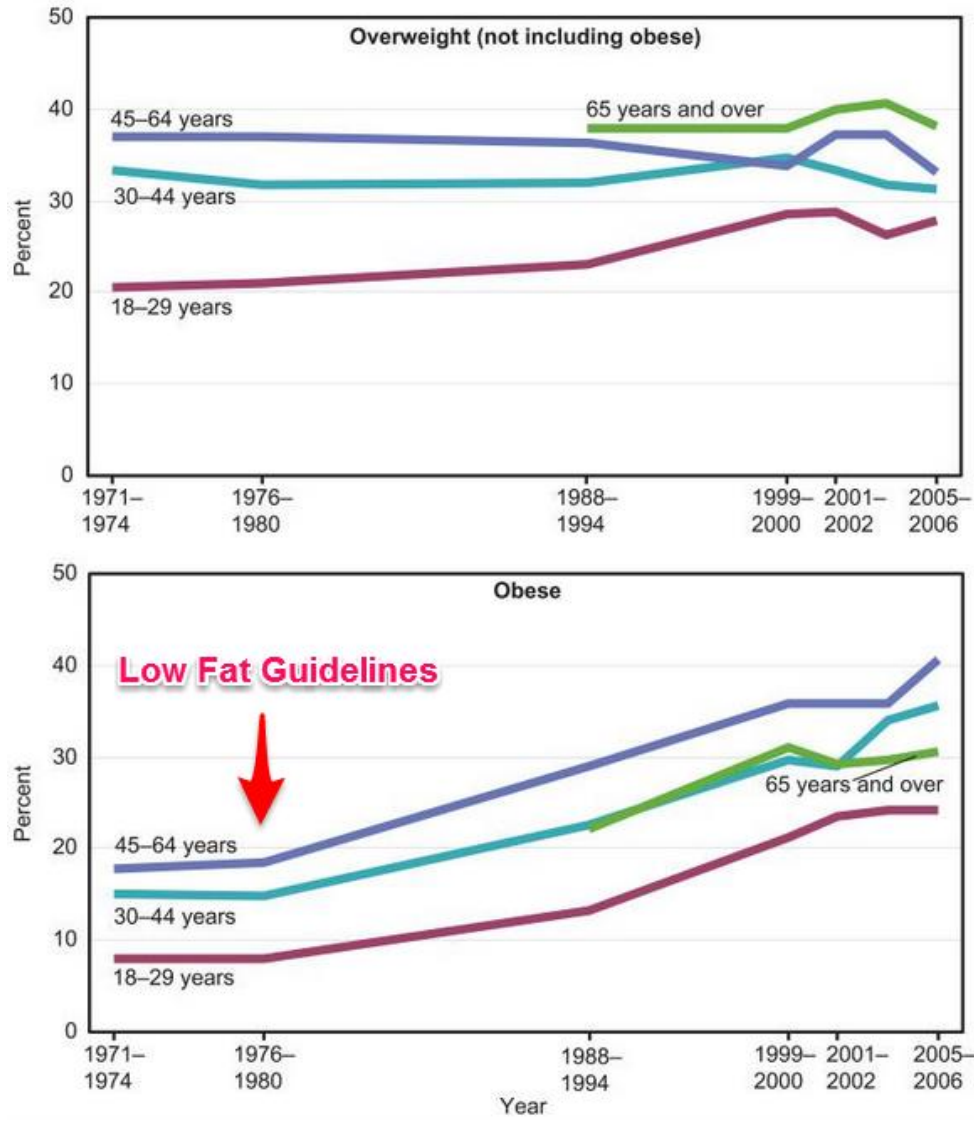


Figure 1.3: The current obesity epidemic began around the same time that low-fat dietary guidelines were issued by the US Government

Source: National Center for Health Statistics (US). Health, United States, 2008: With Special Feature on the Health of Young Adults. Hyattsville (MD): National Center for Health Statistics (US); 2009 Mar. Chartbook.

1.3 Smooth muscle phenotype heterogeneity

Of note is the phenotypic heterogeneity of SMCs in the arterial wall – they do not exist strictly in a ‘contractile’ or ‘synthetic’ phenotype, but rather along a spectrum characterized by the expression of certain marker proteins, and are capable of

transdifferentiation under the appropriate conditions [91]. The inherent heterogeneity can partly be explained by the fact that vascular SMCs are derived from multiple embryonic origins, but this is an incomplete explanation [92]. The picture is complicated further by observations that, in some cases, a portion of the SMCs present in atherosclerotic plaques are actually derived from circulating bone marrow-derived progenitor cells [93], but this is controversial [94,95]. It remains likely, however, that the vast majority of the SMCs in atherosclerotic plaques originate from the arterial media.

The marker proteins expressed by contractile SMCs are, not surprisingly, typically associated with the actomyosin contractile apparatus, and have roles in regulating contractility. With the exception of smoothelin [96], most SMC markers can also be found in myofibroblasts in some, but not all, cases, which further supports the idea of a phenotype spectrum. The earliest expressed SMC marker, which is also considered the primary marker of myofibroblasts, is α -smooth muscle actin (α -SMA), an actin isoform that makes up a large portion of the contractile apparatus in smooth muscle. However, α -SMA is a necessary, but insufficient marker of the contractile SMC phenotype *in vitro* due to similarities between myofibroblasts and the synthetic SMC phenotype.

Mid-stage markers of the contractile phenotype include caldesmon, calponin and smooth muscle myosin heavy chain (SM-MHC). Caldesmon is an actin-binding protein with 2 distinct isoforms, heavy and light (h-CaD and l-CaD), with h-CaD being expressed in SMCs and l-CaD in all tissues, including SMCs. In fact, when SMCs dedifferentiate into the myofibroblast-like synthetic phenotype *in vitro*, they convert to predominantly expressing the l-CaD isoform. Interestingly, caldesmon is part of the cytoskeleton due to its binding and stabilizing actin filaments, but the heavy isoform has additional functionality

by inhibiting myosin ATPase activity, giving it a role in the contractile apparatus in smooth muscle. This difference may be explained by the only structural difference between the light and heavy isoforms, a short, charged spacer linking the actin-binding domain and the myosin-binding domain, which could provide the necessary space for interacting with myosin in SMCs [97].

Similarly, calponin is part of both the contractile apparatus and the cytoskeleton, with actin-binding and myosin ATPase-inhibiting domains, and 2 major isoforms, basic (h1) and acidic (h2) [98]. Like caldesmon, the basic isoform is more strongly expressed in smooth muscle cells, and expression is converted upon dedifferentiation to the synthetic phenotype. Ironically, calponin itself only has a single calponin homology (CH) domain but does not bind actin through this domain, while a doublet of this domain in other proteins (e.g. α -actinin and filamin, and many more) is responsible for actin binding [99]. Instead, a regulatory domain is responsible for actin binding, while yet another domain interacts with and inhibits the ATPase-activity of myosin II. The CH domain enables interaction with other contractile proteins like tropomyosin, Ca^{2+} /calmodulin, as well as the signaling protein ERK [100]. While calponin itself is not a Ca^{2+} -dependent, as the name would suggest, it binds Ca^{2+} /calmodulin, which phosphorylates calponin and inhibits its myosin-binding activity to modulate contractility [101].

Smooth muscle myosin heavy chain is a major functional protein of the contractile apparatus, but not a member of the cytoskeleton. It is the smooth muscle-specific isoform of the heavy chains of myosin II, with two major splice variants, SM1 and SM2. Non-muscle cells express non-muscle MHC (NM-MHC), and upon dedifferentiation to the synthetic phenotype, SMCs shift expression from SM- to NM-MHC [102]. It is likely that

this smooth muscle-specific isoform is required for interactions with the other smooth muscle contractile proteins, like calponin and caldesmon, and these interactions impart unique contractile properties to smooth muscle.

The latest SMC marker recognized, smoothelin, is also purported to be the only truly exclusive SMC marker [96]. It has 2 isoforms, a shorter smoothelin-A and longer smoothelin-B, which are predominantly found in visceral and vascular smooth muscle, respectively [103]. It is an actin binding protein, interacting with filamentous actin through CH domains [104], and evidence from knock-out studies in mice points to a role in the contractile apparatus [105]. However, the complete function of smoothelin remains unknown.

With the exception of smoothelin-B (and only weakly for smoothelin-A), transcription of SMC marker genes is regulated by serum response factor (SRF) and an SRF co-activator, myocardin, binding to CArG boxes in contractile marker gene promoters [106]. MicroRNAs have garnered attention in cardiac and smooth muscle research in the past several years, with the finding they have profound effects on the regulation of cellular phenotype. In particular, expression of the microRNAs miR-143 and miR-145 in SMCs is induced by myocardin/SRF, forming a positive feedback loop due to the effects of miR-143/145 on suppressors of myocardin expression or activity; they also suppress non-myocardin SRF co-activators that would otherwise enable SRF-binding to CArG boxes on genes known to promote dedifferentiation to the synthetic phenotype [107]. MicroRNA-1 is also positively regulated by myocardin [108] and inhibits SMC proliferation, whereas the role of microRNA-133 is less certain [109] because it positively affects some SMC markers (SM-MHC), but decreases expression of others (calponin, transgelin-2 and α -

SMA). However, it has an overall positive effect by reducing SMC migration and proliferation. One thing is clear: the study of SMC phenotype regulation is extremely complex, and it is further complicated and confounded by differences in contexts and even the species of cells used in various studies.

1.4 *In vivo*/animal models

Researchers have developed several animal models for inducing (or resisting) plaque formation reminiscent of that found in humans, most frequently in mice, rabbits and rats. These models are very important for corroborating *in vitro* data and testing potential drugs and treatments for reversing or preventing atherosclerosis.

The earliest work with animal models and atherosclerosis took place primarily in Russia in the early 1900's [110], with a focus on the dietary causes of fatty streak formation in rabbits. However, the use of rabbits as an animal model of human atherosclerosis is specious, because they are exclusively herbivorous in nature and regulate lipids very differently from humans [111].

In recent years, mice have seen much more frequent use in studies of atherosclerosis, especially the apoE^{-/-} knockout mouse. These mice spontaneously forms advanced lesions in the aortic root, as well as in other portions of the arterial tree with a high cholesterol diet. Other transgenic mice have been developed as models dyslipidemia (the SR-BI knockout), familial hypercholesterolemia (the LDLR^{-/-} mouse), and for features of metabolic syndrome (*db/db*, *ob/ob* mice) [111].

Other less-frequently used animal models include rats and hamsters, and the difficult-to-use nonhuman primates and pigs. Rats are, however, frequently used in a balloon injury

model of restenosis, a pathological process with many similarities to atherosclerosis [112,113]. Certain primates (e.g. chimpanzees, rhesus monkeys) and pigs are possibly the ideal animal models for human atherosclerosis, owing to their similar diets, circulatory systems and lipid regulation. Unfortunately, they are also large, expensive to care for, have long lifespans and time to maturation, and their use in biomedical research is ethically challenging.

1.5 *In vitro* models

There are numerous reports of *in vitro* model systems designed to mimic one or more features of the vasculature. These models are used to investigate SMC phenotype and behaviors, including proliferation and migration in response to growth factors and ECM proteins, and the expression of contractile SMC markers. To reflect the *in vivo* reality, SMC differentiation is reported to be enhanced by coculture with endothelial cells [114–116], which most likely induces differentiation and quiescence due the secretion of soluble factors by endothelial cells, most prominently, NO. In order to model changes with arterial stiffness with aging and disease, several groups have used mechanically-tunable hydrogels [64,67,68,117–122]. These reports investigate static mechanical effects on proliferation and cell area, which are positively affected, as well as 2D and 3D migration, and marker expression. Another group has reported that culturing SMCs on an NO-generating polymeric biomaterial [123] reduces proliferation, which recapitulates some features of SMC/endothelial cell coculture.

There have also been numerous reports on more mechanically complex model systems. For instance, the application of cyclic stretch or strain is used as a model of the

mechanical environment produced by pulsatile blood flow [19,62,119,124,125], and various SMC behaviors are reportedly affected, including secretion of ECM proteins and cytokines, proliferation, and cell shape. However, there is disagreement in these literature reports on whether or not cyclic strain is beneficial to SMC phenotype, which is likely a consequence of the specific contexts studied. Other systems model the effects of a damaged, leaky vascular endothelium on the pathogenesis of atherosclerosis by inducing interstitial fluid flow through SMCs encapsulated in collagen I gels [126–129], with findings of significant effects on SMC expression and secretion of MMPs, leading to subsequent degradation and invasion of the collagen gel matrix.

1.6 Mechanotransduction

Generally, cells are able to sense their mechanical environment predominantly through integrin-linked focal adhesion structures [130]. These structures are formed on the cytoplasmic side of integrin clusters and involve dozens of scaffolding and signaling proteins. Cells are able to mechanosense through focal adhesions due to the presence of so-called mechanosensitive proteins such as talin [131]. These proteins are mechanosensitive because they are tethered to both the membrane-anchored adhesion as well as actin stress fibers and, as actomyosin contraction pulls on the adhesions, the proteins are physically unraveled, exposing binding sites for various signaling molecules [131]. Typically, cells seemed to try to balance the internal cytoskeletal tension with the tension at integrin-ECM connections [132], and so a stiffer substrate will result in greater contraction and pull those proteins open to expose signal protein binding sites. For these

reasons, substrate modulus is a major factor that should be controlled in cell biology experiments.

1.7 Hydrogels for studying mechanobiology

As in atherosclerosis, a wide variety of diseases, tissues and individual cell types are affected by mechanical cues from the microenvironment. Mechanical stimuli, which may include tissue modulus, shear stress, and cyclic stretch, are documented to influence cell migration [64,65,133], differentiation [67,134,135], proliferation [136], and other events [137]. The study of mechanobiology benefits from the development of mechanically tunable biomaterials for *in vitro* cell experiments. The biomaterials most commonly used are hydrogels, including synthetic polymers, such as polyacrylamide (PAA) [120,138,139], and poly(ethylene glycol) (PEG) [67], and biologically derived gels, such as alginate [140], Matrigel™ [141], fibrin [142], and type I collagen [143]. Of these, biological hydrogels are the easiest to adapt, as they are commercially available, and inherently contain the integrin-binding and proteolytic domains cells encounter *in vivo*. However, increasing the concentrations of these proteins in order to increase the gel modulus also increases the density of integrin-binding domains, decreases the porosity, and alters degradability [144]. These parameters cannot be easily decoupled, making it difficult to determine which ECM property most significantly affects cell behavior. PAA was the first popularized synthetic hydrogel for cellular mechanobiology [139], in part because one can independently tune the integrin-binding and bulk modulus. However, it is not entirely resistant to protein adsorption [145], and cannot be used to study three dimensional (3D) cell behavior.

PEG-based gels are inexpensive, have independently tunable integrin-binding, bulk modulus, and proteolytic domains [117,146,147], and can be used to study cell behavior in 3D with appropriate modifications [148–150]. However, the mechanical range of PEG-based gels is limited, typically in the range of 0.5-5 kPa when crosslinked with proteolytically degradable groups [67], or 20-500 kPa with diacrylate or dimethacrylate free radical crosslinking [117,151]. PEG and PAA-based hydrogels have been used extensively to study mechanobiology, for example to demonstrate that cells sense the rigidity of their substrate and respond dynamically to changes in tension [133], or that substrate modulus affects stem cell lineage specification [134].

1.8 Hypothesis

To date, biomedical research has generally taken a reductionist approach and used simple *in vitro* models, and *in vivo* animal models that are often of questionable relevance to human physiology and disease. The primary aim of this dissertation work is to begin to bridge the gap between these types of studies by designing a complex *in vitro* model that captures several major features of the *in vivo* vasculature to study SMC phenotype in the context of atherosclerosis. Of course, this approach relies completely on the foundational work of the studies that precede it, and thus those works are absolutely critical despite any shortcomings they may have. The expectation is that piecing together that information to more closely match the *in vivo* microenvironment will reveal interactions, phenotypes, and behaviors that may be unobtainable with the simpler models. Prior works are also essential in helping us understand, interpret, and corroborate the data and observations from a more complex model. Altogether, the fundamental hypothesis of this dissertation is that, with

previous works as a guide, we can design a complex *in vitro* model that recapitulates crucial elements of the *in vivo* cellular microenvironment to reproduce physiologically-relevant phenomena *in vitro*. Finally, these phenomena can be examined in depth to parse the integration of physical, mechanical, and chemical signals and reveal potential regulatory pathways and pathway interactions that may not otherwise be observed in simpler *in vitro* models, and with this new information seek novel drug targets and therapies.

1.9 Objectives for Dissertation

To test the hypothesis proposed in the prior section, I set forth the following objectives for this dissertation work:

1. Design and characterize a superior hydrogel to study mechanobiology, with features:
 - a. Non-fouling
 - b. Can be made very soft or stiff, within a physiologically relevant range
 - c. Inexpensive, simple to make
 - d. Supports attachment of full-length proteins
 - e. Optically transparent
2. Demonstrate that the hydrogel is appropriate for cell culture studies, with requirements:
 - a. Cells must be able to ‘feel’ differences in modulus/stiffness
 - b. Must demonstrate that stiffness can affect cell behavior
3. Design a complex *in vitro* model for SMC phenotype, with features:
 - a. ECM proteins *commonly* associated with intact vascular media and with the atherosclerotic plaque.
 - b. Soluble factors reported relevant to atherosclerosis or maintenance of SMC phenotype.
 - c. Soft and stiff substrates to simulate the stiffening and fibrosis frequently associated with the development of plaques.

4. Use this *in vitro* model to parse the effects of the model features on relevant SMC phenotypes:
 - a. Marker expression
 - b. Proliferation
 - c. Migration
 - d. Invasion
5. Finally, probe signaling pathways predicted to be involved in these behaviors, with a focus on integrins, soluble factors, and how they activate interacting signaling pathways.

1.10 Significance

While there are certainly studies that use relatively complex *in vitro* model systems, the majority of biomedical research is performed on glass or polystyrene, and the effects of extracellular matrix proteins and other mechanical effects are often neglected. This approach has led to a huge amount of disagreement within the literature, and the vast majority of potential therapeutics or treatments that are successfully tested in animal models sadly do not translate to humans. We believe that the valuable information gained from these studies, however, can be used to design increasingly complex *in vitro* models that may vastly improve the odds of making discoveries that are directly applicable to human health and disease. The aim of this dissertation work was to apply this approach to the study of SMC phenotype in the context of atherosclerosis. Through the course of this dissertation research, we have developed and characterized an exceptional synthetic hydrogel for mechanobiology research. We then used this hydrogel platform to study the effect of substrate modulus on the proliferation and mechanotransduction properties of three cancer cell types, the first ever mechanobiology report of this type with these cells. Once proven suitable, we then used this hydrogel as one element of complexity in *in vitro*

models of the vascular microenvironment. Along with this static mechanical feature, we also introduced two mixtures of healthy- and disease-associated ECM proteins and 2 medium supplements containing soluble factors associated with the healthy and disease states. By recapitulating several elements of *in vivo* complexity, we have recreated physiologically-relevant behaviors *in vitro* and provide evidence to reconcile some disputes in the literature and dispute some widely-held notions regarding SMC phenotype. We also propose a detailed, literature-supported hypothetical mechanism by which ECM binding controls SMC behaviors, with potentially widespread implications in many other areas, including cancer. While additional work will be required before we can fully understand these results and confirm our proposed mechanisms, we believe this work will be of great interest to any investigators interested in SMC phenotype and the role of the microenvironment on disease states and cell behaviors.

1.11 References

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CHAPTER 2

PEG-PC SYNTHESIS AND MECHANICAL PROPERTIES

2.1 Abstract

This chapter will detail the development, synthesis and mechanical characterization of a novel hydrogel with significant utility in mechanobiology and disease research. The goal of this work was to create a novel, tunable hydrogel with exceptional anti-fouling properties to control the type and presentation of extracellular matrix proteins. This was achieved by copolymerizing the well-characterized PEG-dimethacrylate hydrogel with a methacrylated phosphorylcholine (PC) monomer. Phosphorylcholine was chosen because it is a very hydrophilic zwitterion, which imparts the ability to resist protein fouling. We find that free radical-copolymerized PEG and PC ('PEG-PC') has a mechanical range of 1 kPa to 10 MPa Young's modulus, which matches or exceeds any previously reported hydrogel, and average mesh sizes that are smaller than typical growth factors or other soluble factors (≤ 5 nm). PEG-PC also has reduced fouling compared to PEG itself, which we show is [PC]-dependent. Finally, we characterize the optical clarity of PEG-PC on coverslips and find that it is completely transparent from approximately 1 kPa to 1 MPa Young's modulus.

2.2 Introduction

The development of tunable biomaterials has driven rapid progress in tissue engineering, either to facilitate or enhance the natural wound healing process, or to act as functional replacement tissues in the human body. Biomaterial platforms have been developed both for eventual *in vivo* application, as well as for *in vitro* model systems for

studying complex biological processes, including cell migration, adhesion, proliferation, and differentiation. Information from these *in vitro* studies drives the design of implantable scaffolds, and promotes understanding of complex biology in environments more physiologically relevant than traditional plastic and glass surfaces. Studying cellular phenomenology and signaling mechanisms in microenvironments that capture key features of the *in vivo* extracellular matrix (ECM) is more efficient and cost-effective than animal studies, and may prove to be more predictive of human clinical outcome.

Mechanical cues from materials and the microenvironment, such as substrate modulus, shear stress, and stretch, influence cell migration [1–3], differentiation [4–6], and proliferation [7] (for review, see ref. [8]). Mechanotransduction, the translation of a mechanical cue from the ECM into a biochemical one, is mediated by focal adhesions [9]. There have been many studies on the effects of substrate modulus on cell spreading [1,10–16], focal adhesion properties [1,11,15,17], proliferation [4,6,11,18–20], migration [1–3,21,22], and phenotype and differentiation [4,5,20,23–25] with several materials platforms [1,12–14,18,20,26–29]. Materials most commonly used to investigate mechanobiology are hydrogels, including synthetic polymers, such as polyacrylamide (PAA) [30–32], and poly(ethylene glycol) (PEG) [4], and biologically derived gels, such as alginate [33], MatrigelTM [34], fibrin [35], hyaluronic acid [14,20,29] and type I collagen [36]. Of these, biological hydrogels are the easiest to adapt, as they are commercially available, and inherently contain the integrin-binding and proteolytic domains cells naturally encounter *in vivo*. However, manipulating the concentrations of these proteins in order to control the gel modulus simultaneously alters the density of integrin-binding domains, porosity, and degradability [22]. These parameters cannot be decoupled easily,

making it difficult to determine which ECM property most significantly affects cell behavior.

With respect to synthetic hydrogels, PAA was the first popularized platform for cellular mechanobiology [32], in part because it was the first system to demonstrate independent tuning of cell adhesion and modulus. While PAA is an important material, it has limitations, such as a narrow mechanical range, and it is not suitable for three-dimensional (3D) studies. PEG-based gels are inexpensive, have independently tunable cell adhesion, bulk modulus, and proteolytic domains [11,37,38], and can be used to study cell behavior in 3D [39–41]. However, the mechanical range of PEG-based gels is limited, typically in the range of 0.5-5 kPa when crosslinked with proteolytically degradable groups [4], or 20-500 kPa with diacrylate or dimethacrylate crosslinking [11,42]. We sought to improve on this limited range of Young's moduli, by combining the commonly used PEGDMA crosslinker with an inexpensive, extremely hydrophilic zwitterion. We report here the synthesis of a hybrid polymer hydrogel, which combines PEG-dimethacrylate (PEGDMA), with the zwitterionic comonomer 2-methacryloyloxyethyl phosphorylcholine (PC). To our knowledge, these "PEG-PC" hydrogels have a mechanical range that matches or exceeds any previously reported hydrogel system, and, importantly, they are simple, inexpensive to produce, and retain optical clarity over most of their mechanical range.

2.3 Materials & methods

2.3.1 Synthesis of PEG-PC hydrogels

In most cases, PEG-PC polymer hydrogel precursor solutions were prepared by mixing PEGDMA (average Mn 750, Sigma-Aldrich, St. Louis, MO), varied between final

concentrations of 7.4 mM and 0.7 M (0.5-55 wt.%, see Figure 2.2E), and 0.6 M (17 wt.%) 2-methacryloyloxyethyl phosphorylcholine (MPC; Sigma-Aldrich) in phosphate buffered saline (PBS). Solutions were degassed for 30 seconds with nitrogen, and sterilized with a 0.2 μm syringe filter (Thermo Fisher Scientific, Waltham, MA). Depending on the desired format, we have used two different free radical initiators for polymerization. To cure under UV light, 0.8 wt.% Irgacure 2959 (from a 20 wt.% stock in 70% ethanol; BASF, Ludwigshafen, Germany) was added, and gel formation was induced with a Spectroline High-Intensity UV Lamp with 365 nm light (Model #SB-100P, Westbury, NY), at approximately 3.5 inches from the gel for 7 minutes. To polymerize hydrogels without UV light, we added 0.05 wt.% ammonium persulfate (APS, from a 20 wt.% stock in water; Bio-Rad Laboratories, Hercules, CA) and 0.125 vol.% tetramethylethylenediamine (TEMED, Bio-Rad Laboratories) and polymerized gels under nitrogen for 10 minutes.

We also performed some experiments with different concentrations of MPC: 1) for mechanical testing, we UV polymerized hydrogels with MPC:PEGDMA molar ratios of 13.9, 7.23 and 2.59 at a constant 20.4 wt.% total polymer (600/43.1, 510/70.5 and 348/135 mM MPC/PEGDMA, respectively), and 2) for comparison of swelling and mechanical properties between molecular weight-matched MPC and a PEG-methacrylate (PEGMA) comonomers, we UV polymerized hydrogels with constant [PEGDMA] (135 mM) and concentrations of MPC or PEGMA of approximately 160, 300 and 600 mM. Due to the poor solubility of PEGMA in water, these latter hydrogels were polymerized in 70% ethanol.

