Annals of Biomedical Engineering, Vol. 38, No. 1, January 2010 *(© 2009)* pp. 2–20
DOI: 10.1007/s10439-009-9811-1

Synthetic Materials in the Study of Cell Response to Substrate Rigidity

STEPHANIE NEMIR and JENNIFER L. WEST

Department of Bioengineering, Rice University, 6100 Main St. MS 142, Houston, TX 77005, USA

(Received 28 May 2009; accepted 23 September 2009; published online 9 October 2009)

Abstract—While it has long been understood that cells can sense and respond to a variety of stimuli, including soluble and insoluble factors, light, and externally applied mechanical stresses, the extent to which cells can sense and respond to the mechanical properties of their environment has only recently begun to be studied. Cell response to substrate stiffness has been suggested to play an important role in processes ranging from developmental morphogenesis to the pathogenesis of disease states and may have profound implications for cell and tissue culture and tissue engineering. Given the importance of this phenomenon, there is a clear need for systems for cell study in which substrate mechanics can be carefully defined and varied independently of biochemical and other signals. This review will highlight past work in the field of cell response to substrate rigidity as well as areas for future study.

Keywords—Substrate rigidity, Substrate stiffness, Durotaxis.

INTRODUCTION

Though early work in a number of laboratories suggested a role for substrate rigidity in regulating cellular behavior,[4,11,31–33,42,43,45,46,51] the first formal study investigating this interaction in a synthetic system in which mechanical and biochemical effects could be independently modified was published in 1997. In their study,[55] Pelham and Wang described changes in cellular properties including shape, spreading, and focal adhesion formation of both normal rat kidney epithelial (NRK) cells and 3T3 fibroblasts with alterations in substrate stiffness. These studies, which used cells grown on collagen-coated polyacrylamide gels with elastic moduli controlled by varying the amount of acrylamide monomer or bisacrylamide crosslinker used, set forth a standard method for investigating cell response to substrate rigidity.

The advantages of this model system, which allowed fairly independent control of substrate mechanical and biochemical properties, led to a quick adoption of polyacrylamide gels as substrates by those studying cell response to substrate mechanics. Previously, researchers investigating cell response to substrate stiffness had largely used natural polymer gels, such as collagen and Matrigel, in which the elastic modulus was altered by changing the concentration of protein within the gel. This approach had the unfortunate side effect of markedly changing the biochemistry experienced by cells grown in or on substrates of different stiffness, thus making it impossible to assess which portion of the cellular behavior was attributable to substrate mechanics. Though some researchers have continued to use biological gels to study cell response to substrate rigidity, this review will discuss only studies in which substrate biochemistry and mechanics were independently controlled.

More recently, in an attempt to develop more cytocompatible substrates that might be used in longer-term studies and potentially implanted as well, some investigators have begun using poly(ethylene glycol) (PEG)-based hydrogels to study cell response to substrate rigidity. These hydrogels allow the same independent control of biochemical and mechanical properties as polyacrylamide gels and have the added benefit of allowing cells to be encapsulated within the polymer network, so that substrate rigidity effects in three dimensions might be analyzed. Whether these hydrogels are able to offer the same range of substrate rigidity as polyacrylamide gels has yet to be determined, as no studies to date have used PEG-based gels with an elastic modulus lower than ~1 kPa, compared with <0.1 kPa achieved in polyacrylamide systems.[22,41]

This review covers several approaches. The first section will review the responses of various cell types cultured in two dimensions on substrates with uniform rigidity. The range of elastic moduli and cell types studied is summarized in Fig. 1. For reference, Fig. 1 also demonstrates the wide range of rigidity found in select human tissues.[1,2,25,29,65,68,77] The second section will look at cell responses to substrate rigidity in three dimensions, while the final section will discuss responses of cells cultured on substrates with non-uniform, patterned rigidity.

Address correspondence to Jennifer L. West, Department of Bioengineering, Rice University, 6100 Main St. MS 142, Houston, TX 77005, USA. Electronic mail: nemir@rice.edu, jwest@rice.edu