To make thin PEG-PC hydrogels with even heights suitable for microscopy, 75 μL aliquots of PEG-PC solution was cured between chemically modified 18 mm glass

coverslips. Methacrylate silanized coverslips served as the base, and covalently attached to the hydrogel during polymerization, whereas hydrophobic coverslips could be easily removed from the final, hydrophilic hydrogels. To create the methacrylate coverslips, slips were treated with O₂ plasma for 10 minutes, reacted in 200 mL of 95% ethanol with 2 vol.% 3-(trimethoxysilyl) propyl methacrylate (adjusted to pH 5.0 with glacial acetic acid; Thermo Fisher Scientific) for 2 minutes with shaking, washed three times in 200 proof ethanol, and finally dried at 120°C for 15 minutes[43]. Hydrophobic coverslips were made with coverslips submerged in Sigmacote (Sigma-Aldrich), shaken for 20 minutes, washed 3 times with 200 proof ethanol, and dried under vacuum. Following polymerization, the Sigmacote cover slips were removed from the gel surface with fine forceps, and the gels were allowed to swell in sterile PBS for 24 hours. Prepared coverslips were stored in foil in a desiccator at room temperature.

To synthesize PEG-PC hydrogels for high throughput applications, 96-well microplates with glass bottoms (no. 1.5 coverslip glass; In Vitro Scientific, Sunnyvale, CA) were treated with O₂ plasma for 10 minutes, reacted with 100 µL/well of 2 vol.% 3-(trimethoxysilyl) propyl methacrylate (Sigma-Aldrich) in 95% ethanol (to pH 5.0 with glacial acetic acid; Thermo Fisher Scientific) for 2 minutes, with shaking, then rinsed 3x with 200 proof ethanol, and dried at 40°C for 30 minutes. To polymerize hydrogels in wells, pre-polymer solutions were prepared with APS and TEMED polymerization as described above, and added to the 96-well plate at 40 µL/well. To remove O₂ in the air that would inhibit polymerization, plates were placed in a vacuum oven at room temperature, pure N₂ gas flushed through the chamber for 5 minutes, and then the chamber sealed for

10 minutes to complete the reaction. Finally, hydrogels were swelled overnight with 100 μL /well PBS.

2.3.2 Hydrogel mechanical and structural characterization

PEG-PC hydrogel cylinders for mechanical compression testing were formed in Teflon molds (5 mm height, 5.5 mm diameter) and swelled in PBS for 48 hours. Post-swelling, hydrogel dimensions were measured with digital calipers, and mechanical compression tests were performed with a TA Instruments (New Castle, DE) AR-2000 rheometer using a 2 μm /second strain rate. The Young's modulus (E) for each hydrogel was calculated by plotting the measured normal force between 0 and 4% strain, and dividing the slope of the best-fit linear regression by the hydrogel cross-sectional area (see Fig. 2.1 for a representative stress-strain curve and example calculation). The Young's modulus was calculated from ≥ 4 samples at each PEGDMA concentration.

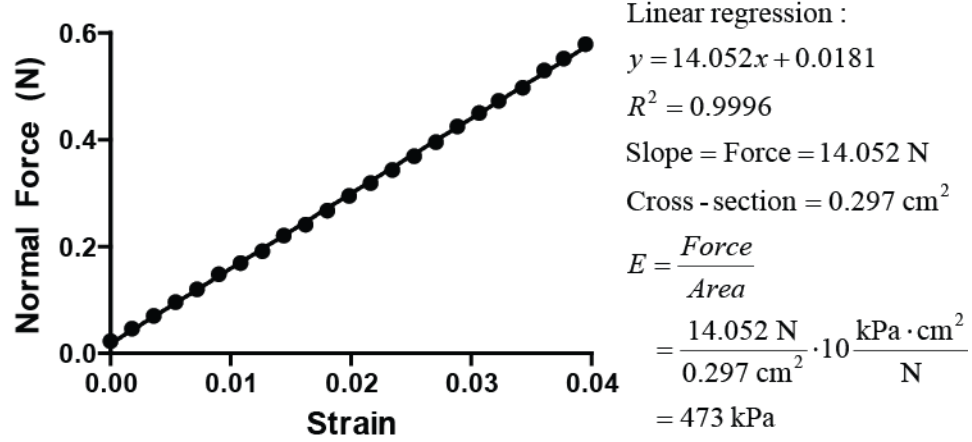


Figure 2.1: Young’s modulus example calculation

Young’s moduli were calculated by uniaxially compressing a cylindrical hydrogel and plotting the measured normal force versus strain from 0 to 4%. The modulus is then computed as the slope of the stress-strain curve divided by the cross sectional area of the hydrogel cylinder.

To determine an approximate average mesh size as a function of PEGDMA crosslinker content with constant PC content, hydrogels were swelled in PBS for 48 hours then weighed, fully lyophilized under vacuum and moderate heating, and weighed again. The average mesh sizes, ξ , of the PEG-PC hydrogels were determined as a function of PEGDMA crosslinker concentration according to the Flory theory, as modified by Canal and Peppas [44]:

$$\xi = v_{2,s}^{-\frac{1}{3}}(\bar{r}^2)^{1/2}$$

where $v_{2,s}$ is the swollen volume fraction of polymer and $(\bar{r}^2)^{1/2}$ is the average end-to-end distance of the PEGDMA crosslinker.

To detect unreacted methacrylate groups, NMR was performed on PEG-PC samples polymerized with APS and TEMED in microcentrifuge tubes with a Bruker Spectrospin DPX300 (Bruker Corporation, Billerica, MA). D₂O was used as the solvent.

To quantitatively measure the observed transparency of PEG-PC versus PEGDMA, hydrogels were made on cover slips, as described above, from 135 mM to 0.7 M PEGDMA with and without 0.6 M PC. After 48 hours of swelling in PBS, the optical densities of hydrogels in clean PBS were measured at 450, 490, 572, 630 and 750 nm with a BioTek ELx800 absorbance microplate reader (BioTek, Winooski, VT).

2.3.3 Quantification of non-specific protein adsorption

We quantified protein adsorption to the hydrogels with an indirect ELISA [45]. Hydrogels were polymerized in 96-well plates, swelled in PBS overnight, and incubated with 10 mg/mL bovine serum albumin (BSA; Sigma-Aldrich) for 20 hours at 37°C. The gels were washed 5x with PBS, incubated with primary antibody to BSA (Life Technologies, Carlsbad, CA) in PBS for 90 minutes, washed again 5x with PBS, and incubated with secondary antibody conjugated to horseradish peroxidase (HRP; Abcam, Cambridge, UK) for 90 minutes. After washing 5x with PBS, the gels were incubated with 0.1 mg/mL 3,3',5,5'-tetramethylbenzidine (TMB; Sigma-Aldrich) and 0.06% hydrogen peroxide (Thermo Fisher Scientific) in 0.1 M sodium acetate (pH 5.5; Sigma-Aldrich) for 1 hour at room temperature with shaking. At 1 hour, an equal volume of 1 M sulfuric acid (Sigma-Aldrich) was added to each well, and the absorbance at 450 nm was measured with a BioTek ELx800 absorbance microplate reader (BioTek).

2.3.4 Statistical analysis

Statistical analysis was performed using Prism v5.04 (GraphPad Software, La Jolla, CA). Data are reported as mean \pm standard deviation, unless otherwise noted. Statistical

significance of the difference between pairs of means was evaluated by computing P-values with unpaired Student's t-tests (with Welch's correction as necessary). $P \leq 0.05$ is denoted with *, ≤ 0.01 with **, ≤ 0.001 with *** and ≤ 0.0001 with ****; $P > 0.05$ is considered not significant ('ns').

2.4 Results

2.4.1 PEG-PC hydrogels are mechanically tunable over a wide range of Young's moduli

We synthesized hydrogels from varying concentrations of PEGDMA with constant PC, and characterized physical properties important for a viable cell culture platform (Fig. 2.2). We quantified the Young's moduli of photopolymerized PEG-PC hydrogels with 0.6 M PC (20 wt.%) in PBS and PEGDMA ranging from 7.4 to 700 mM (0.5 – 55 wt.%) via compression testing (Fig. 2.1, 2.2B). As expected, we found that hydrogel modulus increases with crosslinker concentration from 0.9 ± 0.2 kPa at 7.4 mM PEGDMA to 9300 ± 900 kPa at 0.7 M PEGDMA. This mechanical characterization demonstrates a tunable range of Young's moduli that spans four orders of magnitude, from 900 Pa to nearly 10 MPa. We attribute this wide range of moduli to the inclusion of the PC zwitterion, enabling gelation at very low concentrations of PEGDMA (7.4 mM, or 0.5 wt.%). This range of Young's moduli spans the reported moduli of nearly any tissue type, including brain, tumor tissue, skin, cartilage, and some areas of bone[46].

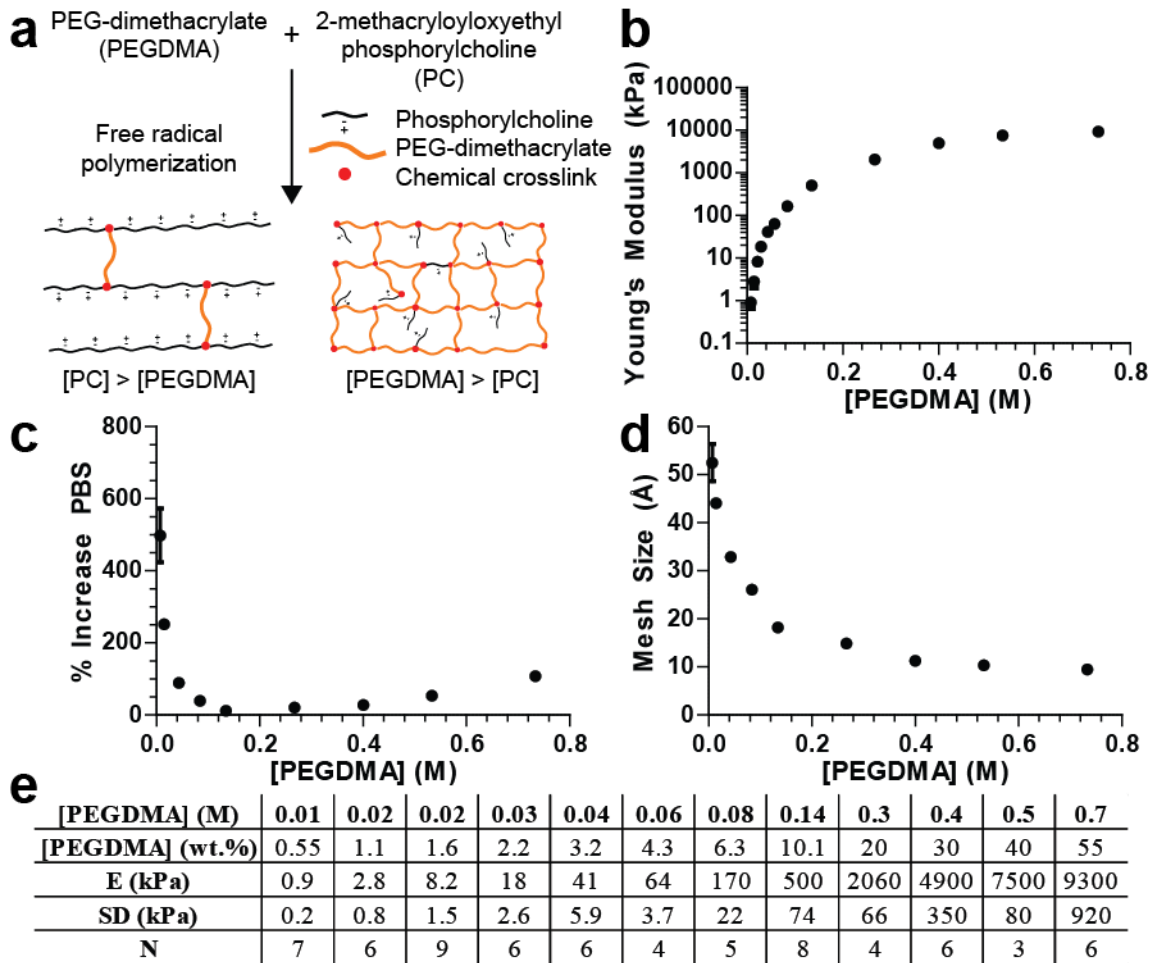


Figure 2.2: PEG-PC hydrogels are mechanically tunable over four orders of magnitude

(a) Schematic of PEG-PC hydrogel structure, which were prepared by free-radical polymerization to form PEGDMA-crosslinked linear PC polymers that entangle and form gels at very low [PEGDMA] concentrations. At very high [PEGDMA], the network structure is dominated by PEGDMA with sparsely distributed PCs. (b) Young's modulus, E , of PEG-PC hydrogels as a function of [PEGDMA] from 7.4 mM to 0.7 M. PC is 20 wt.% (0.6 M). Error bars are SDs. Each adjacent pair is significantly different with $p < 0.001$ or better. (c) PEG-PC swelling behavior in PBS, which is maintained even at very high [PEGDMA]. (d) The average mesh sizes of PEG-PC hydrogels (PC at 0.6 M) as a function of [PEGDMA]. Error bars are SDs ($N \geq 4$). (e) Table detailing all mechanical testing results.

In a single-polymer hydrogel, mesh size and modulus are generally coupled with the total polymer mass fraction. Due to the interesting 2-regime effects we have observed, we also examined the effect on Young's modulus of the molar ratio of [PEGDMA]:[PC]

in hydrogels with constant total polymer fraction (Fig. 2.3). We find that the Young's modulus is linearly and positively associated with [PEGDMA], but negatively associated with [PC] ($R^2 = 0.9997$ and 0.9979 , respectively; data not shown), and the ratio of [PEGDMA]:[PC] is positively and linearly associated ($R^2 = 0.9873$). This result may be explained by 2 mechanisms (or a combination of both): 1) as PC content increases, so does swelling (to be discussed further, below), which results in larger mesh sizes and lower moduli, or 2) increasing PEGDMA increases the number of reactable groups, which creates a more tightly crosslinked polymer network with smaller mesh sizes and higher moduli.

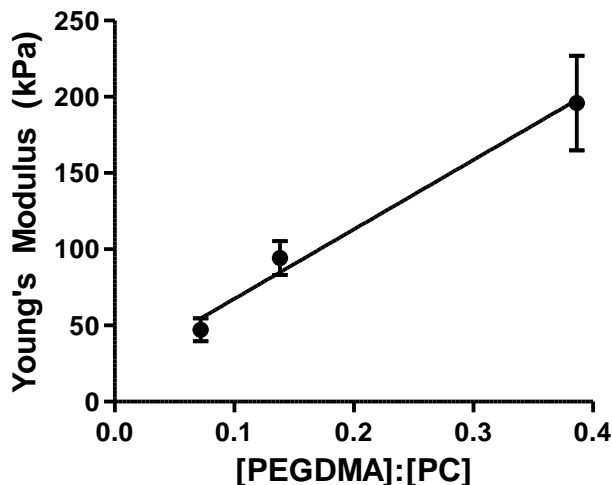


Figure 2.3: The effect of varying [PEGDMA]:[PC] with constant polymer fraction

PEG-PC gels with varied ratios of PEGDMA:PC and constant polymer mass fraction were polymerized and tested with mechanical compression. We find a positive and linear association ($R^2 = 0.9873$) with Young's modulus and [PEGDMA]:[PC] that is likely due to a combination of the effects of PC on swelling and PEGDMA on crosslinking density.

In the mechanobiology field, laboratories typically use either APS and TEMED, or Irgacure, as free-radical initiators of hydrogel polymerization. We observed minor differences in Young's modulus when comparing hydrogels made with one of these two initiators at the same PEGDMA concentration (Fig. 2.4). The Young's modulus of PEG-

PC hydrogels at 0.6 M PC and 7.4 mM PEGDMA polymerized with APS was 5.9 kPa, compared to 900 Pa when polymerized with Irgacure. This trend was consistent at low concentrations of PEGDMA, but differences diminish with increasing PEGDMA content. The mesh size with these same conditions are 4.1 ± 0.1 and 5.3 ± 0.4 nm, respectively. These data indicate that the differences in properties at low [PEGDMA] are likely due to differences in reaction rate or efficiency between the two free radical initiators.

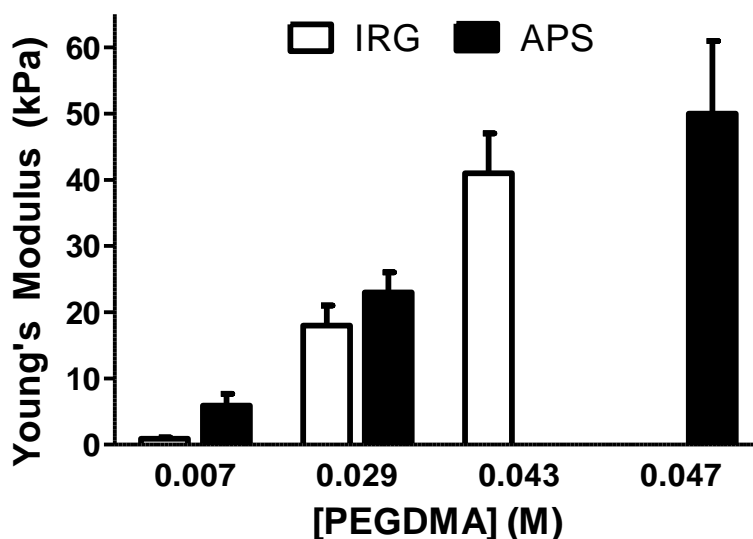


Figure 2.4: Free radical initiator affects PEG-PC modulus at low [PEGDMA]

PEG-PC cylinders of varying [PEGDMA] were polymerized with APS and TEMED and the Young's modulus determined from mechanical compression testing. The difference in hydrogel modulus between polymerization with Irgacure (IRG) and APS/TEMED (APS) narrows as [PEGDMA] increases, likely due to differences in reaction rate or efficiency. Figure adapted with permission from [47], Copyright © 2013 American Chemical Society.

2.4.2 PEG-PC hydrogels have small mesh sizes and structure-dependent swelling properties

The average mesh sizes (ξ) of PEG-PC hydrogels from 7.4 mM to 0.7 M PEGDMA ranged from 5.3 ± 0.4 nm at 7.4 mM crosslinker to 0.95 ± 0.01 nm at 0.7 M crosslinker

(Fig. 2.2D). These sizes approximate values reported for other common synthetic hydrogels such as PEG-diacrylate ($\xi \sim 1.4 - 7$ nm) [48], poly(vinyl alcohol) ($\xi \sim 4 - 32$ nm) [44], or poly(2-hydroxyethyl methacrylate) ($\xi \sim 1.6 - 2.4$ nm) [44]. Significantly, all but the softest PEG-PC has mesh sizes smaller than the predicted hydrodynamic radius of common growth factors, such as FGF-1 and -2, PDGF-BB, EGF and TGF β_1 ($R_{\text{hyd}} \sim 4.4 - 5.1$ nm, predicted from light scattering data and molecular weights [49]), which we expect will prevent their artificial sequestration in the substrate. While the mesh size of 135 mM PEGDMA hydrogels is only 0.5-fold higher than 135 mM PEG-PC (3.2 nm vs 1.8 nm, respectively), the Young's modulus is 10-fold lower (55 kPa vs 500 kPa, respectively [50]), demonstrating the profound effect of the methacrylic PC polymers on the hydrogel mechanical properties, and their impact on the overall gel volume fraction.

Interestingly, the expected strong correlation between mesh size and Young's modulus was found only over a partial range of crosslinker concentrations (Fig. 2.2B, D). There were two different regimes where mesh size and Young's modulus strongly correlated: from 7.4 to 135 mM PEGDMA (Pearson's $R = -0.8383$, $p < 0.05$), and from 0.3 to 0.7 M PEGDMA (Pearson's $R = -0.9572$, $p < 0.05$). Interestingly, these behaviors separate where the weight fraction of PC and PEGDMA are equal in the gel. This finding suggests a change in the fundamental structure of the hydrogel, from one dominated by a PEGDMA-crosslinked methacrylic PC polymer to one dominated by a PEGDMA-crosslinked PEG polymer with sparsely distributed PC pendants (Fig. 2.2A). To investigate whether these regime changes were due to incomplete methacrylate conversion, we collected NMR spectra on the PEG-PC hydrogels. These spectra (Fig. 2.5) indicate complete methacrylate conversion for PEG-PC hydrogels up to 0.4 M PEGDMA. While it

is not possible to directly quantify the methacrylate conversion from these spectra, the post-swelling dry gel masses for these gels are 99% or more of the theoretical polymer masses (data not shown), implying that methacrylate conversion is very high at all concentrations of PEG.

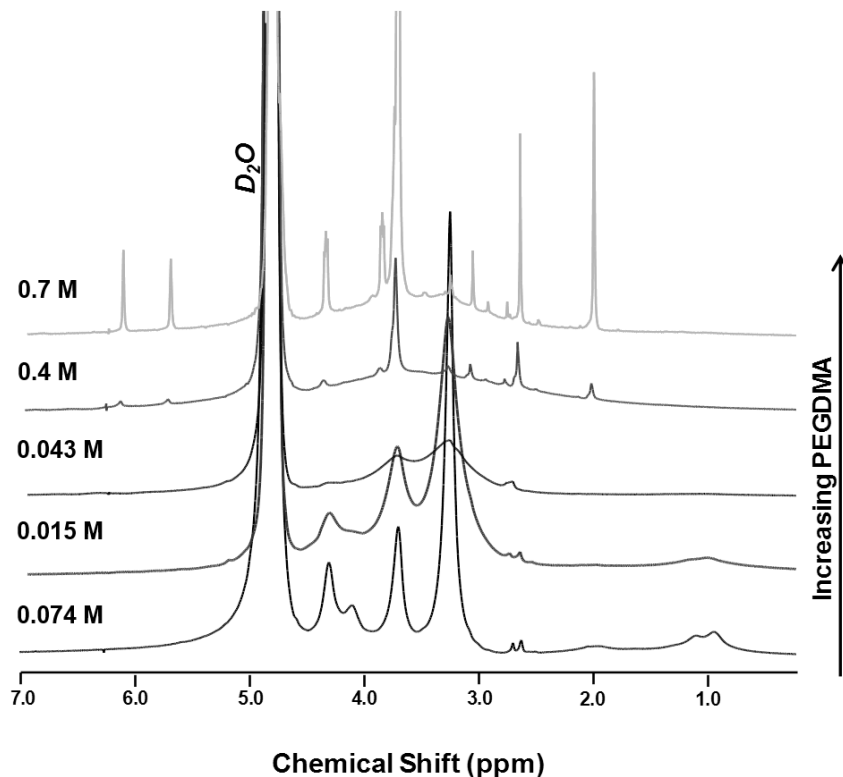


Figure 2.5: NMR spectra of PEG-PC hydrogels

^1H NMR spectra of 5 hydrogel samples prepared with varying concentrations of PEGDMA crosslinker. As PEGDMA, and presumably the crosslink density increases, some residual monomer is noted at 5.5 and 6.0 ppm (vinyl protons). Figure adapted with permission from [47], Copyright © 2013 American Chemical Society.

Given the very high moduli and small mesh sizes of highly crosslinked PEG-PC hydrogels, we speculated that these gels may not swell water typical of PEG gels. Surprisingly, from swelling data, we ascertained that PEG-PC does in fact swell PBS at all crosslinker concentrations (Fig. 2.2C), and we again observed two different regimes of

swelling behavior, separated at the point where the gels undergo a change between a PC and PEG-dominated structure. In examining the stress-strain curves from compression testing, we found that the hydrogels behaved as linear elastic gels at low strain in PC-dominated networks, and non-linearity was observed above this regime change (Fig. 2.6). Recent studies have implied that cells may be able to sense these types of differences in network structure [51]. In contrast to these studies, however, we observed non-linearity at only the highest moduli, outside the mechanical range reported to be discernable by most cell types ($E \leq 500$ kPa).

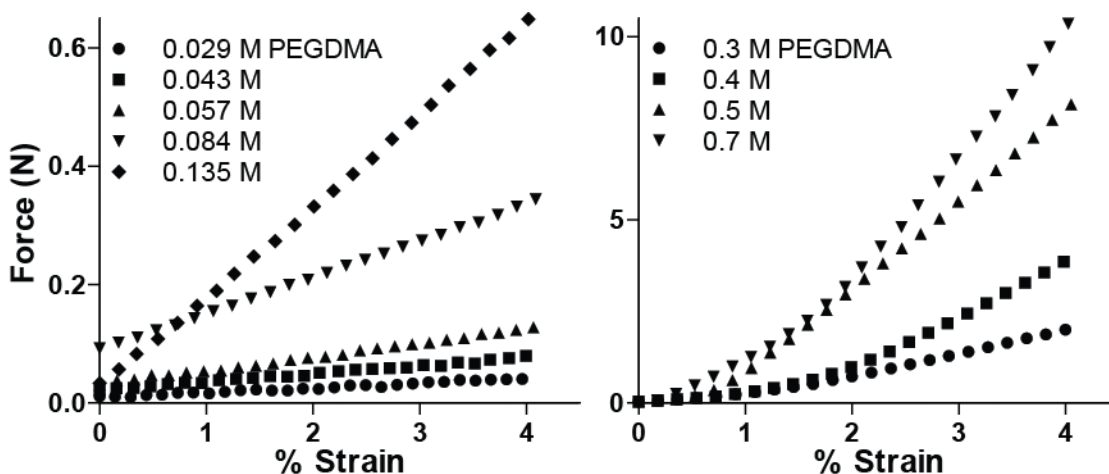


Figure 2.6: PEG-PC hydrogels are linearly elastic up to 0.3 M PEGDMA

At low strain, PEG-PC behaves as a linearly elastic material in the PC-dominated regime ($[PEGDMA] < 0.3$ M), but in the PEGDMA-dominated regime the stress-strain curves are slightly nonlinear. There is evidence that cells feel nonlinear elastic materials differently than linear elastic materials, but the mechanical range of PEG-PC (up to 500 kPa) that is linear elastic covers the vast majority of tissue types. Figure adapted with permission from [47], Copyright © 2013 American Chemical Society.

We also compared the effect of PC on swelling properties with a weight-matched, non-zwitterionic methacrylated comonomer, PEGMA ($M_N \sim 300$) with constant $[PEGDMA]$. These results (Fig. 2.7), presented here as the percent increase in swelling with PC compared to PEGMA, show a linear relationship ($R^2 = 0.9746$) between

comonomer concentration and increased swelling. These data further support the theory that hydrophilic PC groups enhance hydrogel swelling and thereby enable the incredible range of mechanical properties we have reported with PEG-PC.

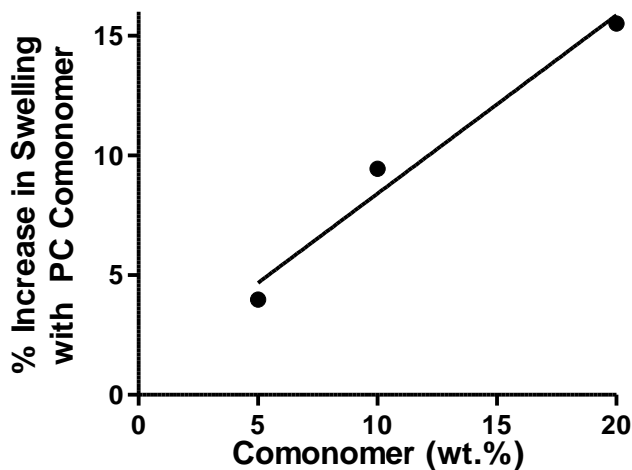


Figure 2.7: Swelling is enhanced by PC comonomer

The role of PC on PEG-PC swelling properties was investigated by comparing against a non-zwitterionic, methacrylated comonomer of similar molecular weight, PEGMA, with varying concentrations and constant [PEGDMA]. The PC-induced increase in swelling over PEGMA linearly increases with comonomer concentration, confirming that PC groups in the hydrogel increase swelling, presumably because of their zwitterionic structure and hydrophilicity.

2.4.3 PC groups reduce non-specific protein adsorption to PEG gels

PEG is an amphiphilic polymer that is resistant to non-specific protein adsorption. We hypothesized that incorporation of extremely hydrophilic PC groups would further enhance resistance to protein adsorption. We tested this by adsorbing BSA to PEGDMA (0.145 M) and PEG-PC (0.6 M PC, 0.054 M PEGDMA) hydrogels that had the same Young's modulus, and, after extensive washing, measuring the adsorbed protein with an indirect ELISA. As expected, the PEGDMA hydrogels had small, but detectable levels of

BSA on the surface, whereas no BSA was detectable on PEG-PC surfaces (Fig. 2.8A), nor on polyacrylamide gels (data not shown).

We also quantified the effect of PC content in hydrogels on protein adsorption by measuring BSA adsorption on hydrogels with constant PEGDMA (0.084 M) and varied PC concentration (0.15, 0.3 and 0.6 M). As expected, BSA levels decreased with increasing PC content (Fig. 2.8B), with half the signal at 0.6 M PC as at 0.15 M. Interestingly, BSA was detected on PEG-PC hydrogels with 0.084 M PEGDMA, but not on the hydrogels with 0.054 M PEGDMA. This suggests that adsorption increases when the ratio of PEGDMA:PC increases, further supporting our hypothesis that PC groups decrease non-specific protein adsorption.

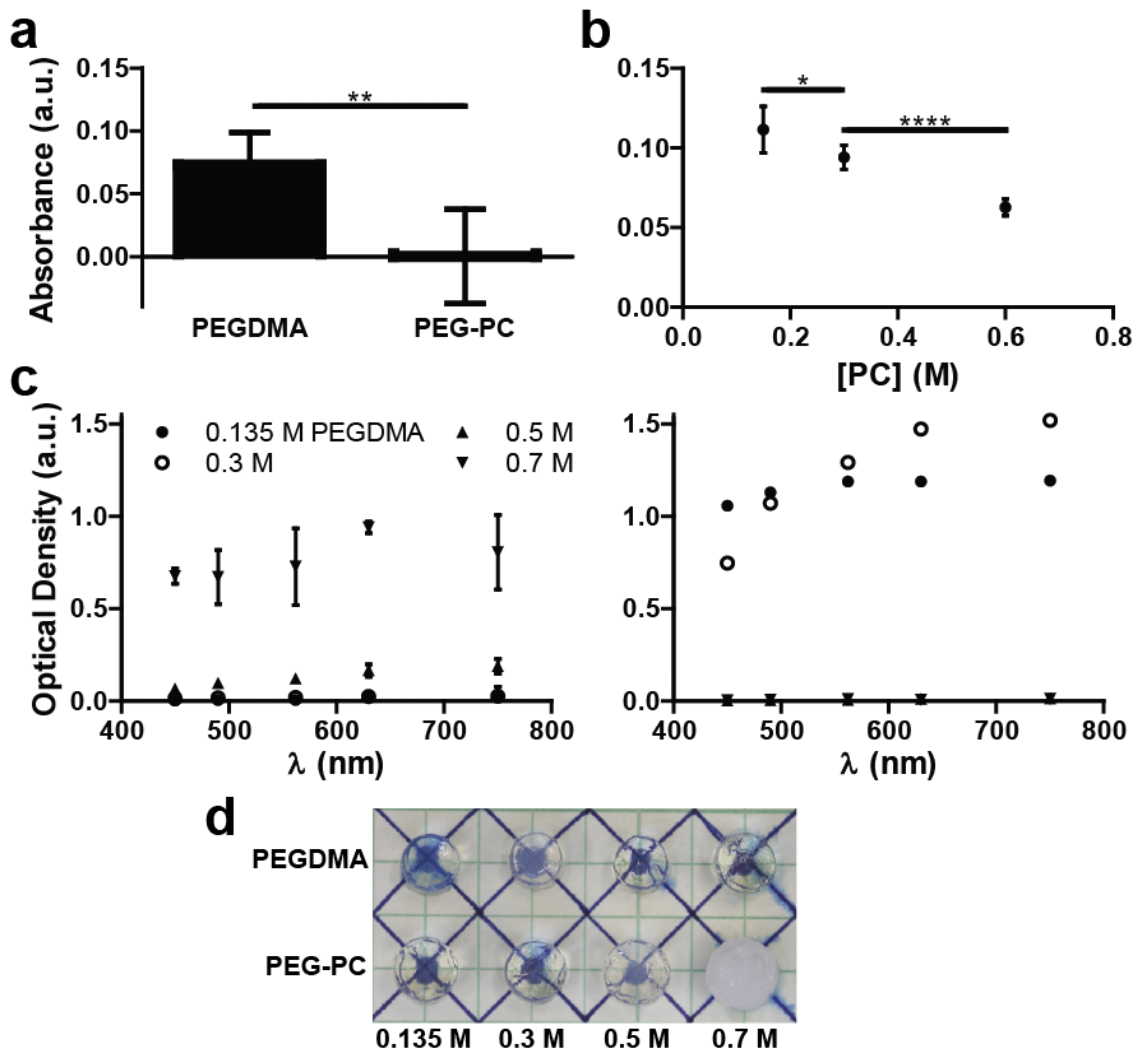


Figure 2.8: PEG-PC hydrogels are non-fouling, and optically transparent at low PEGDMA concentrations

(a) Comparison of adsorption of BSA to modulus-matched PEGDMA (0.145 M) and PEG-PC (0.6 M PC and 0.054 M PEGDMA) hydrogels measured by ELISA with TMB as detection substrate. No BSA was detected (compared to negative controls) on the PEG-PC hydrogel. (b) Adsorbed BSA was measured as a function of PC concentration with constant [PEGDMA] (0.084 M). Adsorption decreases with increasing PC content, demonstrating the ability of PC to prevent fouling. (c) The optical density of PEG-PC (left) and PEGDMA (right) hydrogels on coverslips in PBS was measured at several wavelengths. The optical density of PEG-PC hydrogels are lower than PEGDMA hydrogels at 135 mM and 0.3 M, but the opposite is true at 0.5 and 0.7 M. Error bars are standard deviations ($N \geq 2$). (d) Visual comparison of the optical transparencies of PEG and PEG-PC hydrogels at 0.135, 0.3, 0.5 and 0.7 M PEGDMA. PEG-PC hydrogels are 0.6 M PC. Figure adapted with permission from [47], Copyright © 2013 American Chemical Society.

2.4.4 PEG-PC hydrogels are optically transparent

For a hydrogel platform to be particularly useful for microscopy, the optical clarity (and refractive index) would ideally be close to that of glass or plastic substrates. Both PEG and PAA hydrogels become opaque at high crosslinker concentrations. These PEG-PC hydrogels are completely transparent at all but the very highest cross-linker concentrations of (Fig. 2.8C, D), with opacity emerging when the PEGDMA content reaches 40 wt.%. Optical properties were characterized after polymerizing hydrogels on coverslips, then measuring optical density (OD) at several different wavelengths (Fig. 2.8C). PEG-PC hydrogels are completely transparent (having ODs equal to the polystyrene surface) across all wavelengths up to 0.3 M PEGDMA, only becoming opaque at 0.7 M crosslinker. In contrast, PEGDMA-only hydrogels are opaque at lower crosslinker concentrations. At crosslinker concentrations higher than typically used in the literature, the PEGDMA hydrogels are nearly as transparent as PEG-PC gels.

2.5 Discussion

An ideal biomaterial for studying cell-material interactions should be easily tunable across physical properties relevant to the cell type and tissue of interest. Controllable mechanical properties in biomaterials are extremely valuable for understanding cell behavior in heart disease [52,53] and cancer [54], as cell phenotype [4,5,7,55] and stem cell differentiation [5,56,57] are sensitive to the modulus of the microenvironment. *In vivo*, a vascular SMC in a healthy arterial media experiences a microenvironment stiffness between 2 and 10 kPa [58,59], but in an atherosclerotic plaque, the substrate could have stiffness from tens [60,61] to hundreds of kPa [62]. During metastasis, a cancer cell must

migrate from a stiff, fibrotic tumor environment through more compliant interstitial tissues and blood vessel walls into the blood stream [63]. Matrix crosslinking and stiffness have profound effects on tumor malignancy [64] and cancer cell metastasis [63]. Other ideal features of hydrogels for mechanobiology include independently tunable stiffness and ligand density, hydrophilicity for reduction of non-specific protein adsorption, and optical clarity for modern quantitative microscopy techniques.

Poly(ethylene glycol) (PEG) is a well characterized amphiphilic polymer widely used as a hydrogel crosslinker in biological applications due to its biocompatibility, mechanical tunability, and anti-fouling properties. However, while it is possible to lower the Young's moduli by adding enzymatic degradation sites to the PEG monomers, PEGDMA hydrogels ($M_N \sim 750$) cannot polymerize at concentrations ≤ 5 wt.%, limiting the range of moduli obtainable. Furthermore, PEGDMA hydrogels at most tissue-relevant moduli are opaque (Fig. 2.8C, D), reducing their attractiveness for high-resolution microscopy.

In the present study, we describe and characterize a new PEG-PC hydrogel that improves upon all these properties. PEG-PC hydrogels are composed of a PEGDMA crosslinker, co-polymerized with the PC zwitterion. PC-containing phospholipids are a major component of biological membranes, and PC-polymers exploit biocompatible moieties in synthetic constructs [65]. PC-containing hydrogels and PC-coated surfaces have been exploited for their anti-fouling properties and biocompatibility [65] for use in applications such as contact lenses [66], cell encapsulation [67], and drug delivery [68]. There have been two prior reports of a similar polyMPC hydrogel crosslinked with ethylene glycol dimethacrylate and other crosslinkers [69,70]. However, these authors used a much

higher concentration of PC (2.5 M versus 0.6 M) and very short crosslinkers in comparison to PEG-PC, and the mechanical properties of the materials were not reported.

Hydrogels which incorporate other zwitterion co-monomers have been studied, in particular sulfobetaines such as 1-(3-sulphopropyl)-2-vinyl-pyridinium-betaine [71,72] and N-(3-sulfopropyl)-N-(methacryloxyethyl)-N,N-dimethylammonium betaine [73], but not in cell culture contexts. Another approach to create hydrophilic hydrogels includes the incorporation of hyaluronic acid [14,16,20,29,74]. However, hyaluronic acid has some very undesirable properties, including polydispersity, very high cost, and the requirement of functionalization with organic chemistry. Plus, it is known to affect cell behavior through several different cell-surface receptors [75], which may be undesirable except in instances where the biological effects of hyaluronic acid are specifically being studied. On the other hand, PC has the advantages of being relatively inexpensive (especially compared to hyaluronic acid), biocompatible without affecting cell behavior, and simple integration into any methacrylate-functionalized hydrogel.

We have successfully polymerized PEG-PC hydrogels using UV polymerization with as little as 7.4 mM (0.5 wt.%) PEGDMA crosslinker, and up to 0.3 M crosslinker without diminishing optical clarity (Fig. 2.8C, D). In contrast, PEGDMA hydrogels without PC comonomer cannot polymerize below approximately 70 mM crosslinker, and they are not optically transparent below 0.5 M. Polymerization at such a low crosslinker concentration imparts PEG-PC hydrogels with a wider range of mechanical properties: the Young's moduli of PEG-PC ranges from 900 Pa with 7.4 mM crosslinker to nearly 10 MPa with 0.7 M crosslinker, which is more compliant at the low end of crosslinker concentration than is possible with PEGDMA hydrogels. This range of stiffnesses is greater than

obtainable with PEGDMA gels, and covers a biologically-relevant span that enables reproduction of nearly any biological tissue, e.g. liver (5-55 kPa) [76–78], spinal cord (89 kPa), thyroid (9 kPa), breast tumor (4 kPa), carotid artery (90 kPa), and articular cartilage (950 kPa) [46]. While bone typically has a modulus from 10-20 GPa [79], PEG-PC hydrogels with moduli from 1 – 10 MPa may prove useful for studying osteoclasts and other bone-associated cell types. We have also demonstrated the polymerization of PEG-PC with APS and TEMED free radical initiation, and find that differences in mechanical properties diminish with increasing crosslinker concentration (Fig. 2.4), most likely an effect of different crosslinker concentration-dependent reaction rates or efficiency.

In addition to the exceptional mechanical range and optical properties, we have also demonstrated through BSA adsorption experiments that PEG-PC adsorbs less protein than a PEGDMA hydrogel of the same modulus (Fig. 2.8A), and the amount of adsorbed protein is inversely related to the PC content of the hydrogel (Fig. 2.8B). These results further confirm the non-fouling properties of PC, which enable a greater degree of control over protein presentation than PEGDMA hydrogels.

From our characterization, we conclude that PEG-PC hydrogels outperform PEG hydrogels as a platform for mechanobiology, and this four order-of-magnitude range of Young's moduli exceeds nearly every previously reported polymer or hydrogel reported for cell culture use, including the recent report of a more tunable version of PDMS [28]. We also discovered that the 135 mM crosslinker hydrogels had mesh sizes half that of a 135 mM PEGDMA gel without PC, whereas the Young's modulus is nearly ten times higher [50], implying the inherent structural changes outlined in Figure 2.2. Two reports have demonstrated that hyaluronic acid hydrogels have a similarly wide tunable

mechanical range [14,16], however, they are significantly more expensive to prepare than PEG-PC gels, require a longer preparation time, and hyaluronic acid has inherent bioactivity that can confound interpretation of results.

We propose two distinct phenomena at low and high concentrations of crosslinker to explain the remarkable mechanical properties of PEG-PC. At less than 70 mM (5 wt.%) crosslinker, PEGDMA does not form a gel. However, integration of the PC polymer allows polymerization with as low as 7.4 mM PEGDMA (0.5 wt.%). This is likely due to the overall increase in polymer mass from the PC, which is then crosslinked by the bifunctional PEGs (Fig. 2.2A). Secondly, although PC is charge-neutral in solution due to an intramolecular salt bridge, the PC groups can form dipoles, and it is conceivable that entropic forces (increased water ordering around the PC zwitterions) extend the PEG chains, permitting hydrogel formation at low crosslinker concentration. We have polymerized PEG-PC hydrogels in both water and PBS, and found no differences in the resulting mechanical properties (data not shown), confirming that the mechanical properties of the gels are maintained in the presence of biologically relevant solvents and that salts do not affect polymer conformations. Regardless of the precise interactions responsible, the resulting hydrogels are far more compliant than with PEGDMA alone, due to the lower overall crosslinking density and increased swelling.

At concentrations of PEGDMA above 135 mM, we observed a large change in the trends of mechanical properties as a function of crosslinker concentration. We hypothesized these effects result from a fundamental change in the structure of the hydrogel, specifically by PEGDMA becoming the dominant hydrogel component, as illustrated in the two extremes of very low and very high PEGDMA concentration in Figure

2.2. In this regime, where there is more PEGDMA mass per volume than PC, the dependency of both mesh size and Young's modulus on crosslinker concentration is much weaker than in the low PEGDMA regime.

The proposed ability to form two very different polymer structures with the same two components may also help explain the impressive mechanical range that PEG-PC hydrogels achieve. The structural differences responsible for the regime transition just described may be why we consistently observed linear stress-strain curves in the PC > PEGDMA regime and small portions of nonlinearity (up to 1 or 2% strain) in the PEGDMA > PC regime (Fig. 2.6). This has potential cellular significance due to the recent report that cell phenotype is sensitive to whether a material is linearly elastic or not [51]. However, we could not directly compare results, as we observed non-linearity at low strain in high modulus gels, whereas these authors observed non-linearity at high strain in low modulus gels, implying inherently different networks. Our results on the effect of varying [PC] with constant total polymer fraction, and those comparing swelling properties of PEGDMA gels copolymerized with varying amounts of PC and PEGMA, further confirm the hydrophilicity of PC and its impact on modulus and water uptake. These characteristics lend further support to the hypothesis that entropic forces from PC may be extending PEGDMA polymer chains and thereby increasing mesh size and decreasing modulus, an effect which is presumably diminished in the PEGDMA > PC regime.

2.6 Conclusion

We have developed a new class of hydrogels by combining the tunability and versatility of PEG hydrogels with biomimetic comonomer, PC. These hydrogels have an

extremely wide, 4 order of magnitude range of mechanical properties, with the additional advantages of being inexpensive, simple to synthesize, and optically clear. PEG-PC gels can be used to study mechanobiology across many different cell types, and we encourage others to adapt this system to study cellular responses to ECM modulus.

2.7 References

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CHAPTER 3

CANCER CELL MECHANOTRANSDUCTION VARIES WITH CELL TYPE

3.1 Abstract

In the following chapter, I will demonstrate the utility of PEG-PC hydrogels for use in *in vitro* disease research. This work is a proof-of-concept study to show that PEG-PC hydrogels can be surface-functionalized with full-length extracellular matrix proteins and this promotes cell attachment and adhesion, and to investigate the impact of substrate modulus on proliferation and focal adhesion properties of multiple cancer cell lines (HEP3B, MB-MDA-231, SkBr3). This work also extensively utilizes PEG-PC hydrogels synthesized in a 96-well microplate format for high-throughput screening and other *in vitro* assays. The findings herein are the first reported study on the impact that substrate modulus has on the proliferation of these cancer cell lines, which has relevance to understanding the roles of tumor stiffening and fibrosis *in vivo*.

3.2 Introduction

In modern medicine, there is a somewhat narrow-minded focus on the importance of genetics in understanding development and disease, and for treating disease. While the revolutions in technology that enabled genetic research are technically impressive and critically important, the hyping of the importance of genetics has led many researchers towards the belief that only genetics matter. This is a troubling trend, but it is one which is slowly being pushed back against by work in the field of mechanobiology. It is troubling because the medical community has long known that mechanics has an important role in development and disease [1], and if biomedical researchers continue to ignore or downplay

the importance of the physical microenvironment we may miss opportunities to develop life-saving cures and treatments.

The revelation that mechanics is vitally important in human physiology may be traced back to Wolff's Law from the late 1800's, which essentially states that bone responds to the mechanical load placed upon it through an adaptive response that increases the density of the internal trabeculae [2]. Now, we know that mechanotransduction – the ability of cells to sense and respond to changes in their mechanical environment – is a factor in a very large list of diseases, including many of the most costly diseases such as atherosclerosis, cancer (and metastasis), hypertension, pulmonary fibrosis, irritable bowel syndrome, osteoporosis, and sexual dysfunction (plus many more [1]). Diseases may be caused, or modulated by, defects in the conversion of mechanical signals to chemical signals (e.g. several muscular dystrophies [3], atrial fibrillation [4], osteoporosis [5]), changes in extracellular matrix composition (ECM) or structure (e.g. some muscular dystrophies [6], Marfan's syndrome [7], cancer [8,9]), or alterations to the contractile apparatus within cells (e.g. some types of deafness and blindness, cardiomyopathy [10], sexual dysfunction [11]). While many of the mechanically-related causes or consequences of disease may have a genetic basis, we may be able to discover and develop treatments, or even cures, for many diseases if we can address the underlying mechanical dysfunction, without the need for gene therapy.

A lot of work in the field of mechanobiology has focused on the use of 2D polymeric biomaterials to modulate static mechanics. This approach is based on the observation that integrin-ECM interactions, and the focal adhesion signaling complexes they engender, are the primary means of transducing mechanical signals from the exterior

of the cell into internal signaling. Due to the existence of many different types of ECM proteins and integrin heterodimers, exactly how, and what signals are transduced in any given context is highly complex and depends on cell type (and species), the tertiary structure of the ECM proteins, and potential interactions between soluble factors and integrins and/or ECM proteins. Thus, due in part to the ease with which many cell types are cultured on 2D surfaces, many groups have developed and used tunable polymeric biomaterials to modulate substrate stiffness, especially hydrogels. These materials enable researchers to examine how changes in stiffness may modulate disease states in different microenvironment contexts (e.g. ECM proteins, soluble factors).

Here, we demonstrate the use of our novel PEG-PC hydrogel in a mechanobiology study to examine the influence of substrate stiffness on the proliferation and focal adhesion properties of multiple cancer cell lines. We find that the dependence of proliferation on stiffness varies across cell types, including one cell line which actually proliferates less on stiff substrates. These cell-specific effects are likely due to differences in expression of integrins, focal adhesion-associated signaling proteins, and/or contractile apparatus proteins.

3.3 Materials & methods

3.3.1 Cell culture

All cells and culture media reagents were purchased from Life Technologies, Carlsbad, CA, unless otherwise noted. Human aortic smooth muscle cells (HASMCs) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% penicillin-streptomycin (P/S) and smooth muscle growth supplement (SMGS). Human

hepatocellular carcinoma cells (HEP3Bs, American Type Culture Collection, Manassas, VA), were cultured in modified Eagle's medium (MEM) supplemented with 1% P/S and 10% FBS. Human SkBr3 and MDA-MB-231 (231) breast carcinoma cell lines were generous gifts from Shannon Hughes at the Massachusetts Institute of Technology and Sallie Schneider at the Pioneer Valley Life Sciences Institute, respectively. Both SkBr3 and 231 cell lines were cultured in DMEM supplemented with 1% P/S and 10% FBS.

3.3.2 Synthesis of PEG-PC hydrogels

PEG-PC polymer hydrogel precursor solutions were prepared by mixing PEGDMA (average Mn 750, Sigma-Aldrich, St. Louis, MO), to final concentrations of 29, 47, 84 and 122 mM (2.2 – 9.2 wt.%, see Figure 2.2E), and 0.6 M (17 wt.%) 2-methacryloyloxyethyl phosphorylcholine (MPC; Sigma-Aldrich) in phosphate buffered saline (PBS). Solutions were degassed for 30 seconds with nitrogen, and sterilized with a 0.2 μ m syringe filter (Thermo Fisher Scientific, Waltham, MA). Hydrogels were polymerized by adding 0.05 wt.% ammonium persulfate (APS, from a 20 wt.% stock in water; Bio-Rad Laboratories, Hercules, CA) and 0.125 vol.% tetramethylethylenediamine (TEMED, Bio-Rad Laboratories) and cured in an O₂-free atmosphere.

To make thin PEG-PC hydrogels with even heights suitable for microscopy, 75 μ L aliquots of PEG-PC solution was cured between chemically modified 18 mm glass coverslips with APS and TEMED polymerization, on the bench at room temperature. Methacrylate silanized coverslips served as the base, and covalently attached to the hydrogel during polymerization, whereas hydrophobic coverslips could be easily removed from the final, hydrophilic hydrogels. To create the methacrylate coverslips, slips were

treated with O₂ plasma for 10 minutes, reacted in 200 mL of 95% ethanol with 2 vol.% 3-(trimethoxysilyl) propyl methacrylate (adjusted to pH 5.0 with glacial acetic acid; Thermo Fisher Scientific) for 2 minutes with shaking, washed three times in 200 proof ethanol, and finally dried at 120°C for 15 minutes[12]. Hydrophobic coverslips were made with coverslips submerged in Sigmacote (Sigma-Aldrich), shaken for 20 minutes, washed 3 times with 200 proof ethanol, and dried under vacuum. Following polymerization, the Sigmacote cover slips were removed from the gel surface with fine forceps, and the gels were allowed to swell in sterile PBS for 24 hours. Prepared coverslips were stored in foil in a dessicator at room temperature.

To synthesize PEG-PC hydrogels for high throughput applications, 96-well microplates with glass bottoms (no. 1.5 coverslip glass; In Vitro Scientific, Sunnyvale, CA) were treated with O₂ plasma for 10 minutes, reacted with 100 µL/well of 2 vol.% 3-(trimethoxysilyl) propyl methacrylate (Sigma-Aldrich) in 95% ethanol (to pH 5.0 with glacial acetic acid; Thermo Fisher Scientific) for 2 minutes, with shaking, then rinsed 3x with 200 proof ethanol, and dried at 40°C for 30 minutes. To polymerize hydrogels in wells, pre-polymer solutions were prepared with APS and TEMED polymerization as described above, and added to the 96-well plate at 40 µL/well. Oxygen was purged by placing plates in a vacuum oven at room temperature and flushing pure N₂ gas through the chamber for 5 minutes, then sealing the chamber for 10 minutes to complete the reaction. Finally, hydrogels were swelled overnight with 100 µL/well PBS.

3.3.3 Protein functionalization

To facilitate cell adhesion to the gels, proteins were covalently attached to the hydrogel surfaces. Hydrated gels on coverslips were transferred to 12-well tissue culture dishes and treated twice with sulfo-SANPAH (0.3 mg/mL in pH 8.5 HEPES buffer; ProteoChem, Denver, CO) under UV light for 15 minutes. The gels were washed by twice pipetting sterile PBS directly over the surface and shaking for 10 seconds, followed immediately by incubation with protein cocktails. For gels in 96-well plates, 100 μ L of 0.6 mg/mL sulfo-SANPAH was added to each well and the plates exposed to UV light for 20 minutes and then briefly washed three times with HEPES buffer. To demonstrate versatility, we also showed that acrylate-poly(ethylene glycol)-succinimidyl valerate (PEG-SVA; Laysan Bio, Arab, AL) can crosslink proteins by adding it to the PEG-PC pre-hydrogel solution at 0.11 wt.%. This method incorporates an amine reactive group into the bulk of the hydrogel instead of isolating the reaction at the surface.

After reacting the gel surfaces with the heterobifunctional crosslinker, hydrogels presented a highly amine-reactive functional group for covalent linkage to a variety of integrin-binding proteins. We made two different mixtures of integrin-binding proteins (protein “cocktails”), which consisted of either integrin-binding ECM proteins that are found in typical basement membranes (70% collagen III, 15% collagen IV and 15% laminin at 10 μ g/cm²), or in inflammation and wound healing (50% collagen I and 50% fibronectin at 10 μ g/cm²). The proteins used were type I collagen (rat tail) and laminin (mouse) (both from Life Technologies), recombinant human collagen III (FibroGen, San Francisco, CA), recombinant human collagen IV (Neuromics, Edina, MN), and human plasma fibronectin (EMD Millipore, Billerica, MA). Protein cocktails were made in sterile

PBS and adjusted to pH 3 to prevent collagen gelation. Post-protein reaction, hydrogels were washed 3x over an hour in sterile PBS with shaking, and then UV sterilized for 60 minutes before cell seeding.

3.3.4 Quantification of protein coupling

We quantified protein coupling to the hydrogels with an indirect ELISA[13]. Hydrogels were polymerized on coverslips, swelled in PBS overnight, and coupled with varying concentrations of recombinant human collagen III (Fibrogen, San Francisco, CA) with sulfo-SANPAH, as described above. The gels were washed 5x with PBS, incubated with primary antibody to collagen III (clone 1E7; Santa Cruz Biotechnology, Dallas, TX) in PBS, at 37°C for 90 minutes, washed again 5x with PBS, and incubated with secondary antibody conjugated to horseradish peroxidase (HRP; Abcam, Cambridge, UK) at 37°C for 90 minutes. After washing 5x with PBS, the gels were incubated with 0.1 mg/mL 3,3',5,5'-tetramethylbenzidine (TMB; Sigma-Aldrich) and 0.06% hydrogen peroxide (Thermo Fisher Scientific) in 0.1 M sodium acetate (pH 5.5; Sigma-Aldrich) for 40 minutes at room temperature with shaking. Immediately, an equal volume of 1 M sulfuric acid (Sigma) was added to each well, and the absorbance at 450 nm was measured with a BioTek ELx800 absorbance microplate reader (BioTek, Winooski, VT).

3.3.5 Focal adhesion quantification and imaging

All cell lines were seeded in serum-free DMEM (with the exception of HEP3Bs, which were seeded in MEM) on 18, 26, 165 and 400 kPa PEG-PC hydrogels coupled with 10 $\mu\text{g}/\text{cm}^2$ collagen I. After 48 hours, cells were rinsed 2x with warm PBS, fixed in fresh

4% formaldehyde, and blocked with AbDil (2% BSA in Tris-buffered saline with 0.1% Triton X-100, TBS-T). Vinculin was immunofluorescently labeled with a monoclonal mouse anti-vinculin antibody (Sigma-Aldrich) and an anti-mouse FITC secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). F-actin was fluorescently labeled with Alexa Fluor 555-conjugated phalloidin (Life Technologies), and cell nuclei were labeled with DAPI (MP Biomedicals, Santa Ana, CA). Antibody incubations were performed for 1 hour in AbDil, in the dark, and cells were thoroughly washed between labeling steps with TBS-T. Each sample was equilibrated with ProLong Gold antifade reagent (Life Technologies) for 5 minutes before imaging. Images were taken with a 63x oil immersion objective, on a Zeiss Axio Observer Z1 microscope (Carl Zeiss, Oberkochen, Germany), and ImageJ [14] was used to adjust the brightness and contrast for clarity in figures. With unmodified images, ImageJ's built-in measurement functions were used to quantify focal adhesion area and circularity. A minimum of 42 focal adhesions were manually traced from a minimum of 8 cells per condition.

3.3.6 Cell proliferation

In 96-well glass-bottomed plates, PEG-PC hydrogels with Young's moduli of 18, 26, 165 and 400 kPa were polymerized with APS and TEMED. Sulfo-SANPAH was used to attach collagen I at $10 \mu\text{g}/\text{cm}^2$, as described above. 231s and SkBr3s were seeded at 6,000 cells/well in serum-free DMEM with 1% P/S; HEP3B cells were seeded at 6,000 cells/well in MEM media with 10% FBS and 1% P/S. In all cases, the media was exchanged for serum-containing media (10% FBS) 24 hours after seeding and was replenished every two days. Five days after seeding, the MTS-based assay CellTiter96 AQueous (Promega,

Madison, WI) was added to each well at 20 μL /well to indirectly measure cell proliferation via mitochondrial redox activity. After 4 hours of incubation, the absorbance was read at 490 nm with a BioTek ELx800 (BioTeK) absorbance microplate reader. In order to demonstrate how cell number changes as a function of hydrogel modulus, the data is presented as a fold-increase in absorbance compared to the softest (18 kPa) hydrogel tested.

3.3.7 Statistical analysis

Statistical analysis was performed using Prism v5.04 (GraphPad Software, La Jolla, CA). Data are reported as mean \pm standard deviation, unless otherwise noted. Statistical significance of the difference between pairs of means was evaluated by computing P-values with unpaired Student's t-tests (with Welch's correction as necessary). When one-way ANOVAs were performed, the statistical significance of pairwise differences was determined with the Tukey post-test. $P \leq 0.05$ is denoted with *, ≤ 0.01 with **, ≤ 0.001 with *** and ≤ 0.0001 with ****; $P > 0.05$ is considered not significant ('ns').

3.4 Results

3.4.1 Protein surface density can be controlled with sulfo-SANPAH

To demonstrate that sulfo-SANPAH coupling of proteins to PEG-PC is robust and controllable, we coupled PEG-PC hydrogels (84 mM PEGDMA) with 5, 10 and 20 $\mu\text{g}/\text{cm}^2$ (theoretical) of collagen III and indirectly measured the relative concentrations with an ELISA assay (Fig. 3.1). We found that the ELISA signal is linearly proportional to the theoretical concentration of collagen III protein ($R^2 = 0.9622$). This result confirms the

coupling of proteins to PEG-PC with sulfo-SANPAH, and that this process can be precisely controlled to achieve different surface concentrations of proteins.

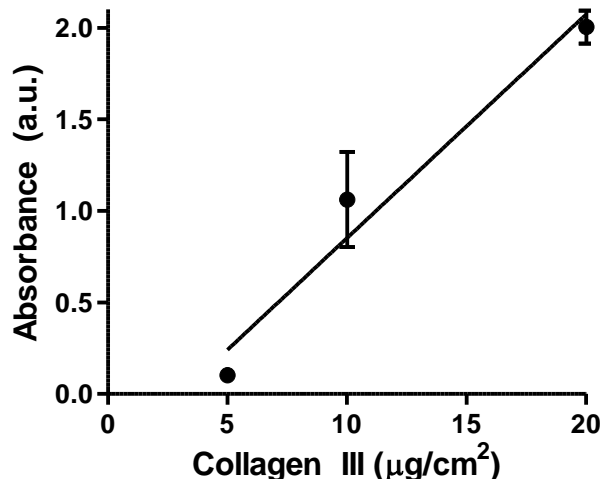


Figure 3.1: Control of PEG-PC protein surface concentration with sulfo-SANPAH

With an ELISA assay, we demonstrate that the surface concentration of proteins coupled to PEG-PC with sulfo-SANPAH is controllable, and linearly proportional to protein concentrations in solution.

3.4.2 Cells adhere and spread to protein-coupled PEG-PC surfaces

We covalently attached integrin-binding ECM proteins to the surface of PEG-PC hydrogels with two methods: sulfo-SANPAH and PEG-SVA. Both methods enabled cell attachment and spreading on the PEG-PC surfaces (Fig. 3.2A) with multiple cell types (Fig. 3.2B), and different protein mixtures (Fig. 3.2C). As shown in Figure 3.2A, no cell attachment or spreading occurs on PEG-PC surfaces without sulfo-SANPAH or PEG-SVA modification, demonstrating again that PEG-PC hydrogels are non-fouling, and useful for studies in which parsing the roles of integrin-binding vs. mechanical properties is desired. With coupling, however, extensive cell spreading was observed consistently for all cell types we tested (examples shown are left: smooth muscle, right: liver carcinoma).

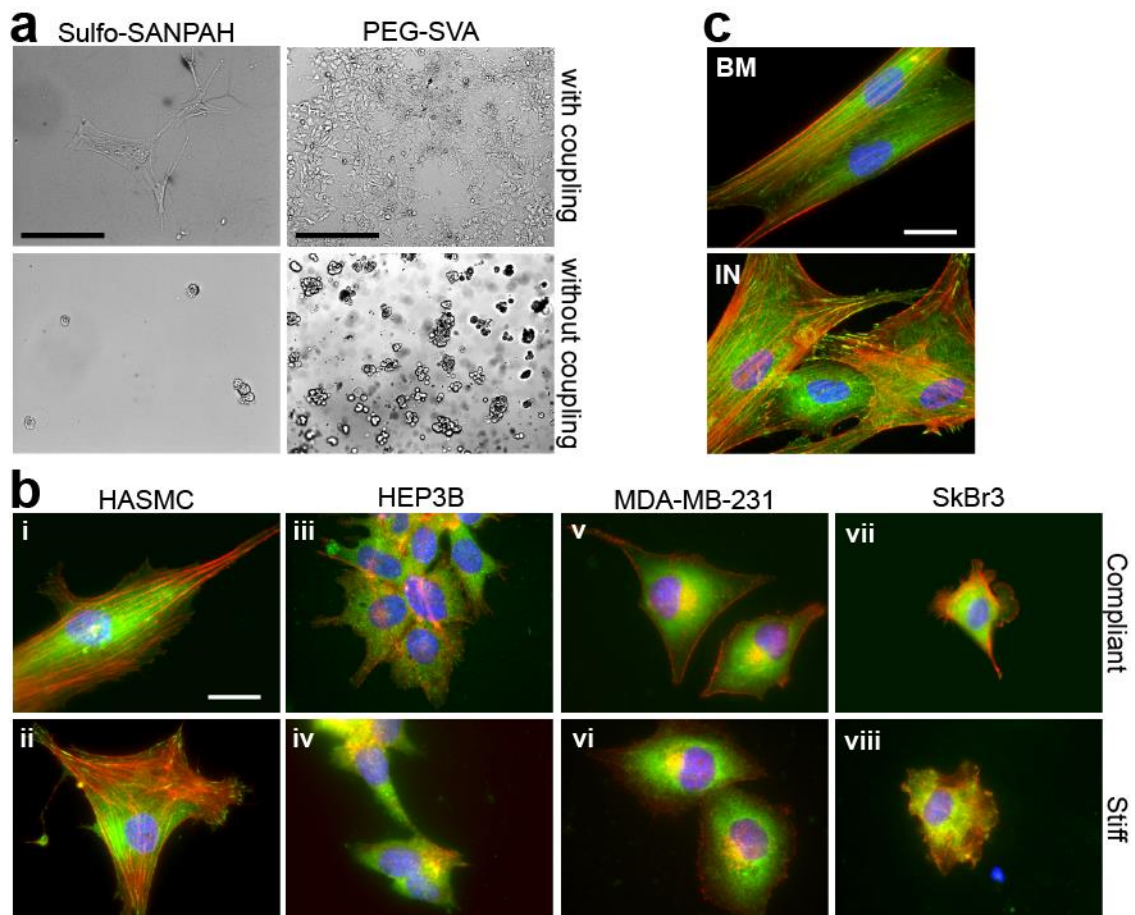


Figure 3.2: Modulus and integrin-binding on PEG-PC gels modulates cell morphology

(a) (Left) HASMCs on PEG-PC (84 mM PEGDMA) treated with (top) or without (bottom) sulfo-SANPAH and collagen I at $10 \mu\text{g}/\text{cm}^2$, scale bar is $100 \mu\text{m}$. (Right) HEP3B cells on PEG-PC (22 mM PEGDMA) with (top) or without (bottom) PEG-succinimidyl valerate (PEG-SVA) and 65% collagen III, 23% collagen I and 2% fibronectin at $5 \mu\text{g}/\text{cm}^2$, scale bar is $200 \mu\text{m}$. (b) i - ii: HASMCs on PEG-PC (compliant = 15 and stiff = 84 mM PEGDMA, 3 and 170 kPa, respectively, except for SkBr3 for which stiff = 0.15 M PEGDMA and 400 kPa) with collagen I. Vinculin = green, F-actin = red, and DNA = blue. iii - iv: HEP3Bs. v - vi: MDA-MB-231s, and vii - viii: SkBr3s. Scale bar is $20 \mu\text{m}$. (c) HASMCs on basement membrane-like ECM (70% collagen III, 15% each collagen IV and laminin at $10 \mu\text{g}/\text{cm}^2$ total) and on inflammatory ECM (50% each fibronectin and collagen I at $10 \mu\text{g}/\text{cm}^2$ total). Scale bar is $20 \mu\text{m}$. Figure adapted with permission from [15], Copyright © 2013 American Chemical Society.

3.4.3 Substrate modulus controls focal adhesion maturity

Going further, we demonstrated that cells sense differences in the stiffness of PEG-PC hydrogels coupled with $10 \mu\text{g}/\text{cm}^2$ collagen I by performing experiments with three human cell types: liver carcinoma (HEP3B) and two breast cancer cell lines (SkBr3 and MDA-MB-231) (Fig. 3.2B). After 48 hours of culture, cells were fixed and stained for actin (red), DNA (blue) and vinculin (green). Vinculin staining was used to identify focal adhesions [16], which were counted and analyzed with ImageJ (Fig. 3.3A). We manually traced focal adhesions at 4 stiffnesses to quantify the number of focal adhesions visible per cell (Fig. 3.3B), the average focal adhesion area (Fig. 3.3C) and elongation (Fig. 3.3D). Focal adhesion circularity was quantified with ImageJ as a measure of adhesion maturity, with elongation (circularity^{-1}) being associated with increased adhesion stability (Fig. 3.3D) [17].

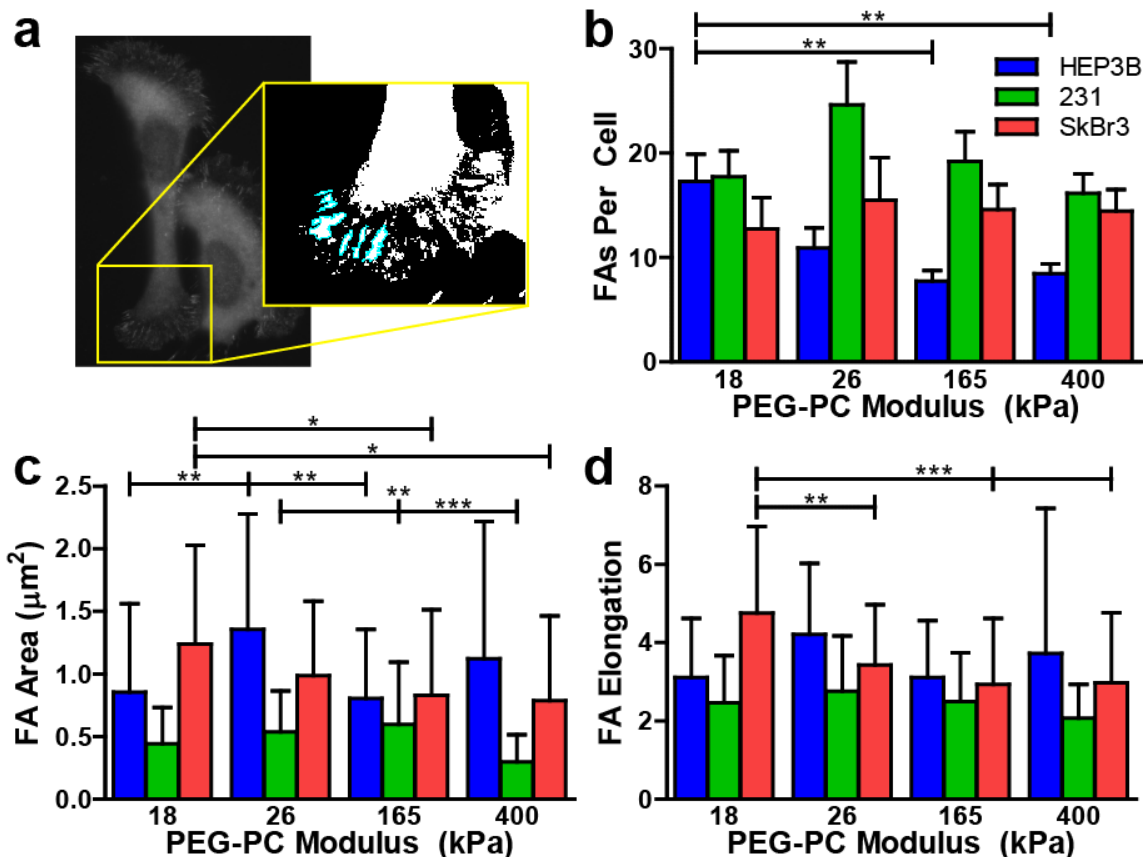


Figure 3.3: PEG-PC modulus control of focal adhesions is cell-specific

(a) Example of image thresholding and focal adhesion tracing performed in ImageJ of an SkBr3 cell with labeled vinculin. (b) Average number of focal adhesions (FA) counted per cell as a function of PEG-PC modulus with $10 \mu\text{g}/\text{cm}^2$ collagen I. HEP3B cells had significantly fewer focal adhesions on the 165 and 400 kPa PEG-PC gels than on the 18 kPa gels, as measured with one-way ANOVA and the Tukey post-test for significance. (c) Average focal adhesion area on 18 – 400 kPa PEG-PC with $10 \mu\text{g}/\text{cm}^2$ collagen I. (d) Average focal adhesion elongation (departure from circularity) on 18 – 400 kPa PEG-PC with $10 \mu\text{g}/\text{cm}^2$ collagen I. SkBr3 focal adhesion elongation decreased by 37% from 18 to 400 kPa. Figure adapted with permission from [15], Copyright © 2013 American Chemical Society.

We observed that focal adhesion area decreased with Young’s modulus in SkBr3 and 231 cells (Fig. 3.3C; areas on the stiffest PEG-PC were reduced approximately 37 and 45% from the softer PEG-PC hydrogels, $p < 0.05$ and $p < 0.01$, respectively), and SkBr3 elongation also decreased with increasing stiffness. Interestingly, HEP3B focal adhesion

area is biphasic (Fig. 3.3C; area on 26 kPa PEG-PC is 65% larger than on 18 and 165 kPa, $p < 0.01$), and they were also the only cell line to have a significantly different number of focal adhesions per cell. Both HEP3Bs and MDA-MB-231s had very few cells with actin stress fibers, but many cells had prevalent, actin-rich filopodia (Fig. 3.2B iii-iv, v-vi). These results demonstrate the ability of PEG-PC hydrogel mechanical properties to modulate cytoskeletal organization, and revealed that mechanosensitivity is cell line-specific.

3.4.4 The effect of substrate modulus on proliferation is cell type-specific

We investigated the role of substrate stiffness on the proliferation of three types of cancer cells. HEP3Bs, SkBr3s and 231s were each seeded on PEG-PC gels across a range of modulus with $10 \mu\text{g}/\text{cm}^2$ collagen I. We found that 231 proliferation increases by approximately 25-35% (over the reading on 18 kPa hydrogels), for hydrogels between 18 and 26 kPa, before saturating (Fig. 3.4A). HEP3Bs were most sensitive to substrate modulus, with average proliferation increasing about 60% from 18 kPa to 26 kPa, and then another 60% from 26 kPa to 165 kPa (Fig. 3.4C). Finally, SkBr3s proliferation decreased by 25% between 26 kPa and 165 kPa, and another 25% between 165 kPa and 400 kPa (Fig. 3.4B).

We connect these proliferation results with our analysis of focal adhesions using simple statistical methods. For instance, HEP3B cells proliferated substantially more on stiff substrates, but they had fewer focal adhesions on these substrates; in contrast, SkBr3 cells proliferate less on the stiffer substrates, with smaller focal adhesions. Finally, 231 cell proliferation increased marginally with stiffness, and their focal adhesions were also

minimally affected by substrate modulus. These three contrasting trends imply differing degrees of interconnectedness of adhesion, contractile and proliferation signaling pathways in these cell types. Although the mechanisms of this behavior are outside the scope of this particular study, we quantified the correlations, if any, between focal adhesion number, area, elongation, and cell proliferation for each cell type. Using the Spearman correlation coefficient we find that SkBr3 focal adhesion area and elongation are strongly correlated with proliferation ($R = 1$ and 0.8 , respectively), and HEP3B focal adhesion number is inversely correlated with proliferation ($R = -0.8$).

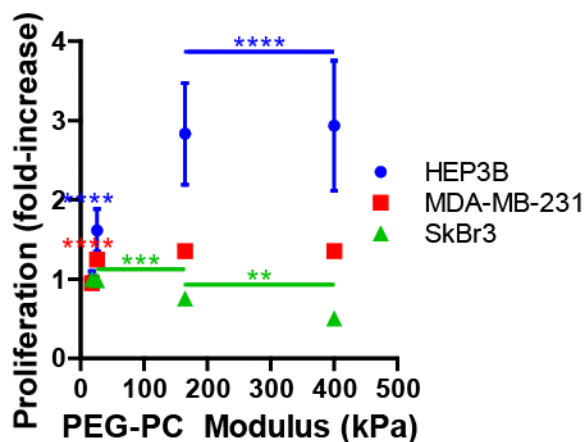


Figure 3.4: PEG-PC modulus affects cellular proliferation

HEP3B (●), MDA-MB-231 (■), and SkBr3 (▲) cells were grown on PEG-PC from 18 to 400 kPa for 5 days and their proliferation quantified with CellTiter96. Results are presented as arbitrary absorbance units normalized to the 18 kPa condition for each cell type (reported as “1”). 231s and SkBr3s display minor dependence of proliferation on substrate modulus, but with opposing trends. However, HEP3B proliferation is strongly and positively affected by substrate modulus. Error bars are standard error, with N=4, for 231s, N=3 for SkBr3s and N=2 for HEP3Bs. Figure adapted with permission from [15], Copyright © 2013 American Chemical Society.

3.5 Discussion

In the mechanobiology field, researchers are interested in the availability of biocompatible materials with tunable mechanical properties that can probe how cells sense mechanical forces, and the role that mechanical forces may have in disease and development. Moreover, materials for these applications must be capable of being functionalized with adhesive ligands, be they full-length ECM proteins or adhesive peptide sequences. In this work, we have investigated the suitability of PEG-PC as a hydrogel for these applications. In addition to the exceptional mechanical properties described in Chapter 2, we have shown here that full-length proteins can be coupled to PEG-PC surfaces with sulfo-SANPAH and UV light, or alternatively by the addition of PEG-SVA to the hydrogel bulk polymer (Fig. 3.2A). The attachment of ECM proteins supports cell adhesion

and spreading on PEG-PC (Fig. 3.2), and the amount of protein coupled with sulfo-SANPAH can be controlled by adjusting the protein concentration in solution during the reaction (Fig. 3.1).

As proof of concept in mechanobiology, we cultured several cell lines (HASMCs, HEP3Bs, and several breast cancer cell lines: SkBr3 and 231 shown here, BT-549 and others not shown) on PEG-PC gels from 3 to 400 kPa Young's moduli functionalized with collagen I, and quantified the effect of substrate stiffness on cell morphology and focal adhesion number, size and elongation. The relationship between focal adhesion properties and substrate modulus was cell type-dependent (Fig. 3.3B-D), as was the relation between focal adhesion properties and proliferation. Mechanobiology reports with these cell lines is limited, but one group reported a decrease in the relative adhesivity of 231s with increasing stiffness [18]. This report supports our findings that focal adhesion area decreases with stiffness, but a direct comparison cannot be drawn due to experimental differences.

Comparing our focal adhesion quantification with proliferation has revealed cell type-specific differences in mechanotransduction. These different trends highlight how cell type-specific differences in mechanotransduction machinery reported elsewhere (integrins, adhesion proteins, actin, myosin and many more [19,20]) can result in very different mechanosensing properties and subsequent changes in pathway signaling. To our knowledge, this is the first report on the effects of 2D substrate stiffness on proliferation in these cell types. Other, previously available mechanotransduction data on these cell lines is limited, and our results may guide future research. We propose that PEG-PC hydrogels

are ideal for studies of mechanobiology or mechanotransduction, development, and stiffness-associated disease states such as cancer and atherosclerosis.

3.6 Conclusions

We have shown that PEG-PC can be functionalized, with control, to support cell adhesion and spreading in 2D, and demonstrated the mechanosensing of 3 cancer cell lines over a range of ~380 kPa Young's modulus. All 3 cell lines had varying responses to changes in stiffness, as reflected in their proliferation rates and focal adhesion shape, size and number. These results, plus PEG-PC's exceptional mechanical, optical and fouling properties, prove it to be an excellent choice for future studies in these areas.

3.7 References

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CHAPTER 4

EXTRACELLULAR MATRIX COMPOSITION IS THE MAJOR DRIVER OF SMC PHENOTYPE AND BEHAVIORS

4.1 Abstract

The aim of the work described in this chapter is to gain a deeper understanding of how the changing microenvironment affects smooth muscle cell (SMC) phenotype and behaviors in atherosclerosis. We have designed a complex *in vitro* model to recapitulate characteristic changes in the composition of the surrounding extracellular matrix (ECM), soluble factors, and vessel wall stiffness that SMCs undergo in this disease. We found that ECM composition is the primary regulator of cell behaviors, and modulates the effects of soluble factors, whereas stiffness had an unexpectedly modest role. Surprisingly, in direct conflict with previous studies, we did not find that proliferation and migration are inversely related to SMC marker expression. Cell signaling studies revealed that marker expression is mediated chiefly via focal adhesion kinase (FAK) signaling, with additional evidence of ECM-specific differences in their regulation downstream of FAK. Finally, our findings suggests that longstanding disagreement regarding the distinction between proliferative and migratory phenotypes in SMCs is well-explained by the changing ECM composition, and we propose hypothetical, integrin-driven models to explain this switchover. From these results we conclude the importance of increasing the complexity of *in vitro* models to carefully match critical features of *in vivo* microenvironments, which will induce physiological behaviors and aid in identification of novel drug targets and treatments.

4.2 Introduction

Heart disease is the leading cause of mortality worldwide, accounting for over 30% of all deaths [1]. Smooth muscle cells (SMCs) play an important role in atherosclerosis, the pathological condition that leads to most cerebrovascular- and cardiovascular disease-related deaths. These normally quiescent, immobilized cells are induced to migrate from the arterial media to the developing plaque where they may rapidly proliferate and contribute to plaque volume and instability. During this invasion process, SMCs are subjected to significant changes in their local microenvironment, including the composition of the surrounding extracellular matrix (ECM), the availability of soluble factors, and the stiffness of the vessel wall.

Due to their role in arterial mechanics, SMCs – along with fibroblasts, a closely related cell type – have been the subject of many studies in the fields of mechanotransduction and biomechanics. Of major interest to these fields are the effects of substrate compliance and ECM proteins on aberrant SMC behavior in atherosclerosis, particularly proliferation and migration. These characteristics are typically tested with biocompatible hydrogels [2–5] having tunable mechanical properties and the ability to be coupled with adhesive ECM proteins. With these *in vitro* model systems, some common trends have emerged. Typically, proliferation is positively correlated with substrate compliance in SMCs [6,7] and other cells [8], but reports vary based on the material and cell type [9,10]. ECM proteins and other adhesive ligands are also capable of regulating proliferation and migration of SMCs [2,11] and other cell types, and of modulating the effects of soluble growth factors (GFs) and cytokines [4,12,13].

However, many of these *in vitro* studies do not reproduce, or in some cases, conflict with conclusions derived from *in vivo* studies. For example, SMCs reside on a spectrum of phenotypes, yet only two phenotypes are typically described: in a healthy, intact arterial media, SMCs are differentiated and ‘contractile,’ whereas proliferative SMCs in an atherosclerotic lesion are in the dedifferentiated, ‘synthetic’ phenotype. Further, SMC proliferation and marker expression (and therefore differentiation) are typically regarded to be inversely related [14–17], but conflicting reports [18–21] raise serious questions regarding the true diversity of SMC phenotypes and the interrelation between the extracellular environment, phenotype, and pathological behaviors. Despite the comparatively simplistic designs of the *in vitro* models used, these prior studies have nevertheless revealed substantial and vital information concerning the effects of local stimuli on cellular phenotype and behaviors that are the foundation of the work described here.

Given that the arterial microenvironment is much more complex and heterogeneous than a typical *in vitro* model, we hypothesize that some – and possibly many – conflicts between *in vitro* and *in vivo* findings are due to the loss of signaling complexity that occurs when a complex *in vivo* microenvironment is reduced and simplified for *in vitro* studies. We believe that conflicts in the literature such as these, as well as the high failure rate of drug candidates in pre-clinical and phase I clinical trials, can potentially be explained by significant differences between human biology, *in vitro* models, and animal models.

In response, we investigated the diversity of SMC phenotypes and behaviors with a polymeric biomaterial system designed to recapitulate not only multiple major features (physical, chemical, and mechanical) of the *in vivo* microenvironment, but also the changes

in these features that SMCs experience during invasion of a plaque. We used poly(ethylene glycol) dimethacrylate-phosphorylcholine (PEG-PC) hydrogels [9,22], described in Chapter 2, as a tunable biomaterial substrate, and coupled them with mixtures of integrin-binding ECM proteins representative of the changing arterial microenvironment. We forced SMCs into the contractile phenotype with a series of media changes, and then quantified their plasticity toward the synthetic phenotype and pathological cell behaviors as modulated by substrate stiffness, ECM/integrin binding, and soluble factors.

Foremost, our results show that ECM/integrin binding is the most significant factor in regulating SMC behaviors and modulating the effects of soluble factors. Our data also strongly suggests that literature conflicts regarding the distinction between migratory and proliferative phenotypes may be best explained by the changes in the surrounding ECM composition, which is natively captured in *in vivo* studies, but not in simpler *in vitro* models. Strikingly, many of our results do not agree with some widely-accepted notions about SMC phenotype *in vitro*, most notably our finding that SMC proliferation is *not* inversely correlated with contractile marker expression in our system, nor is marker expression related to SMC morphology. We also found that marker expression is primarily regulated by focal adhesion kinase (FAK) signaling through phosphatidylinositol 3-kinase (PI3K) and Akt. Finally, we present a detailed, hypothetical model that fits very well with observations from this and many other reports, and describes a plausible mechanism by which integrin binding controls the switch from a migratory to proliferative phenotype. This study represents an early step towards closer replication of the *in vivo* microenvironment, and we hope our work will inspire others to carefully consider the effects of numerous relevant stimuli on SMC behavior, with any tunable platform.

4.3 Materials & methods

4.3.1 Cell culture

All cells and culture media reagents were purchased from Life Technologies, Carlsbad, CA, unless otherwise noted. Human aortic smooth muscle cells (HASMCs) from passages 2 through 8 were routinely cultured in Medium 231 supplemented with 1% penicillin-streptomycin (P/S) and smooth muscle growth supplement (SMGS; contains 4.9% FBS, 2 ng/mL human basic fibroblast growth factor, 0.5 ng/mL human epidermal growth factor, 5 ng/mL heparin, 2 μ g/mL recombinant human insulin-like growth factor-I, and 0.2 μ g/mL bovine serum albumin). 1% P/S was used in all media types. Experiments were performed by trypsinizing confluent HASMCs on tissue culture plastic, resuspending in Dulbecco's modified Eagle's medium (DMEM) and 10% fetal bovine serum (FBS) and seeding on ECM-coated PEG-PC samples. Cells were synched into the contractile phenotype 24 hours post-seeding by briefly washing with serum-free DMEM and then treating with serum-free DMEM for 48 hours, then stimulating with 2.5 ng/mL recombinant human TGF β ₁ (R&D Systems, Minneapolis, MN) in serum-free DMEM for an additional 48 hours. Finally, media was changed to DMEM supplemented with either SMGS ('growth medium'), or smooth muscle differentiation supplement (SMDS; contains 30 μ g/mL heparin and 1% FBS; 'differentiation medium') for 48-96 hours, depending on the experiment.

4.3.2 PEG-PC hydrogel polymerization and contact mechanics measurements

As a base for 2D PEG-PC hydrogels, 15 mm no. 2 glass coverslips (Thermo Fisher Scientific, Waltham, MA) were treated with a UV Ozone ProCleaner for 10 minutes

(BioForce Nanosciences, Las Vegas, NV), and reacted with 50 mL of 95% ethanol (adjusted to pH 5.0 with glacial acetic acid; Thermo Fisher Scientific) with 2 vol.% 3-(trimethoxysilyl) propyl methacrylate (Sigma-Aldrich, St. Louis, MO) for 2 minutes with shaking, washed three times in 200 proof ethanol, and dried at 120°C for 20 minutes [23]. Prepared coverslips were wrapped in aluminum foil and stored in a desiccator at room temperature.

PEG-PC polymer hydrogel precursor solutions were prepared by mixing PEGDMA (average M_n 750, Sigma-Aldrich), between final concentrations of 14.7 mM and 0.135 M (1.1 - 10.1 wt.%), and 0.6 M (17 wt.%) 2-methacryloyloxyethyl phosphorylcholine (Sigma-Aldrich; Fig. 4.1) in phosphate buffered saline (PBS). Solutions were sterilized with a 0.2 μ m syringe filter (Thermo Fisher Scientific) and degassed for 30 seconds with grade 5.0 nitrogen gas. Free-radical polymerization was induced with the addition of 0.125 vol.% tetramethylethylenediamine (TEMED, Bio-Rad Laboratories, Hercules, CA) and 0.05 wt.% ammonium persulfate (APS, from a 20 wt.% stock in water; Bio-Rad Laboratories), then 50 μ L aliquots of polymer solution were pipetted onto methacrylate-silanized coverslips and topped with untreated coverslips. These reactions were performed on the bench at room temperature, with empirically-determined optimal polymerization times of 22, 20, 19 and 18 minutes for 14.7, 43.1, 83.8 and 135 mM PEGDMA, respectively. At the specified times, the gels were immediately moved to 6-well plates (3 gels/well) and covered with PBS. The next day, top coverslips were carefully removed with fine forceps.

To quantify the elastic moduli of the hydrogels directly on the coverslips, a piezo-controlled linear actuator (Burleigh Inchworm Nanopositioner) was used to bring a polystyrene-coated steel cylindrical probe (cross-sectional radius \sim 0.75 mm) into contact

with a sample. Upon contact and reproach, the relative displacement, δ , and resulting force, P , were measured with a custom-designed load cell (Fig. 4.1a). In addition, the contact radius, a , established between the probe and the gel was confirmed to equal the radius of the probe with a Zeiss Axiovert 200M microscope (Carl Zeiss, Oberkochen, Germany). Tests were conducted at a crosshead velocity of 0.5 $\mu\text{m/s}$ and the total displacement for each test was fixed at 30 μm .

To quantify the elastic modulus, the compliance, $C = \frac{\partial\delta}{\partial P}$, was determined from the linear force-displacement curves (Fig. 4.1a), and the modulus, E , determined by [24]:

$$E = \frac{(1 - \nu^2)}{2Ca} \left\{ 1 + 1.33 \left(\frac{a}{h}\right) + 1.33 \left(\frac{a}{h}\right)^3 \right\}^{-1}$$

where $\frac{a}{h}$ is the ratio of the contact radius to the thickness of the hydrogel. Fully swollen hydrogels are often considered to be incompressible under relevant time scales of loading [25], such that the Poisson ratio, ν , is typically in the range of 0.4-0.5. We present the results in Figure 4.1 as both an effective modulus, $E^* = \frac{E}{1-\nu^2}$, and as a Young's modulus, E , with an estimated Poisson's ratio of 0.5.

4.3.3 Protein functionalization to hydrogel surfaces

Two protein cocktails were prepared in PBS (Fig. 4.2): 'BaM' (basement membrane representation) containing 50% (w/v) collagen IV (recombinant human; Sigma-Aldrich) and 50% laminin (mouse), and 'InF' (inflammatory ECM representation) containing 50% monomeric collagen I (rat tail) and 50% fibronectin (human plasma; EMD Millipore, Billerica, MA). Glacial acetic acid was added to the InF cocktail at 0.14 vol.% to prevent collagen fibril formation.

Hydrated gels were treated with sulfo-SANPAH (0.3 mg/mL in pH 8.5 HEPES buffer; ProteoChem, Denver, CO) under UV light for 10 minutes at ~3 in. They were then washed with PBS 2x and incubated with 75 μ L of cocktail for a 3 μ g/cm² theoretical surface concentration. After 20 h, gels were transferred to fresh wells, washed 3x over 30 min in PBS, and UV sterilized for at least 30 min prior to cell seeding.

4.3.4 Cell proliferation assays

We used the common alamarBlue (resazurin [26]) assay to quantify relative cell populations. An alamarBlue stock solution was made by adding resazurin sodium salt (Sigma-Aldrich) to 0.1 wt.% in PBS and then sterile-filtering with a 0.2 μ m filter (Thermo Fisher Scientific). The assay was performed by replacing the media on samples with a solution of 10% alamarBlue stock and 90% DMEM containing 10% FBS and 1% P/S. The sample plates were then incubated at 37°C/5% CO₂ for 2 hours, 2 x 100 μ L aliquots were transferred to 96-well plates and fluorescence measured with a Spectramax M5 microplate reader (Molecular Devices, Sunnyvale, CA) using 550 nm (excitation) and 585 nm (emission) wavelengths. To assess the impact of soluble factors, ECM composition and stiffness on SMC proliferation, HASMCs were seeded at approximately 5,600 cells/cm² on samples with Young's moduli from approximately 40 to 94 kPa in 24-well tissue culture plates. 24 hours post-seeding, initial cell adhesion was assessed on a set of technical replicates with the alamarBlue assay. The final cell proportions of the remaining technical replicates were assayed following phenotype syncing and 96 hours of treatment with either differentiation or growth medium, and we have reported these results as the fold-difference between the first and final days with 5-6 independent biological replicates per condition.

4.3.5 Cell migration assays

To assess the impact of soluble factors, ECM composition and stiffness on SMC 2D motility, cells were sparsely seeded on samples in 12-well plates, synced towards the contractile phenotype, then immobilized in the wells of 24-well plates with 5-minute epoxy (Devcon, Danvers, MA) and either growth or differentiation medium pipetted into the wells. Cell motility was observed with a Zeiss Axio Observer Z1 (Carl Zeiss) inverted microscope (5x objective, brightfield; images taken for 12 hours at 15 minute intervals). Cell positions were manually tracked with ImageJ [27] using the Manual Tracking plugin [28], and we calculated the average velocity, displacement, and chemotactic index of each tracked cell by fitting to a random walk model in MATLAB with code provided by Aaron Meyers. We have reported the results as the population average velocities and chemotactic indices for each condition, from 3-5 independent biological replicates and a minimum of 100 cells per condition.

4.3.6 Cell invasion assays

To assess the impact of soluble factors, ECM composition and stiffness on SMC invasion into collagen I gels, we performed experiments in 24-well plates, as described above, with 40 and 94 kPa Young's modulus PEG-PC, but immediately after phenotype syncing we polymerized collagen I gels on each sample. This was performed by mixing either differentiation or growth medium 1:20 with sterile 1 M NaOH (Thermo Fisher Scientific), and mixing this solution 1:2 with 3 mg/mL type I collagen (rat tail; Life Technologies) for a 2 mg/mL collagen I solution. All solutions were pre-chilled and kept on ice. This well-mixed solution was then pipetted over each sample (300 μ L/well) and the

plates incubated at 37°C/5% CO₂ for 5 minutes. After polymerization, 600 µL of warm differentiation or growth medium was slowly pipetted onto each collagen I gel and the plates returned to the incubator. After 48 hours, the media solutions were very carefully aspirated off the gels and replaced with fresh media. After another 48 hours, the samples were imaged with a Zeiss Axio Observer Z1 inverted microscope, by taking images with 30 µm Z-slices at 5x and a 10x objective in brightfield. The approximate depth of invasion into the collagen gels was determined manually by counting the number of Z-slices from the surface of the PEG-PC gel to the focal plane of invaded cells. We report these results as the average cell invasion depth, with a minimum of 3 independent biological replicates and 140 cells per condition.

4.3.7 SMC marker Western blotting

To assess the impact of soluble factors, ECM composition and stiffness on SMC marker expression, we seeded HASMCs at approximately 11,000 cells/cm² on samples in 24-well plates and cultured them as described in the preceding sections. Note that, to eliminate the possibility that differences in expression are due to differences in time in culture, for all media-type comparisons the samples were maintained in culture for the same length of time, i.e. in the comparison between serum-starved and TGFβ₁ treated samples, the TGFβ₁ treated samples were serum-starved for 48 hours and TGFβ₁ treated for 48 hours, whereas the serum starved samples were in serum-free media for 96 hours. At various time points, samples were briefly rinsed with ice-cold PBS, and immediately lysed, on ice, for 5 minutes with freshly-prepared, ice-cold Tris-Triton lysis buffer containing 10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-

100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate and freshly-added protease inhibitors (cOmplete Mini EDTA-free protease inhibitor tablets, Roche Applied Sciences, Penzberg, Germany). To increase total protein concentration, lysis buffer volumes were kept low (< 100 μ L/well) and/or 2-3 replicates of each condition were pooled with the same aliquot of lysis buffer. If not to be used that day, lysates were snap frozen in liquid nitrogen and stored at -80°C.

The protein concentrations of freshly prepared lysates, or previously-frozen lysates thawed on ice, were measured with the Pierce BCA protein assay in a microplate format (Thermo Fisher Scientific) using a BioTek ELx800 absorbance microplate reader (BioTek, Winooski, VT), and the sample protein concentrations normalized by dilution with ice-cold Tris-Triton buffer containing freshly-added protease inhibitors. Each sample, typically containing 6-10 μ g total protein, was then mixed with Laemmli sample buffer (4x; Boston BioProducts, Ashland, MA), heated in boiling water for 5 minutes, loaded on 4-20% SDS-PAGE gels (10 well, 50 μ L/lane Mini-Protean[®] TGX precast gels, Bio-Rad Laboratories) along with a lane for the Precision Plus Protein Kaleidoscope protein standard (Bio-Rad), and run at 200 V for 30 minutes in SDS-PAGE running buffer (25 mM Tris, 190 mM glycine, 0.1% SDS).

The proteins were then transferred to PVDF membranes in Western blot transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol) at 110 V for 90 minutes on ice. After transfer, blots were blocked for 2 hours at room temperature, with agitation, using either 5 wt.% bovine serum albumin (BSA; Sigma-Aldrich) in Tris-buffered saline with 0.1 vol.% Triton X-100 (TBS-T) for calponin detection, or in TBS-T containing 5 wt.% nonfat milk for smoothelin detection. After blocking, the blots were incubated overnight at 4°C, with

agitation, in BSA blocking buffer containing primary antibody to calponin (clone EP798Y, 1:10000; Abcam, Cambridge, MA), or in milk blocking buffer with primary antibody to smoothelin-B (clone H-300, 1:200; Santa Cruz Biotechnology, Dallas, TX). The next day, the blots were washed 5x with TBS-T, then incubated with HRP-conjugated secondary antibody (anti-rabbit polyclonal, Abcam) in blocking buffer for 2 hours at room temperature, with agitation, then washed 5x with TBS-T. Finally, detection was performed with enhanced chemiluminescence (ECL) by mixing equal volumes of a solution containing 2.5 mM luminol (3-aminophthalic hydrazide, 250 mM stock in DMSO; Thermo Fisher Scientific) and 0.396 mM p-coumaric acid (90 mM stock in DMSO, Thermo Fisher Scientific) in 100 mM Tris buffer, pH 8.6, and a solution containing 0.018% hydrogen peroxide (Thermo Fisher Scientific) in 100 mM Tris buffer, pH 8.6, incubating blots in this solution for exactly 1 minute and then imaging with a G:BOX (Syngene, Frederick, MD). Finally, the calponin blots were washed 3x with TBS-T and probed for cyclophilin B as an internal control (primary Ab from Thermo Fisher Scientific, 1:10000) with the same procedure.

4.3.8 Immunofluorescent staining of HASMCs

To fix cells for immunofluorescent staining immediately following phenotype syncing, as well as immediately following phenotype syncing plus 48 hours of treatment with differentiation or growth media, samples were rinsed 2x with warm PBS, incubated in fresh 4% formaldehyde for 20 minutes, and blocked with AbDil (2% BSA in Tris-buffered saline with 0.1% Triton X-100, TBS-T) for 1 hour. Calponin was immunofluorescently labeled with a primary antibody to calponin (clone EP798Y ; Abcam)

and an anti-mouse FITC secondary antibody (Jackson ImmunoResearch Laboratories), or smoothelin was labeled with a primary antibody to smoothelin-B (clone H-300; Santa Cruz Biotechnology) and an anti-rabbit FITC secondary antibody (Jackson ImmunoResearch Laboratories). F-actin was fluorescently labeled with Alexa Fluor 555-conjugated phalloidin (Life Technologies), and cell nuclei were labeled with DAPI (MP Biomedicals). Antibody incubations were performed for 1 hour in AbDil, in the dark, and cells were thoroughly washed between labeling steps with TBS-T. Each sample was equilibrated with ProLong Gold antifade reagent (Life Technologies) for 5 minutes before imaging. Images were taken with a 63x oil immersion objective, on a Zeiss Axio Observer Z1 microscope (Carl Zeiss).

4.3.9 Reverse transcriptase PCR to confirm smoothelin expression

We confirmed the expression of smoothelin mRNA with reverse transcriptase PCR (RT-PCR). HASMCs were grown to confluence on tissue culture plastic in complete growth medium, total RNA collected with the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich), and the concentration of RNA measured with a NanoDrop ND-1000 (Thermo Fisher Scientific). We then mixed 1 µg of total RNA with RNasin Plus (Promega), oligo(dTs) and RNase-free water according to the manufacturer's instructions, denatured secondary structures with heating at 70°C for 5 minutes, chilled on ice for 5 minutes, and finally added a reverse transcriptase master mix (1x reaction buffer, RNasin Plus, dNTPs and RevertAid Reverse Transcriptase, Thermo Fisher Scientific) to the total RNA and incubated at 42°C. After 60 minutes, the reaction was heat inactivated at 70°C for 10 minutes. The reaction product was amplified with standard PCR using JumpStart Taq DNA

polymerase (Sigma-Aldrich) and primers designed against the SMTN-B gene (accession Y13492, forward: CACTCATGTCACCAGCTTCAG, reverse: CTCTGATCCAGCATCTTGTCC). Finally, the PCR reaction product was separated on an agarose gel along with a nucleotide ladder (New England BioLabs, Ipswich, MA) and visualized with an InGenius UV gel imager (Syngene).

4.3.10 Characterization of HASMC morphology

To assess the impact of soluble factors, ECM composition and stiffness on spreading area and morphology, HASMCs were sparsely seeded on ECM-coated PEG-PC samples and, after 48 hours of treatment with the specified media type, washed with warm PBS and fixed for 20 minutes with warm 4% formaldehyde in PBS, at room temperature. The fixed samples were then washed 3x with PBS, then incubated with Cellomics Whole Cell Stain Blue (Thermo Fisher Scientific) according to the manufacturer's instructions, for 90 minutes. The samples were again washed 3x with PBS, then imaged with a Zeiss Axio Observer Z1 inverted microscope with a DAPI filter and a 10x objective. Finally, isolated cells were manually traced in ImageJ, and the areas, aspect ratios (AR) and other properties measured with the built-in "Measurement" tool. We report these results as cell averages from a minimum of 3 independent biological replicates and 100 cells per condition.

4.3.11 Cell signaling time course with MAGPIX multiplex analysis

To assess the impact of soluble factors, ECM composition and stiffness on several major intracellular signaling pathways, sample lysates were collected during 3 separate

time courses (Fig. 4.8) and analyzed using a MAGPIX instrument (Luminex Corporation, Austin, TX) and a 9-plex magnetic bead cell signaling multiplex assay kit (48-680MAG; Merck Millipore, Billerica, MA) to simultaneously measure the activities of the following phospho-proteins: CREB (pS133), ERK (pT185/pY187), NF κ B (pS536), JNK (pT183/pY185), p38 (pT180/pY182), p70 S6K (pT412), STAT3 (pS727), STAT5A/B (pY694/699), and Akt (pS473). Lysis was performed with MAGPIX lysis buffer, containing 50 mM Tris-Cl, pH 7.1, 1% NP-40, 10% glycerol, and 150 mM NaCl, with freshly-added phosphatase inhibitors (Phosphatase Inhibitor Cocktail II, 2x working concentration; Boston BioProducts) and protease inhibitors (cOmplete Mini, EDTA-free), plus (all from Thermo Fisher Scientific) additional sodium pyrophosphate (1:100 from 0.1 M stock), β -glycerophosphate (1:40 from 1 M stock), phenylmethanesulfonyl fluoride (1:500 from 0.5 M stock), leupeptin (1:1000 from 10 mg/mL stock) and pepstatin A (1:1000 from 5 mg/mL stock). To minimize protease and phosphatase activity, lysis and sample handling was performed in a 4°C environment. At each time point, sample plates were rinsed briefly with ice-cold PBS, put on ice and lysed with buffer for 5 minutes. Sample lysates were then pipetted over the gel surface several times, collected in chilled microcentrifuge tubes, snap frozen in liquid nitrogen, and stored at -80°C. On the day of each MAGPIX experiment, samples were thawed on ice, protein levels quantified with the Pierce BCA assay as described above, and concentrations normalized with fresh lysis buffer. The MAGPIX assays were then performed in accordance with the kit manufacturer's instructions. We report these results as the average net mean fluorescence intensity (MFI) of 2-4 biological replicates.

4.3.12 Kinase inhibition experiments

To assess how inhibition of 4 kinases of interest modulate SMC marker expression, we seeded HASMCs on 40 and 94 kPa ECM-coated PEG-PC samples at approximately 11,000 cells/cm², as described above. After 48 hours of serum-starvation, kinase inhibitors were co-administered during TGFβ₁ treatment. We inhibited Akt with the allosteric inhibitor sc-66 [29] at 1 μg/mL (20 mg/mL stock in DMSO; Abcam), FAK with FAK inhibitor 14 [30] at 100 μM (100 mM stock in DMSO; Merck Millipore), and PI3K with LY294002 at 50 μM (50 mM stock in DMSO; Merck Millipore). With the exception of FAK inhibitor 14, inhibitors were present during the first hour after the media change, and then replaced with inhibitor-free media. FAK inhibitor 14, however, was kept in the media during the entire 48 hours. After 48 hours, samples were lysed with Tris-Triton buffer and Western blotted as described above.

Proliferation experiments in differentiation medium were performed as described above with inhibitors to Akt or ERK (FR180204 at 20 μM final concentration; Sigma-Aldrich) administered during the first hour of differentiation medium stimulation, then additionally for one hour during the medium change at 48 hours.

4.3.13 Statistical analysis

Statistical analysis was performed using Prism v5.04 (GraphPad Software, La Jolla, CA). Data are reported as mean ± standard error, unless otherwise noted. Statistical significance of the difference between pairs of means was evaluated by computing P-values with unpaired Student's t-tests. P ≤ 0.05 is denoted with *, ≤ 0.01 with **, ≤ 0.001 with *** and ≤ 0.0001 with ****; P > 0.05 is considered not significant ('ns').

4.4 Results

4.4.1 PEG-PC mechanical properties and *in vitro* microenvironments

We used indentation to quantify the effective modulus of samples that were prepared on glass cover slips identically to those used in biological experiments. As expected for a linear elastic material loaded with a flat punch geometry, the relationship between force and displacement is linear for all compositions tested (Fig. 4.1a). Interestingly, the PEG-PC mechanical properties measured in this way were rather different than those described in Chapter 2 [9]. It is unexpected that different testing methods would produce such large differences, so we suspect the softer gels may be due to the APS and TEMED curing, differences in curing times (which were optimized for adhesion to cover slips), or surface effects from curing between cover slips. There was no significant dependence on displacement rate as observed from the load-displacement response at different crosshead velocities ($v = 0.1 \mu\text{m/s}$ and $0.5 \mu\text{m/s}$, not shown). For ease of comparison to the more commonly used Young's modulus, we estimated a Poisson ratio of 0.5 to translate these effective moduli to Young's moduli.

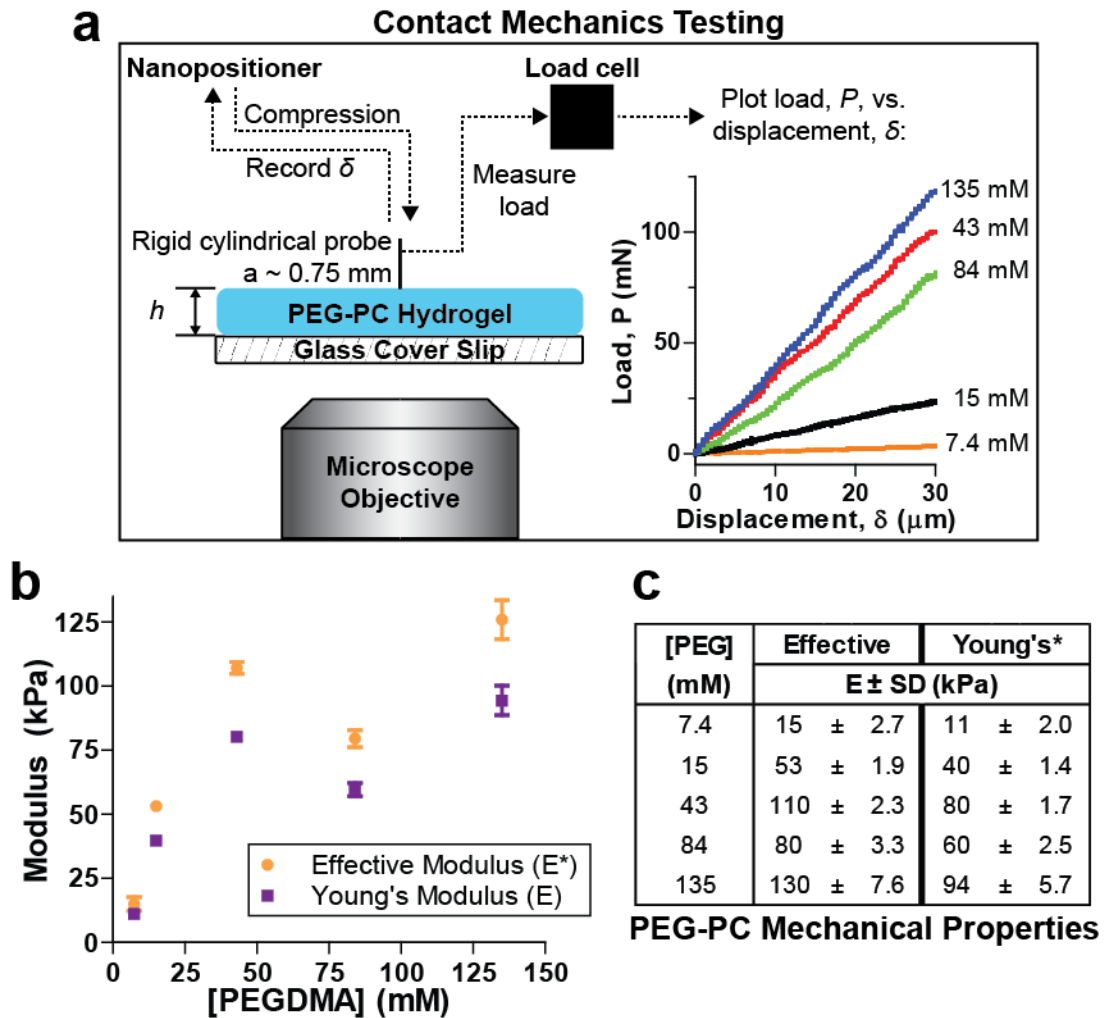


Figure 4.1: *in situ* PEG-PC contact mechanics testing

(a) PEG-PC hydrogel elastic modulus on glass coverslips was measured with contact mechanics by bringing a cylindrical probe into contact with the gel surface and measuring the force, P , versus displacement, δ . Data is plotted with compressive force and displacement values being positive. (b) The effective modulus, E^* , was measured over a range of [PEGDMA] concentrations from a minimum of three samples per condition and used to calculate a Young's modulus, E , using an estimated Poisson's ratio of 0.5. Calculated and estimated moduli are plotted versus [PEGDMA]. (c) Tabulated PEG-PC data.

To create a biomaterial environment that captured key biochemical and physical environments, we coupled the tunable mechanics of the PEG-PC gel system with ECM proteins representative of the proteins to which SMCs are attached in a healthy medial layer

(basement membrane, or BaM) or those representative of an inflammatory plaque (inflammatory or InF) (Fig 4.2a). These insoluble cues were combined with soluble factors also commonly found in these two scenarios. Unless otherwise noted, in all experiments we also ‘synced’ cell populations towards the contractile phenotype with a series of media changes prior to stimulation with growth or differentiation mediums (Fig. 4.2b).

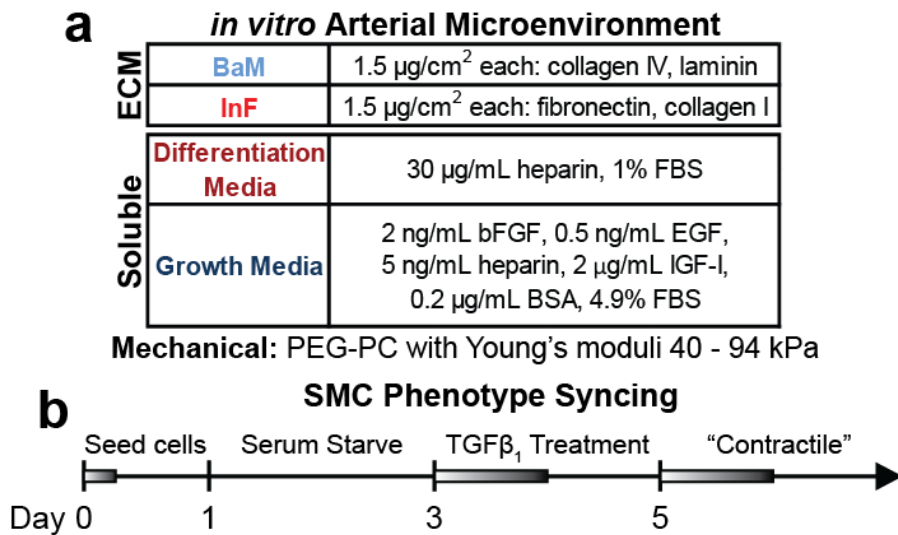


Figure 4.2: Description of *in vitro* model and general experimental design

(a) Two ECM protein mixtures representative of the major integrin-binding adhesive proteins found in an intact arterial media (BaM) and an atherosclerotic plaque (InF) were used in combination with two mixtures of soluble factors (differentiation and growth medias) and PEG-PC substrates with Young’s moduli 40-94 kPa to model the changes that typically occur during the progression of atherosclerosis. (b) Time line of phenotype syncing protocol performed for all experiments (unless otherwise noted).

4.4.2 alamarBlue accurately measures differences in HASMC number

We confirmed that the alamarBlue assay is suitable for use in this cell type by seeding serial dilutions of cells on plastic and collagen III-coated PEG-PC and performing the alamarBlue assay after 24 hours of adhesion. We report that, with a 2 hour incubation,

this assay produces an excellent standard curve with respect to the number of seeded cells, on both PEG-PC and tissue culture plastic (Fig. 4.3), linear regression $R^2 = 0.9970$ and 0.9869 , respectively). In the same experiment, we also compared against the popular, and much more expensive, CellTiter96 AQueous One (Promega) assay, but got subpar results ($R^2 = 0.8788$ and 0.7711 for plastic and gels, respectively. Data not shown). We conclude that the very low cost and excellent linearity of the alamarBlue assay in the range of cell numbers used with these proliferation experiments make it a suitable choice for this cell type.

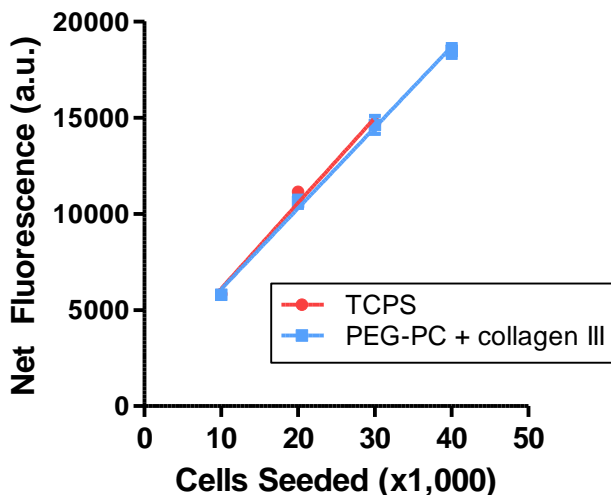


Figure 4.3: alamarBlue produces an excellent cell number standard curve

We demonstrated the linearity of the alamarBlue assay on TCPS and PEG-PC by seeding serial dilutions of HASMCs and performing the alamarBlue assay 24 hours later. Representative example shown. Note that it is not possible to compute an actual cell number without creating a standard curve for every proliferation assay and assuming that all seeded cells adhere and do not proliferate.

4.4.3 Proliferation is primarily regulated by ECM composition

We quantified proliferation of contractile SMCs on gels from 40 to 94 kPa, after 4 days of culture in growth and differentiation media (Fig .4.4). Substrate modulus had a

positive effect on cell proliferation in differentiation medium, and with the BaM configuration in growth medium. This effect was most pronounced with the combination of the BaM ECM and differentiation medium (Fig. 4.4b). Substrate modulus had a less pronounced effect on proliferation on the InF configuration with growth medium, and played no significant role with differentiation medium. We infer that the ECM-specific responses are mediated by differences in integrin activation, in agreement with a previous study comparing fibronectin, collagen, and laminin-coated gels [31]. Heparin is well known to inhibit the effects of certain GFs on SMC proliferation [32,33], further supporting this hypothesis. However, the several soluble factors in the growth medium are apparently capable of overriding any growth inhibitory effects mediated by the BaM configuration, resulting in no differences in proliferation with this condition. Furthermore, modulus has a large effect on cell population from 40 to 60 kPa (with the exception of InF ECM in differentiation medium), but the effect is not further enhanced with increasing modulus.

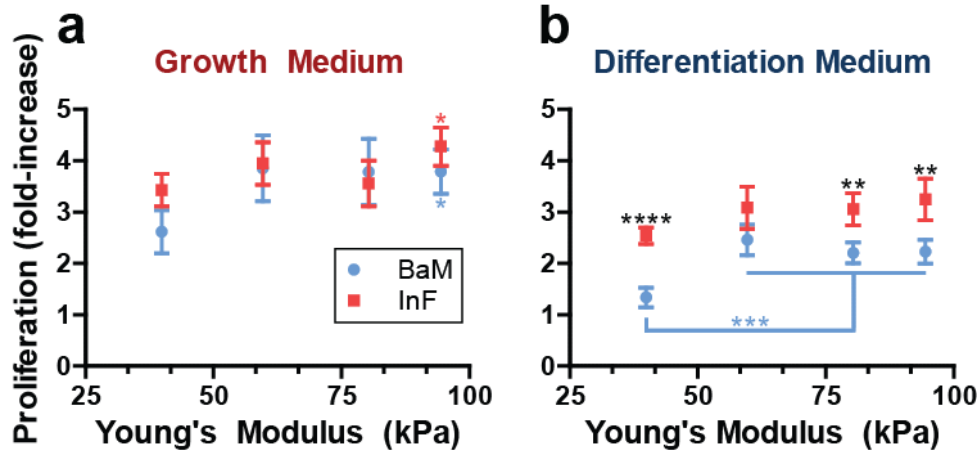


Figure 4.4: SMC proliferation is modulated by stiffness, ECM, and soluble factors

SMC proliferation was measured as a function of hydrogel modulus (x-axis) on both ECM configurations (BaM, ●, and InF, ■). The results are reported here as the fold-change in cell populations between day 1 (24 hours post-seeding) and day 9 (post-syncing and 96 hour stimulation with growth (a) or differentiation (b) medias). In (a), colored *'s denote the difference in proliferation is significantly different than the softest condition. In (b), black *'s indicate a significant difference in proliferation between ECM compositions at that stiffness, and blue *'s indicate significant difference from stiffer BaM conditions. Error bars are \pm SEM with N = 5-6 independent biological replicates.

4.4.4 ECM composition is the most significant driver of 2D and 3D motility

As models of the early stages of SMC invasion into a developing plaque, we evaluated both the 2D random migration and 3D invasiveness of SMCs as modulated by ECM composition, soluble factors, and stiffness (Fig. 4.5). We found that ECM composition and soluble factors have a very significant impact on average cell speeds and migration directionality (Fig. 4.5a-b). SMC speed has been reported as biphasic with respect to substrate modulus [5], which we did not observe in our initial experiments; however, after introducing an even softer condition (11 kPa), we found that cells on BaM in differentiation medium migrated much slower than the other stiffnesses, demonstrating that the biphasic regime for BaM and differentiation medium was not spanned in our original experiments. Unexpectedly, the average speed on BaM is significantly higher than

on InF with either medium supplement, but the chemotactic index on InF is higher. This latter result indicates that cells on BaM move erratically and non-directionally. Growth medium generally increased both random migration speed and the chemotactic index on both ECM configurations.

Given these surprising results on 2D surfaces, we hypothesized that integrin binding was significantly influencing cell adhesion, and could therefore impact 3D invasion as well. With a collagen gel invasion assay, we found that invasion depth is greater with SMCs from the BaM ECM in growth medium, with a modest increase in invasion depth on the soft versus stiff substrates (Fig. 4.5c-d). Invasion depth in differentiation medium was overall shallow, but greater for the soft BaM condition. Given the observed weaker adhesion/spreading on BaM, it is possible that invasion depth is greater due to ease of detachment; however, since the SMCs are given 4 days to invade the collagen matrix, BaM may also induce phenotypic changes that affect the SMCs long after leaving the BaM surface.

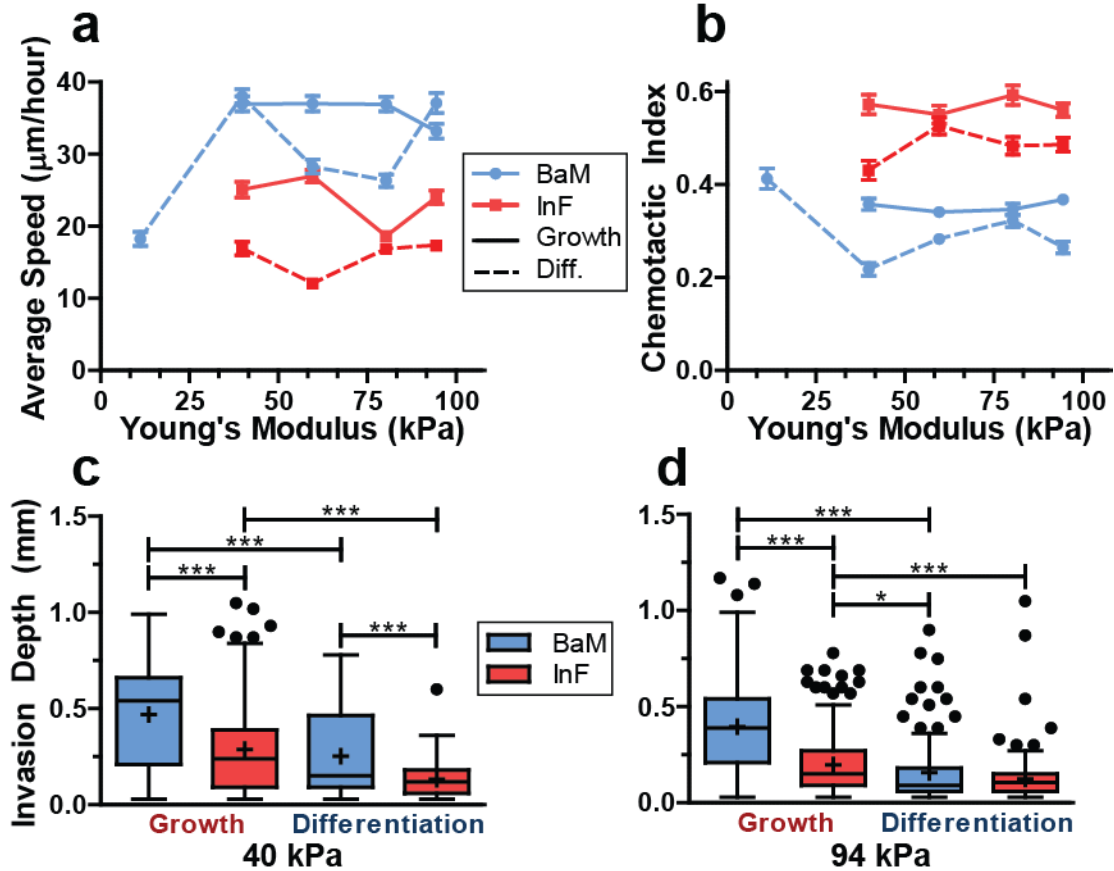


Figure 4.5: SMC motility is modulated by ECM and soluble factors

SMC average migration speed (a) and chemotactic index (b) during stimulation with growth and differentiation mediums (solid and dashed lines, respectively) was quantified as a function of Young's modulus (x-axis) on both ECM configurations (BaM, ●, and InF, ■). Error bars are \pm SEM with $N > 100$ cells and at least 3 independent biological replicates. Statistical significance is not labeled because all discernible differences have p-values < 0.05 or better. In a collagen gel invasion assay, SMCs were seeded on the softest (c) and stiffest (d) conditions with BaM and InF ECM configurations (blue and red, respectively) and with both growth and differentiation mediums as chemoattractants (x-axis). Tukey box plot with $N > 100$ cells per condition and 3-4 independent biological replicates, except for 2 replicates on soft BaM in differentiation medium.

4.4.5 SMC size and morphology is primarily dictated by ECM composition

Since SMC phenotype is reported to be correlated with shape and size [34], we quantified cell morphology on our various surfaces (Fig. 4.6). We found that cells are approximately 2-3-fold larger in area on InF compared to BaM (Fig. 4.6a), consistent with

a synthetic phenotype on these inflammatory proteins. Average cell aspect ratio (AR) was used to evaluate the ‘spindle’-like shape of SMCs, with high ARs indicating an elongated, spindle-like morphology, indicative of the contractile phenotype. We found that the average cell AR has little dependence on modulus or ECM composition after syncing (Fig. 4.6b), which is further validation that this priming step does chemically “sync” the otherwise heterogeneous cells into a consistent, differentiated cell population, even though they are seeded onto different stiffness gels with different integrin-binding conditions. Remarkably, we observed that the average AR of cells on BaM in growth medium increases with substrate modulus, and it is higher overall than on InF with either growth or differentiation media stimulation (Fig. 4.6b). Thus, while aspect ratios are synced during TGF β ₁ treatment, the contractile phenotype appears to be better maintained, at least with respect to cell morphology, on stiff gels with basement membrane proteins, regardless of the ensuing medium conditions.

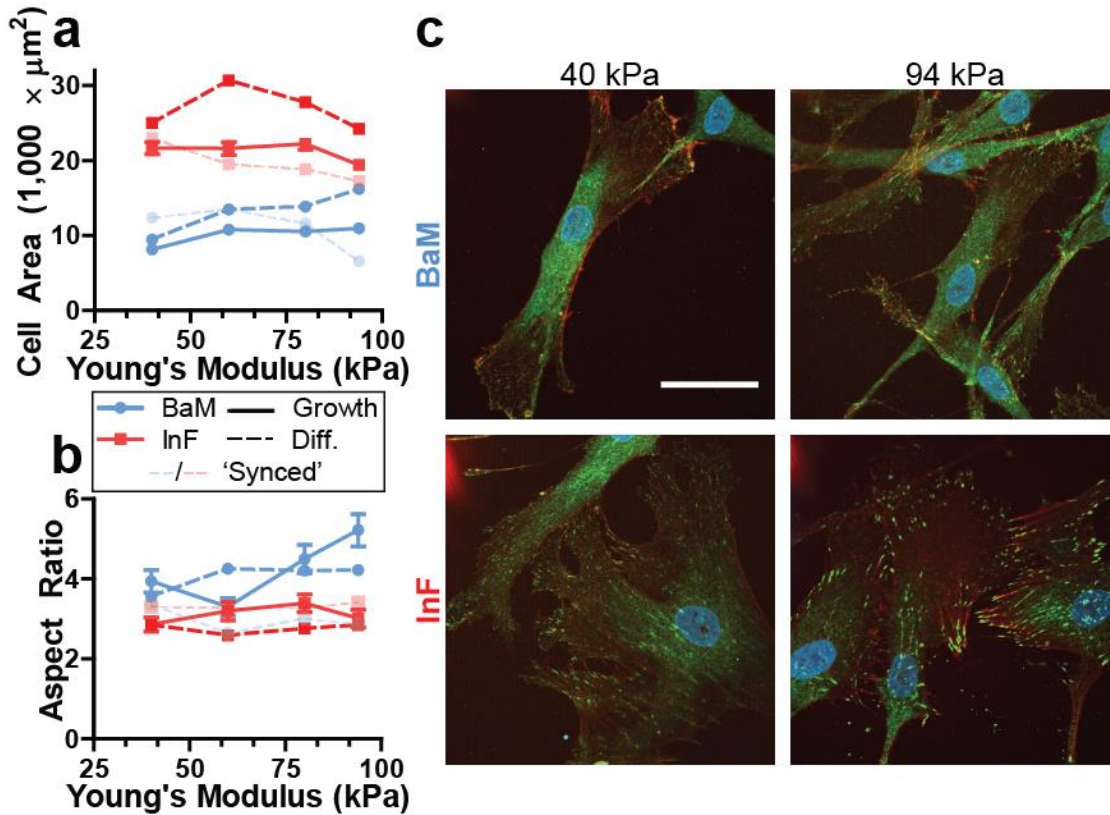


Figure 4.6: SMC adhesion and morphology is dictated by ECM composition

(a,b) Cell spreading area (a) and aspect ratio (b) were quantified with manual tracing and ImageJ's 'Measurement' tool as a function of hydrogel modulus (x-axis) and ECM composition (BaM, ●, and InF, ■) after phenotype syncing (light colored dashed lines) and stimulation with growth or differentiation mediums for 48 hours (solid and dashed lines, respectively). Error bars are \pm SEM with $N > 100$ cells and 3 independent biological replicates. Statistical significance is not labeled because all discernible differences have p-values < 0.05 or better. (c) Immunofluorescent staining of vinculin (green), actin stress fibers (orange), and nuclei (DAPI) of SMCs on the softest and stiffest substrates with both ECM compositions in growth medium after 48 hours of culture. Scale bar is 50 μ m.

4.4.6 Soluble factors and modulus significantly affect marker expression

To connect SMC motility and proliferation to prototypical markers of SMC differentiation, we evaluated the expression of calponin and smoothelin via Western blotting (Fig. 4.7a). Synthetic SMCs secrete ECM and are classically characterized by the downregulation of several contractile phenotype-associated marker proteins (caldesmon,

calponin, SM-MHC, smoothelin and more [35–37]). These proteins interact with the actin cytoskeleton and have roles in regulating actomyosin contractility, and are therefore involved in mechanotransduction. In agreement with previous reports [38], we found that treatment with TGF β ₁ during the syncing steps increased expression of both calponin and smoothelin after serum starvation. The differentiation medium supplement (SMDS) is intended to promote SMC differentiation, while the growth medium induces proliferation and dedifferentiation. In agreement with these expectations, we found that, relative to TGF β ₁ treatment, calponin expression is modestly up- and down-regulated by differentiation and growth medium, respectively (Fig. 4.7a, top). Smoothelin expression, however, increases with differentiation and *further* increases with growth medium (Fig. 4.7a, bottom). Calponin expression increases on stiff substrates and InF with differentiation medium, but is not affected by stiffness in the other contexts, whereas smoothelin expression is enhanced on stiff substrates with TGF β ₁ and differentiation medium, or with growth medium on InF. Any effect of stiffness was most pronounced on InF, and expression of both markers was greater on InF than BaM on soft substrates, but otherwise there was little-to-no difference in expression between the integrin-binding conditions. These results, in combination with our proliferation and migration results (Figs. 4.4, 4.5), *directly contradict* the notion that SMC marker expression and behaviors indicative of the synthetic phenotype are inversely related.

Given the reported scarcity of smoothelin in cultured SMCs [35], we visualized actin fibers and calponin or smoothelin with immunofluorescence (Fig. 4.7b). These results confirmed antibody specificity for stress-fiber localized proteins, and also that smoothelin co-localizes to the nucleus [39]. We further confirmed expression of the smoothelin-B

mRNA with reverse transcriptase PCR in SMCs grown on TCPS in FBS-containing growth medium (Fig. 4.7c).

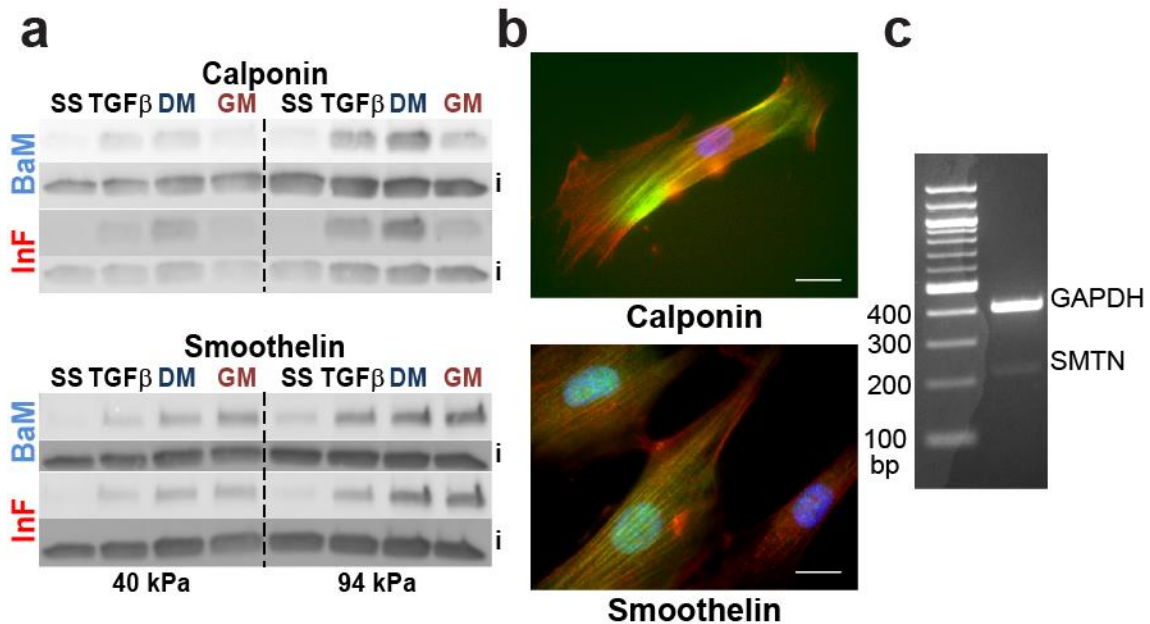


Figure 4.7: SMC marker expression is more dependent on soluble factors than ECM

(a) Representative Western blots for calponin (top) and smoothelin (bottom) after each syncing step and stimulation with growth or differentiation mediums. SS: Serum-starve, TGF β : TGF β treatment step, DM: switch to differentiation medium, GM: switch to growth medium. Internal control bands (labeled ‘i’) for normalization are cyclophilin B. (b) Immunofluorescent staining of calponin (top) and smoothelin (bottom) in green with actin (red) and DAPI (blue). Scale bar is 20 μ m. (c) Reverse transcriptase PCR to confirm expression of SMTN-B mRNA.

4.4.7 FAK controls SMC plasticity, in part through PI3K/Akt

To understand the possible signaling mechanisms driving the phenotypic diversity of the SMCs we had observed thus far, we quantified the activation of several major signaling proteins during adhesion, TGF β ₁ treatment, and stimulation with differentiation medium, with a phospho-protein multiplex assay (Fig. 4.8). We observed characteristic spikes in phosphorylation of ERK and Akt on each modulus tested, and phosphorylation

was generally higher on the InF configuration. We detected little to no phosphorylation of Akt during TGF α_1 stimulation (not shown), and again ERK peaked at early time points and was enhanced by the InF configuration. Interestingly, ERK showed higher phosphorylation on the stiffer hydrogel during TGF β_1 stimulation (Fig. 4.8b). Finally, we saw opposing trends in Akt and ERK phosphorylation during stimulation with differentiation medium (Fig. 4.8c). Akt activity was initially low, suggesting that differentiation medium does not directly activate Akt, but by 24 hours the phosphorylation level was significantly greater, with a positive influence from stiffness and the InF ECM. However, ERK had the characteristic early spike in activity which subsides to a baseline after approximately 1 hour, and activity was once again greater on the InF ECM without being affected by substrate stiffness.

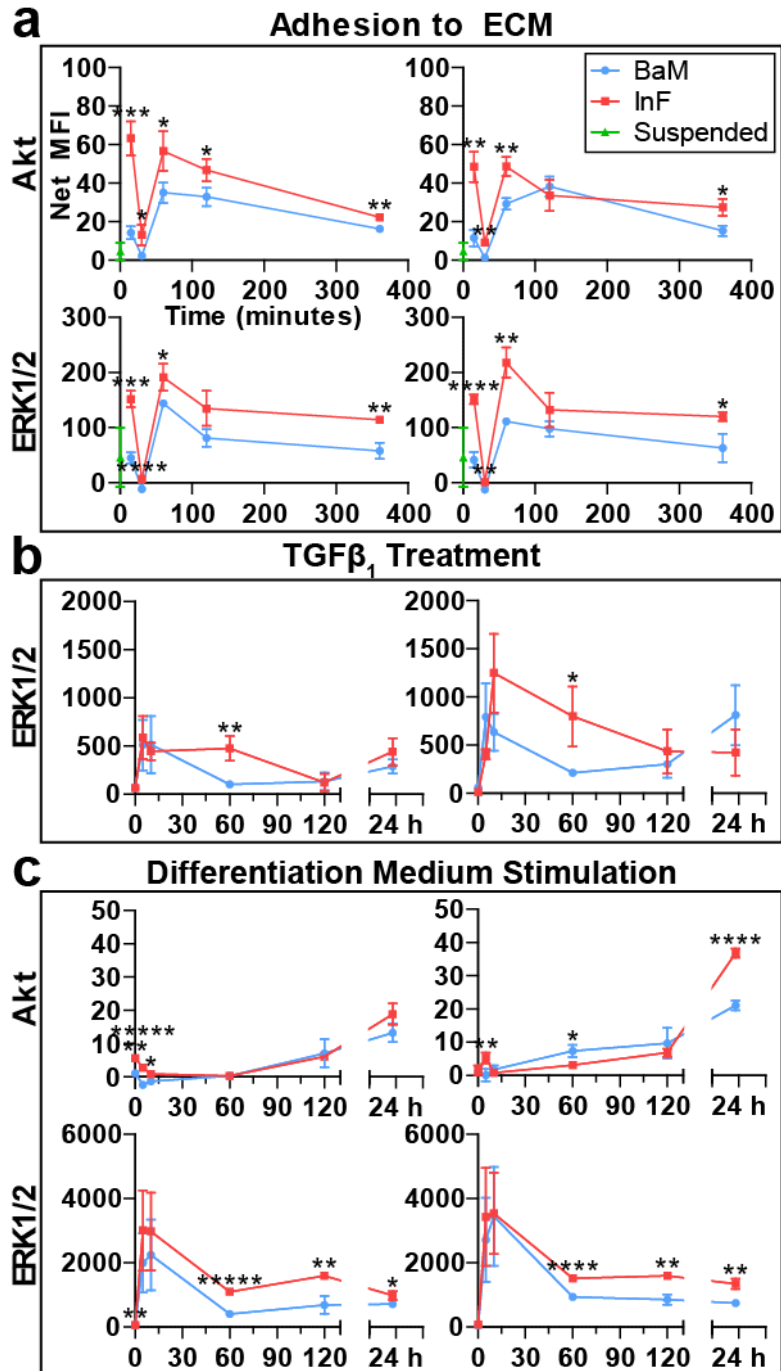


Figure 4.8: Cell signaling time courses

(a-c) Phosphorylation of Akt and ERK, when occurred, are plotted as a function of time (x-axis) directly upon adhesion to substrates (a), during TGF β_1 stimulation (b), and stimulation with differentiation medium (c). Error bars are \pm SEM from N = 2-4 independent biological replicates.

We then performed experiments with inhibitors to signaling proteins that are associated both with Akt and integrin activity to draw a connection between our observed SMC phenotypes and ECM composition (Fig. 4.9). We inhibited FAK, PI3K and Akt with small molecule pharmacological inhibitors, and found that FAK was the strongest upstream driver of SMC differentiation on all conditions. In fact, inhibition of FAK completely abrogated expression of both calponin and smoothelin (Fig. 4.9a). However, while inhibition of PI3K or Akt reduced expression of calponin, it had little to no effect on smoothelin expression. Interestingly, though we did not find any substantial differences in expression between the two ECM configurations, it is of note that cells on InF remained well-spread and appeared relatively normal over 48 hours, but cells on the BaM composition underwent rounding and detachment, prohibiting us from obtaining data for the 40 kPa BaM condition with Akt or FAK inhibitors. These results imply that regulation of smoothelin and calponin expression diverges downstream of activated FAK, though it appears that PI3K and Akt may still have role. We also performed proliferation experiments with ERK and Akt inhibitors in differentiation medium, but Akt inhibition for 96 hours caused total cell loss through detachment and/or apoptosis on both ECM configurations, and the effects of ERK inhibition were inconsistent and differences were statistically significant (Fig. 4.9b). Despite this, we still suspect a role for ERK in mediating SMC proliferation in our model, but we speculate that the inhibitor was ineffective because the working concentration was not verified and optimized. In addition, there are few or no reports of FR180204 being used with SMCs, suggesting we may have had different results with the more commonly-used inhibitor PD98059.

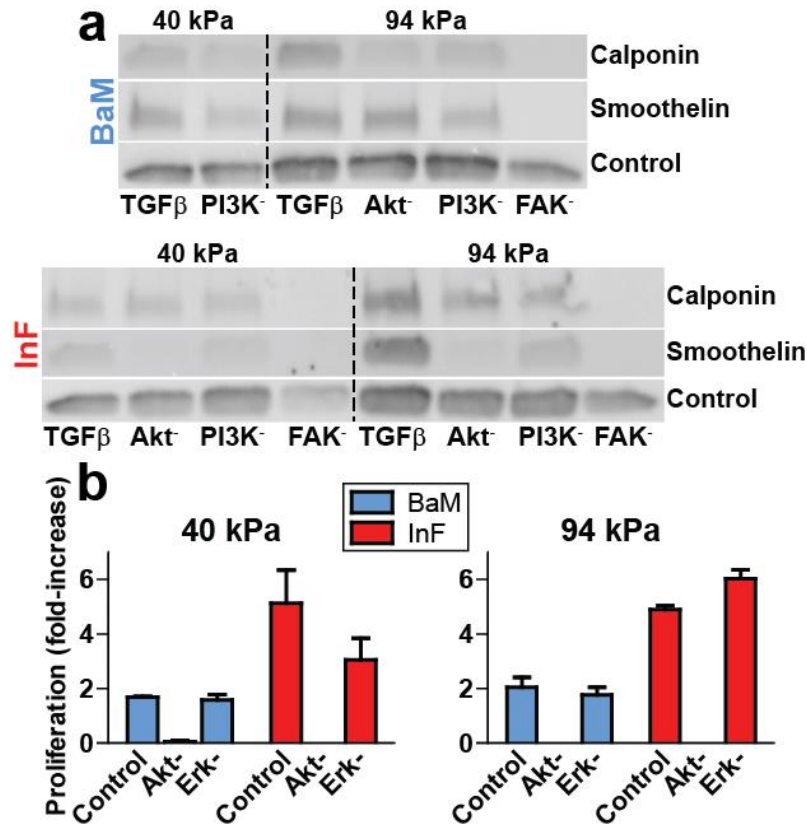


Figure 4.9: Effects of kinase inhibition on SMC marker expression and proliferation

(a) During the final phenotype syncing step (TGFβ treatment for 48 hours), inhibitors to PI3K (50 μM LY294002), Akt (1 μg/mL sc-66), and FAK (100 μM inhibitor 14) were co-administered for the first hour (with the exception of FAK inhibitor 14, which was present for the duration), and after 48 hours lysates were collected and subsequently Western blotted for calponin and smoothelin. Internal control bands are cyclophilin B. Note that Akt and FAK inhibition for 48 hours resulted in complete loss of cells on the soft BaM condition and thus we were unable to examine expression under those conditions. (b) Proliferation experiments with differentiation medium were performed as described previously, with the addition of inhibitors to Akt (1 μg/mL sc-66) and ERK (20 μM FR180204). Error bars are ± SEM with N = 3 independent biological replicates.

4.5 Discussion

SMCs are observed to rapidly convert from the contractile to the synthetic phenotype when removed from the *in vivo* microenvironment and cultured on plastic. This phenotype plasticity has been heavily studied due to similarities between the synthetic phenotype observed *in vitro* and that of the SMCs found in atherosclerotic plaques. The

presence of synthetic SMCs in atherosclerosis is a maladaptive consequence of the phenotype plasticity necessary for SMCs during development and wound healing, which must be capable of phenotype switching for maintaining vascular wall integrity (contractile) and for vascular wall remodeling during development, growth, and normal wound repair (synthetic). Inspired by the role of SMCs in vascular mechanics, many groups have investigated the effects of various types of mechanical stimuli on SMC phenotype, including oscillatory flow [40], stretch [41–43], interstitial flow [44,45], and laminar versus nonlaminar flow [46]. However, most previous *in vitro* studies of SMC phenotype use relatively simple models, and we believe many new insights may be gained by trying to more closely match the *in vivo* microenvironment with more complex *in vitro* models.

With this approach in mind, we have designed a 2D biomaterial system that reflects several major, physicochemical changes in the *in vivo* extracellular microenvironment that are purported to occur during the progression of atherosclerosis and restenosis. Our observations demonstrate that interplay between the various components of a complex microenvironment can modulate phenotype and behavior in complicated, unexpected ways. In fact, many assumptions from the SMC literature on/in other *in vitro* environments do not hold here, which we suspect is due the focus on single physicochemical cues, instead of the combinatorial approach we have taken here.

One of the major failed assumptions revealed by our data is that expression of SMC marker proteins is not inversely related to proliferation as commonly reported [15,17,20,47]. In fact, expression of calponin, and smoothelin to a lesser extent, was overall greater with inflammatory than basement membrane proteins on soft substrates. In contrast, proliferation was significantly greater on InF than BaM in differentiation medium, and

unchanged in growth medium (Figs. 4.4, 4.7). This data suggests that the signaling pathways regulating SMC proliferation and marker expression are not strictly antagonistic, or possibly even coupled at all. However, the proliferation trends in differentiation medium indicate that the effects of soluble factors on proliferation in these contexts are modulated by integrin activity [48,49].

In contrast, soluble factors had unexpected effects on marker expression that were only modestly modulated by ECM composition – applying TGF β ₁ to serum deprived cells significantly increased expression of both markers, as expected [38,50], and calponin was upregulated and downregulated by differentiation and growth medias, respectively, as expected. However, smoothelin expression was slightly increased with either supplement, suggesting differences in regulation. Stiffness consistently positively affected expression of both proteins, which is counterintuitive but in agreement with prior reports [6,7,51].

In agreement with expected effects of these ECM proteins on SMC phenotype, we found that both supplements induced a contractile, spindle-shaped morphology on BaM and a large, rhomboidal shape associated with the synthetic phenotype [52,53] on InF (Fig. 4.6). Remarkably, these traits did not correspond to differences in expression of calponin or smoothelin, despite their purported status as components of the SMC contractile apparatus. Though a spindle shape is typically associated with contractile phenotype and marker expression, they are not strictly coupled [54], which our data corroborates. Conversely, we have frequently observed the so-called “hill-and-valley” pattern of cell adhesion with TGF β ₁ treatment on the BaM composition (Fig. 4.10c-d) which is usually associated with SMCs cultured *in vitro*, and hence the synthetic phenotype. Other groups have reported similar results with TGF β ₁ treatment on plastic [55] and with prolonged

serum-deprivation [56]. Lending further support to veracity of our results is the similarity of SMC morphologies on BaM treated with growth or differentiation medium to those observed on Matrigel [57], which is comprised primarily of collagen IV and laminin.

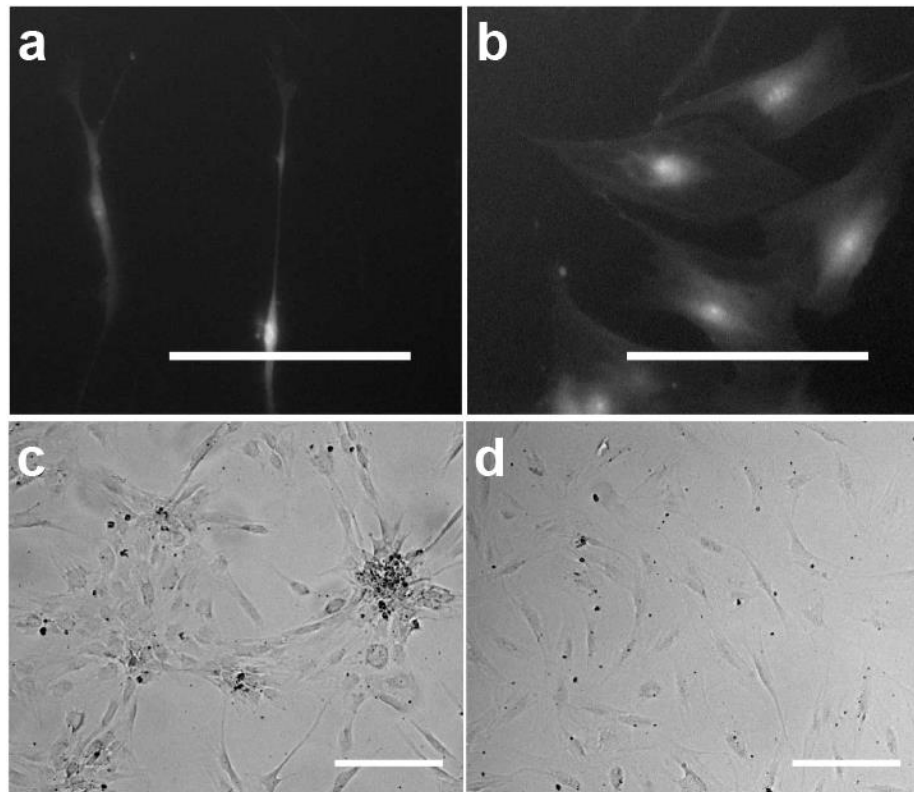


Figure 4.10: SMC morphology is dictated by ECM composition and soluble factors

Despite little or no difference in contractile marker expression between the BaM and InF ECMs, we observed major differences in morphologies after stimulation with growth or differentiation medium, including a spindle-like shape (high AR, Fig. 4.6b) on BaM (a, 94 kPa BaM after growth medium stimulation), and rhomboidal morphologies on InF (b, 80 kPa InF after differentiation medium stimulation). Conversely, while differences in AR were negligible immediately following phenotype syncing, we consistently observed the synthetic phenotype-associated “hill-and-valley” morphology on the BaM ECM (c), but not on InF (d, both on 94 kPa substrates). Scale bars are 200 μm .

Of equal interest is the observation that migration and invasion characteristics are disconnected from SMC marker expression. However, upon further reflection, the BaM

and growth medium condition models the process of detachment from the basement membrane and migration towards chemoattractants in a developing plaque (Fig. 4.5c-d). Thus, we speculate that SMCs in this model may be reflective of an invasive phenotype characteristic of the early stages of atherosclerosis. Finally, our initial experiments showed that the effect of stiffness on 2D migration speed did not follow the previously reported biphasic trend *in vitro* [5]. We initially explained this by our combinatorial protein approach, as this previous study was done on fibronectin alone, and collagen IV has been previously reported to strongly enhance SMC motility relative to the other ECM proteins used here [58]. We investigated this further by performing additional migration experiments with BaM on 11 kPa PEG-PC in differentiation medium, and possibly identified the range of elastic moduli over which SMCs migrate biphasically in this microenvironment.

These and our proliferation results are counter to the oft-repeated notions that SMC behaviors associated with atherosclerosis (proliferation, migration, a rhomboidal morphology) are inversely related to SMC marker expression, but from our work and other reports we now know that the relationship between marker expression and phenotype is highly complex. Numerous factors are likely to affect these characteristics, including species of origin, cell density, cell age, and anatomical origin. For instance, coincident proliferation and contractile marker expression has been reported in neonatal rat SMCs [59], in developmental contexts [60–62], in rat SMCs of different ages and from intimal hyperplasia [54], and even in human SMCs exposed to TGF β ₁ *in vitro* [17]. Conversely, while synthetic SMCs in advanced atherosclerotic or restenotic lesions express very low levels of marker proteins [63], and SMCs in lesions generated by the commonly used rat

balloon-injury model are proliferative [64], this does not necessarily imply they are proliferative in lesions in humans [65].

Synthetic SMCs are also typically found to be more migratory [34] and invasive, but we find large differences in migration speed and invasion depth between the ECM compositions, despite there being little difference in differentiation marker expression. We conclude from this series of experiments that the relationship between differentiation markers and phenotypic characteristics is highly dependent on the physicochemical context, and it would be unwise to consider the previously reported correlations between markers and behaviors as hard and fast rules.

Measurement of several signaling protein activity levels over time implicates Akt and ERK1/2 signaling pathways in regulating these phenomena, the activities of which are overall greater on InF than BaM (Fig. 4.8). Increased stiffness also enhanced signaling under certain conditions, but relatively modestly. We were surprised to not detect any p38 or JNK activity, which have reported roles in SMC migration [66,67], proliferation [67], and the response to TGF β ₁ [50,68]. However, these studies are themselves contradictory [66,67], and so we propose that differences in cell types and contexts explain the lack of p38 and JNK activity in our model.

This analysis was followed up with kinase inhibition experiments to identify potential connections between cell signaling pathways and integrins/focal adhesion complexes. The effects of Akt and FAK inhibitors were similar on soft BaM during TGF β ₁ treatment, with total loss of cells by 48 hours, but PI3K inhibition only caused significant rounding. Cell rounding was also dramatic with all inhibitors on stiffer BaM, but cells were not completely detached. Remarkably, inhibitors had little visible effect with InF during

TGF β ₁ treatment, with the interesting exception that FAK inhibition dramatically reduced cell-cell contacts (not shown).

We also measured the effect of kinase inhibition during TGF β ₁ treatment on marker expression and, despite apparently significant effects on the actin cytoskeleton and/or adhesion structures, were surprised to find little relation between markers and detachment/rounding. The most intriguing finding is that FAK inhibition completely abrogates expression of both markers, implicating FAK as a key regulator of contractile marker expression in 2D. Furthermore, expression of calponin and smoothelin appear to be regulated by distinct, but possibly overlapping signaling pathways that diverge downstream of FAK. This conclusion is derived from the finding that smoothelin is modestly affected by PI3K inhibition, but barely affected by Akt inhibition, whereas calponin is strongly downregulated by inhibition of either on BaM. On InF, however, calponin is more strongly regulated via Akt than PI3K, which suggests that Akt may be activated through integrin-linked kinase (ILK) independently of, or in addition to PI3K [69].

The likely explanation of how integrin binding in our system regulated stiffness sensitivity and sensitivity to the medium conditions is the crosstalk between integrin binding and colocalized growth factor receptors (GFRs). Numerous studies (for review, see [70]) have reported a concomitant increase in migration and proliferation after treatment with soluble factors or mechanical stimulation, and our data agrees with this notion. However, it has long been hypothesized that migration and proliferation of synthetic SMCs in atherosclerosis are temporally distinct phenomena [71,72]. We believe this seeming contradiction between *in vitro* data and physiological intuition is an

illustrative example of the limitations necessarily imposed by simpler models. Our data indicates that, as the ECM surrounding SMCs changes from a basement membrane composition to that found in a plaque, there is a switch from migratory behavior to a high rate of proliferation. This data altogether is *in vitro* evidence that proliferation and migration may be inversely related *with respect to particular ECM compositions*, a conclusion that fits well with our understanding of the pathology of atherosclerosis.

From our data and previous work, we propose hypothetical signaling mechanisms in Figures 4.11 and 4.12 that may explain the impact of ECM composition on SMC phenotype and behaviors, and how these processes are modulated by soluble factors. As described above, our inhibitor experiments indicate that FAK regulates the expression of SMC markers, and suggest that calponin and smoothelin regulation may diverge downstream of FAK. These experiments also indicate that calponin expression may be mediated primarily via PI3K/Akt on BaM and ILK/Akt on InF, whereas smoothelin may be regulated via disparate, or possibly partially overlapping pathways. We additionally propose that activation of different integrin heterodimers – most likely $\alpha_v\beta_3$ on InF and $\alpha_2\beta_1$ on BaM – well explains the physiologically relevant phenomena we observed. The much greater spreading area and slower migration of SMCs on InF, as well as a higher prevalence of stress fibers and vinculin-rich focal adhesions, is an indicator of significant FAK autophosphorylation [73], which in turn recruits and activates c-Src and enhances overall FAK signaling [74]. In fact, β_3 integrin itself provides Src homology 2 (SH2) binding domains for direct association of c-Src independent of actin and focal adhesion assembly [75]. The higher prevalence of these signaling moieties on InF may lead to greater proliferation by accumulated signaling through the c-Src/ERK1/2 and/or FAK/ILK/Akt

pathways, and greater spreading through FAK-mediated enhancement of RhoA/ROCK and stress fiber formation. In contrast, while $\alpha_2\beta_1$ integrin activation on BaM may generate many of the same signaling motifs [48], the weaker adhesion we observed suggests less FAK/c-Src signaling, resulting in dampened proliferation.

We also propose a mechanism to explain our observed ECM-controlled switch from a migratory to a proliferative phenotype. Prior work with fibroblasts revealed that these cells are motile and proliferative at low and high concentrations of PDGF, respectively, a change that appears to be mediated by switching between clathrin-mediated endocytosis (CME) and raft/caveolin-mediated endocytosis (RME) of the PDGF receptor [76]. We hypothesize that changes in integrin activation could facilitate a similar mechanism in SMCs. In fact, SMC binding to fibrillar collagen via α_2 integrin has been shown to inhibit cholesterol biosynthesis [77], which impairs RME and would likely inhibit proliferation. Heparin has also been shown to induce its anti-proliferative effects, in part, via inhibition of RME and ligand-less EGFR activation [78], which may be partially responsible for the suppressed proliferation of SMCs on InF in differentiation medium. Other evidence for this hypothesis in the literature include a report that insulin signaling in primary adipocytes is RME-dependent [79], as is the mitogenic effect of PDGF-BB on human bladder SMCs [80].

This proposal also fits well with the finding that migration is random and non-directional on BaM, because CME is responsible for rapid, localized recycling of GFRs at the plasma membrane that enables a fast cellular response to transient microgradients of chemoattractants [81]. The adhesion properties on BaM also suggest faster turnover of FAs, which is likely to support rapid migration on soft substrates. This latter mechanism

would seem to also apply to $\alpha_2\beta_1$ integrin binding to monomeric collagen I on InF, but this is likely mitigated by the additional presence of fibronectin, which has been found to dramatically increase phosphorylation of paxillin [82], decrease migration, and increase spreading [83].

Of potentially major significance is that the association between RME and mitogenic signaling provides yet another plausible mechanism by which statins exert their atheroprotective effects. Statins are specific inhibitors of HMG-CoA reductase, a rate-controlling enzyme in the cholesterol biosynthetic pathway, and therefore reduce cholesterol concentrations in the plasma membrane and inhibit receptor signaling by inhibiting RME [84]. Another side effect of disrupting this pathway is impairment of protein prenylation, which is a requirement for plasma membrane localization of Ras superfamily members [85], and is likely one way how statins inhibit proliferation (Ras) and disrupt actin dynamics/migration (Rho) [86].

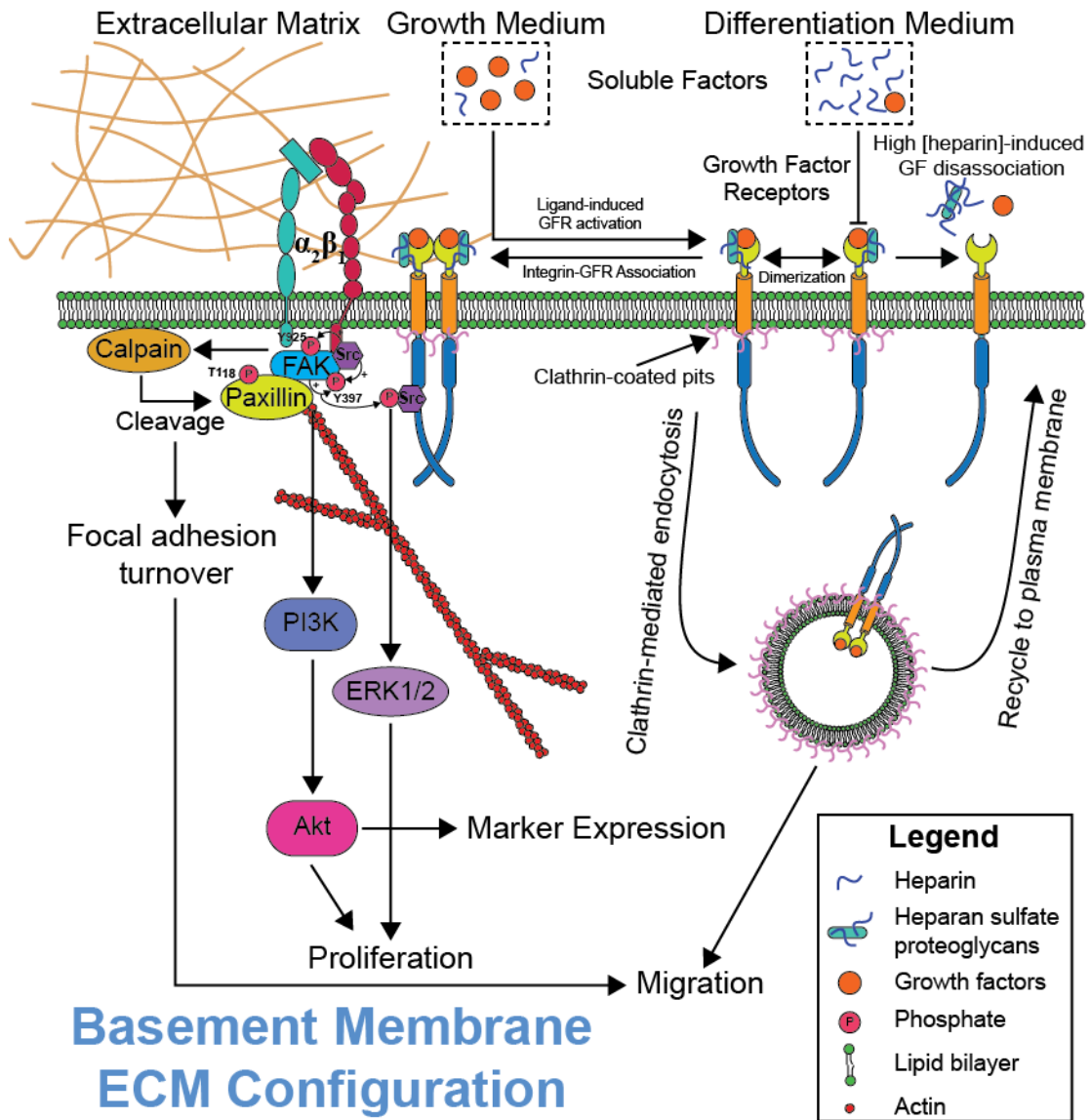


Figure 4.11: Proposed integrin-mediated signaling mechanisms on BaM ECM

On the BaM ECM, we predict that specific signaling motifs driven by integrin-binding – with $\alpha_2\beta_1$ integrin as a prime candidate – induce a migratory and invasive phenotype with low proliferative capacity. We hypothesize that a migratory phenotype is favored on BaM due to weak adhesion, high focal adhesion turnover, and CME enabling a rapid response to chemoattractant microgradients. Conversely, proliferation is poorly supported due to a paucity of robust actin stress fibers, the favoring of CME over RME, and possible inhibition of cholesterol biosynthesis and protein prenylation.

Soluble factors play a role in these phenomena by activating integrin-associated GFRs, which further enhance mitogenic signaling via c-Src and/or Akt. Despite the relative

scarcity of soluble factors in differentiation medium, proliferation is greater on InF due possibly to overall greater basal activation of mitogenic signaling induced by ligand-free association of integrins and GFRs. Other possible contributory factors include the induction of autocrine GF production by heparin [87] or collagen I [88], supported by our finding that Akt activity increased after 24 hours in differentiation medium on the stiff condition. Proliferation is lower on BaM, however, due to a lack of many of these effects and/or potential sequestration of TGF β ₁ in the pericellular matrix by collagen IV [89], which has been shown to potentiate the GF inhibition of heparin [90]. Finally, despite the lack of significance between the fold-change in cell populations, we believe that SMCs on InF in growth medium may actually proliferate more than on BaM due to the mechanisms described above, but this is obfuscated due to greater spreading and attachment of SMCs on InF, resulting in faster saturation of the surface and cell-cell contact inhibition.

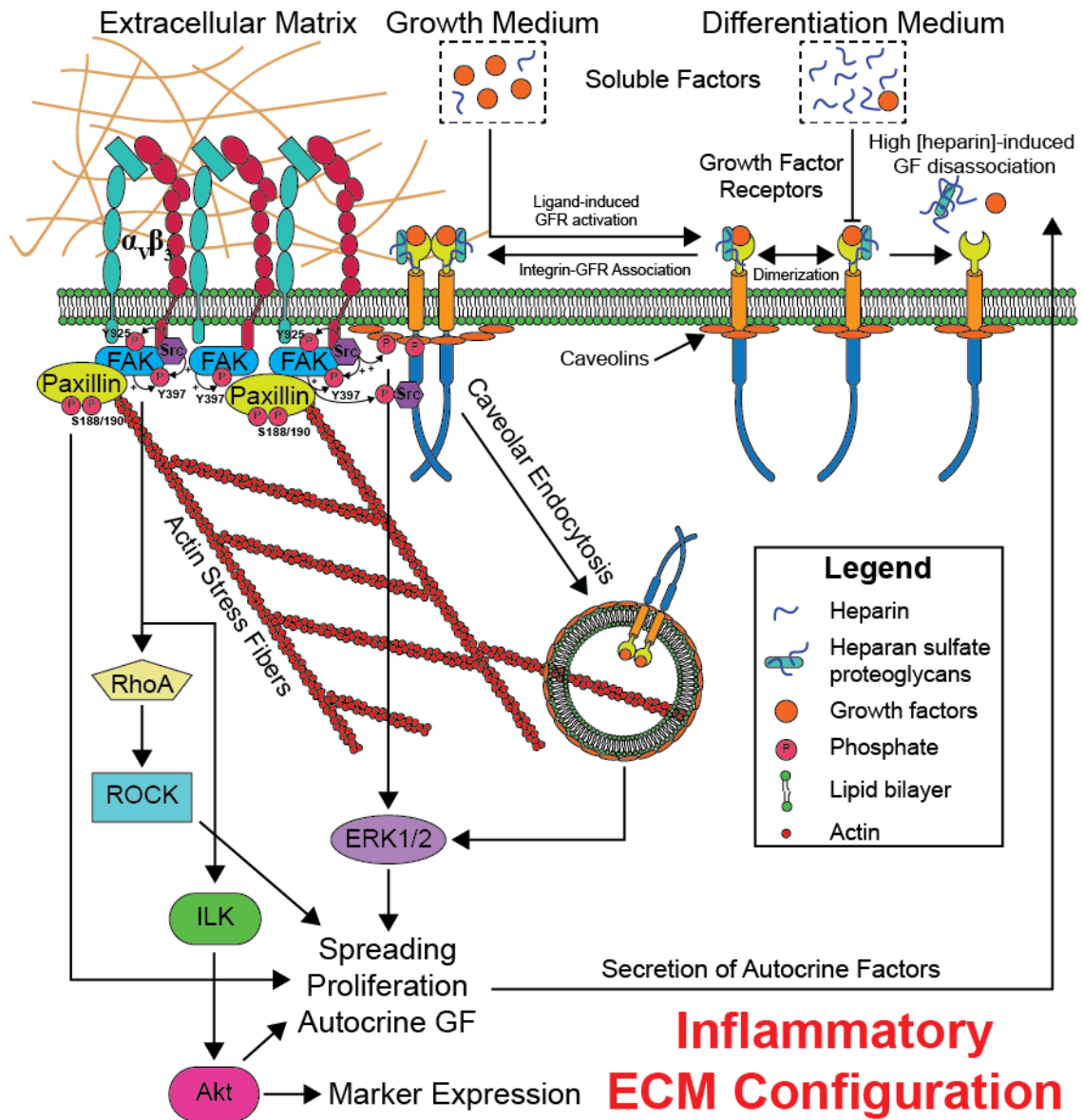


Figure 4.12: Proposed integrin-mediated signaling mechanisms on InF ECM

On the InF ECM, we predict that specific signaling motifs driven by integrin-binding – with $\alpha_5\beta_1$ integrin as a prime candidate – induce a proliferative phenotype with low migratory capacity. We hypothesize that a proliferative phenotype is favored on InF due to strong adhesion, a robust actin cytoskeleton, and a combination of integrin-GFR association and RME enabling a concentrated response to GFR activation. Conversely, migration is poorly supported due to low focal adhesion turnover.

4.6 Conclusion

We present here a simple *in vitro* biomaterial system that can parse out the complexity of smooth muscle cell phenotype and signaling while under multi-factorial exposure to differences in substrate modulus, integrin binding, and soluble factor stimulation. The novelty in our approach was initially syncing cell populations on multiple combinations of substrate stiffness and ECM composition, and then probing their susceptibility to the synthetic phenotype when dosed with different soluble factor cocktails. This simple change from previous methods allowed us to see the true phenotypic diversity of SMCs, and helped resolve some contradictions that exist between *in vitro* and *in vivo* studies. Coupled with our signaling analysis, we demonstrated that integrin binding is the most significant driver of SMC phenotype, and it dictated SMC stiffness sensing and sensitivity to soluble factors. This was modulated primarily by FAK phosphorylation, and we propose FAK's downstream targets are regulated by the integrin heterodimer bound and its ability to recruit GFRs and Src into the focal adhesion complex. In sum, our results demonstrate the need to examine any biophysical cue in proper context, and studies of mechanosensing in isolation (including our own) may result in incomplete conclusions.

4.7 Future work & considerations

In the prior discussion we touched upon many areas of potential interest to explore in the future. To elaborate on these suggestions, we propose two equally important directions: 1) Validate the proposed signaling models with the current *in vitro* platform using advanced cell biology techniques, and 2) Add additional complexity to the *in vitro*

platform to identify other potentially important mechanisms. Next, we will discuss the pros and cons of each and potential methods for moving forward in these directions.

4.7.1 Validate the proposed signaling models

Since the results in this current work are very interesting and reveal an array of SMC behaviors and phenotypes rarely observed *in vitro*, it is important to validate the proposed mechanisms behind the significant differences in proliferation, motility, and cell morphology reported here. Perhaps the most crucial component of our hypothetical signaling model is the role of CME and RME in promoting migration and proliferation, respectively. Their roles may be partially confirmed or denied through the use of inhibitors to each type of endocytosis, including siRNAs towards proteins involved in the processes [76], small molecule drugs [91], and statins to inhibit RME by depleting cholesterol in the plasma membrane [92]. Of course, the choice of inhibitor is important, as non-specific effects could confound interpretation of the results, and thus siRNAs against proteins or enzymes validated to be directly involved in vesicle formation may be the best choices. Other options to further validate the significance of these endocytic pathways include immunofluorescent staining and, in the case of RME, detergent extraction and separation of cytosolic, membrane, and lipid raft fractions and subsequent immunoblotting for proteins of interest i.e. integrins and growth factor receptors. We can then use these tools to test our model predictions that inhibiting CME will increase the chemotactic index and decrease the speed of SMCs on the BaM composition, and inhibiting RME will decrease proliferation on InF. While it would be necessary to carefully avoid confounding effects because statins can inhibit SMC proliferation independent of their effects on cholesterol

biosynthesis [93], it would be of great interest to the medical field if we were to find that statins also inhibit proliferation of SMCs by inhibiting RME.

We must also elucidate the integrin activity patterns on each ECM composition to confirm our suspicions that $\alpha_2\beta_1$ and $\alpha_v\beta_3$ are major regulators of the behaviors on BaM and InF, respectively. This can be investigated with a combination of Western blotting, immunofluorescent staining, and integrin inhibition experiments. Immunofluorescent staining is likely the most straightforward method, because it permits direct visualization of integrins in the plasma membrane, whereas Western blotting will inform us of integrin expression, but not activation. Immunofluorescent staining does have the disadvantage of not being so easily and rigorously quantified. Integrin blocking or inhibition can also be useful, but it is important to know *how* the method of inhibition acts on the targeted integrin. Together, however, these methods would likely be sufficient to confirm or deny involvement of our integrins of interest in these phenomena.

In addition to differences in integrin expression, we suspect there is a role for Src and EGF receptor (and/or other GFRs) transactivation in these phenomena, which is itself likely modulated by integrin expression patterns and endocytosis. EGFR transactivation via Src is a somewhat common theme in SMC and cancer research, and integrin modulation of this type of activity could certainly help explain the differences in proliferation we observed. While it would likely be best to investigate this possibility by Western blotting phosphorylated and total forms of EGFR and Src during different time points of TGF β_1 or stimulation with growth or differentiation medium, it is difficult to get sufficient protein yields from samples in the formats used to successfully visualize phosphoproteins via Western blot. This could be alleviated with the use of very sensitive secondary antibodies

(i.e. with infrared emitting conjugates), but another approach is, again, immunofluorescent staining and co-localization. If either method shows a difference in these activities, the application of inhibitors to EGFR (or other GFR) or Src, or transfection with siRNAs, should then be used to confirm that these are the signaling proteins responsible for the observed behaviors.

Another potential mechanism of interest that could help explain our proliferation and signaling data is the autocrine production of GFs or cytokines such as VEGFs, PDGFs, TNF α , or several interleukins-of-interest. This type of phenomena has been observed in SMCs treated with TGF β ₁ [94], and it is possible that integrin-ECM interactions could modulate this process. With the wide availability of multiplex panels for GFs and cytokines, the MAGPIX instrument is an excellent tool to study this type of phenomena, and assaying non-phosphorylated proteins in cell culture supernatants is fast and easy. If it were discovered that one ECM composition does in fact promote secretion of relevant soluble factors, we would next test different integrin inhibitors to see if the secretion is reduced, add and remove ECM proteins to replicate or eliminate the effect, or alter the ECM concentration to check for ‘dose’-dependency of the effect.

Similarly, given that the BaM composition promotes deeper collagen gel invasion with growth medium stimulation, we hypothesize that this combination of factors is inducing synthesis, secretion and/or activation of one or more matrix metalloproteinases (MMPs), the class of enzymes primarily responsible for degrading ECM proteins during 3D cell migration. Since MMPs are synthesized in an inactive form, it is necessary to test for MMP activity, with gelatin zymography for some MMPs, or through the use of

cleavage-activated fluorophores and other reporter molecules, with findings confirmed with the use of integrin or MMP inhibitors.

4.7.2 Extend *in vitro* model to recapitulate more complex features of the *in vivo* microenvironment

Having demonstrated that greater complexity can yield physiologically-relevant behaviors *in vitro*, a natural next step is to further extend the complexity of our *in vitro* model to replicate additional features of the *in vivo* microenvironment. One simple change is the addition of softer and stiffer PEG-PC substrates to cover a wider range of measured arterial stiffnesses. We originally chose PEG-PC compositions that ranged from ~10 – 500 kPa as measured by uniaxial compression testing (see Chapter 2), but the indentation mechanical testing method employed here to measure the effective elastic modulus on glass cover slips provided dramatically different Young's moduli of ~40 – 94 kPa. While this is unexpected, and it is unclear as to why the new testing or polymerization methods yielded such different results, it may explain why stiffness had a much more modest effect than anticipated. Nevertheless, since we found that ECM is a major driver of SMC behaviors, and substrate stiffness is sensed through integrins and focal adhesions, it could be informative to further examine the effects of stiffness on SMC behavior and phenotype by repeating the more interesting experiments – especially proliferation in differentiation medium and 2D migration – on softer and stiffer PEG-PC substrates (~10 kPa and ~150-500 kPa, respectively) and characterizing the changes in focal adhesion and cytoskeletal properties. These experiments may yield additional information regarding the role of

integrins and the cytoskeleton in mediating the phenomena we examined, and may provide additional supporting evidence of our endocytosis hypothesis.

Since there are hundreds of physical and chemical factors implicated in atherosclerosis, additional complexity could easily be introduced in the form of a huge number of potential soluble factors and relevant ECM proteins. In the microenvironment of an intact media there are layers of collagen III in a mesh-like network, layered bundles of collagen I fibers, and sheets of elastic laminae (see Chapter 1, Fig. 1.2). However, it is unclear if SMCs are actively bound to these other ECM proteins via integrins, or if they are merely in proximity. Regardless, including these features in our BaM model may be technically infeasible, as we have tried unsuccessfully to covalently attach collagen I and III fibers to PEG-PC with sulfo-SANPAH. However, it would likely be trivial to extend our 3D invasion model to account for these additional layers of ECM by phenotype syncing SMCs on the BaM ECM composition and polymerizing a layer of collagen III directly on top, followed by a layer of collagen I. Ideally, polymerization conditions (i.e. pH, temperature, monomer concentration, and lysyl oxidase concentration) would be tested and optimized to produce a fibrous network that closely matches the properties found in the *in vivo* vascular media including fiber diameter, crosslinking density, and possibly even fiber alignment. With this more advanced model we could determine if collagen III and fiber morphology impairs or promotes SMC invasion. Even better would be if we could tweak the BaM microenvironment to achieve greater confluency and more closely-match the high density of SMC packing in the arterial media, possibly by using a higher surface density of the BaM ECM proteins. Additionally, it would more physiologically relevant if SMCs

on BaM could be induced to produce their own basement membrane proteins on their apical surface if they do not already, which would first be tested with immunofluorescent staining.

While it is a major part of the arterial media, elastin fiber polymerization and crosslinking into elastic laminae is a complicated multi-step process [95] and it is unclear how, or if, it is possible to synthesize or induce production of native-like elastic laminae *in vitro*. However, it would be interesting to test if any of our experimental conditions induce elastin production and crosslinking, which could be confirmed by detecting elastin and markers for elastin crosslinking, desmosine and isodesmosine, with an ELISA or immunofluorescent staining.

Additionally, since disordered, fragmented elastin is reportedly found in atherosclerotic plaques, we could further investigate the role of elastin by testing the effects of different elastin forms – tropoelastin, native soluble elastin, and the cleavage product elastin-derived peptide [96] – on the various behaviors and phenotypes observed with our *in vitro* models. We could also easily introduce other common soluble factors such as interleukins, tenascin C, TNF α , numerous proteoglycans, and nitric oxide to investigate their involvement and modulation of pathological behaviors. Of particular interest are the soluble factors secreted by endothelial cells, macrophages, and foam cells, which are hypothesized to produce the chemoattractants that induce SMC migration towards a plaque. One approach to this is to use endothelial- or macrophage-conditioned media in place of, or as a supplement to, the other medias used in our studies. However, it is preferable to have a convenient way to evaluate the soluble factors in the conditioned medium, and the conditions in which the cells are grown will certainly have an effect on the soluble factors they produce, both of which could hamper this approach.

Of course, an overabundance of complexity could quickly become detrimental, because it may be impossible or infeasible to perform every experimental iteration necessary to parse out the relative contributions and effects of each additional feature. Luckily, advancements in technology are driving down the costs of experimental lab work and making it possible to study very complex biological systems faster, better and more easily than ever before, for instance Luminex's xMAP technology. In conjunction with biostatistics, high-throughput '-omics' techniques also enable the examination of hundreds, or even thousands, of targets of interest.

Proteomic techniques, commonly performed with a combination of 2D electrophoresis and mass spectroscopy, have identified numerous proteins that are differentially regulated in atherosclerotic plaques compared to a healthy media [97], as well as in comparisons between plaques histologically-identified as stable or unstable [98]. However, most (but not all [99,100]) proteomics studies related to atherosclerosis use sectioned tissues from animals or human patients, therefore including proteins produced by any resident cell type, including immune cells and endothelial cells, and cannot provide any specific information regarding the roles of SMCs. The same is typical of transcriptome studies, which have identified many genes associated with atherosclerosis [101,102], but the mRNA transcripts could be produced by any of the resident cells.

While the types of studies described above are very useful for identifying potential markers of varying disease states, we are more interested in applying these techniques with our complex *in vitro* model system to get a 'big picture' view of how specific element(s) of the microenvironment regulate gene transcription and protein expression in SMCs and subsequently modulate their behavior. In such an approach it would be appropriate to first

consider how various combinations of environmental stimuli modulate the transcriptome, in part because it is much easier to measure the mRNA levels of thousands of genes with RNA-seq technology than it is to quantify the levels of the same number of proteins with mass spectroscopy. While not strictly necessary, in the case of SMCs it would be beneficial to perform a whole-transcriptome analysis with sequencing because several microRNAs are associated with the contractile and synthetic phenotypes [103–105]. With this data we can deduce potential regulatory pathways and identify proteins of interest that may have never been considered *a priori*, then follow up with proteomics techniques to confirm and evaluate the expression of any interesting proteins identified from the transcriptome analysis. Finally, any interesting findings can be further confirmed with inhibitor studies and low-throughput techniques including Western blots and qRT-PCR, as necessary.

In addition to extending the essential components of our current *in vitro* model, there are other areas of interest that could be examined with some significant changes to our model. One of these areas of interest is dynamic mechanical forces, especially oscillatory stretch like that which SMCs experience from pulsatile blood flow. However, it is unclear how, or if, PEG-PC could be adapted for use in the “Flexcell” devices commonly used for such studies, or otherwise how the *in vitro* model could be modified to exert these forces on SMCs. The addition of fluid flow, or oscillatory shear stress, is also of interest. While technically it is the endothelial cells of the vascular intima that are primarily subjected to fluid flow, this is still of interest with SMCs because they may experience these forces following endothelial denudation, as can occur with stent implantation or balloon angioplasty. These features can and have been modeled in microfluidic devices, but with limited physiological complexity [106]. It would

undoubtedly be technically challenging, but it should be feasible to introduce the complexity of our *in vitro* system into a microfluidic model.

Another extremely interesting feature that would be relatively straightforward to integrate with our *in vitro* model is co-culture with endothelial cells, which has previously been shown to profoundly affect SMC phenotype [107–109]. This could be achieved indirectly with endothelial cell conditioned media, or by seeding endothelial cells directly on top of confluent SMCs. However, since endothelial cells and SMCs in healthy arteries are physically separated by connective tissue (mostly collagen), it would be more relevant for SMCs and endothelial cells to be separated by a thin layer of polymerized collagen, or possibly a synthetic, degradable hydrogel such as PEG-4-maleimide [110]. Ideally, fluid flow would also be introduced. The more complex 3D invasion model incorporating both collagen I and III described above might be suitable for this purpose, with vascular endothelial cells seeded on top of the collagen I gel. This would certainly be technically challenging, but if feasible in practice it could ultimately prove to be a useful *in vitro* model of the pathogenesis of atherosclerosis. For instance, with such an *in vitro* model we could introduce oxidized LDL particles and monocytes to a subconfluent layer of endothelial cells and see if SMCs are induced to invade and form an artificial plaque. Of course, to be truly useful, systems of this complexity must be designed carefully, with reproducibility and ease-of-use established as a high priority from the start.

4.8 References

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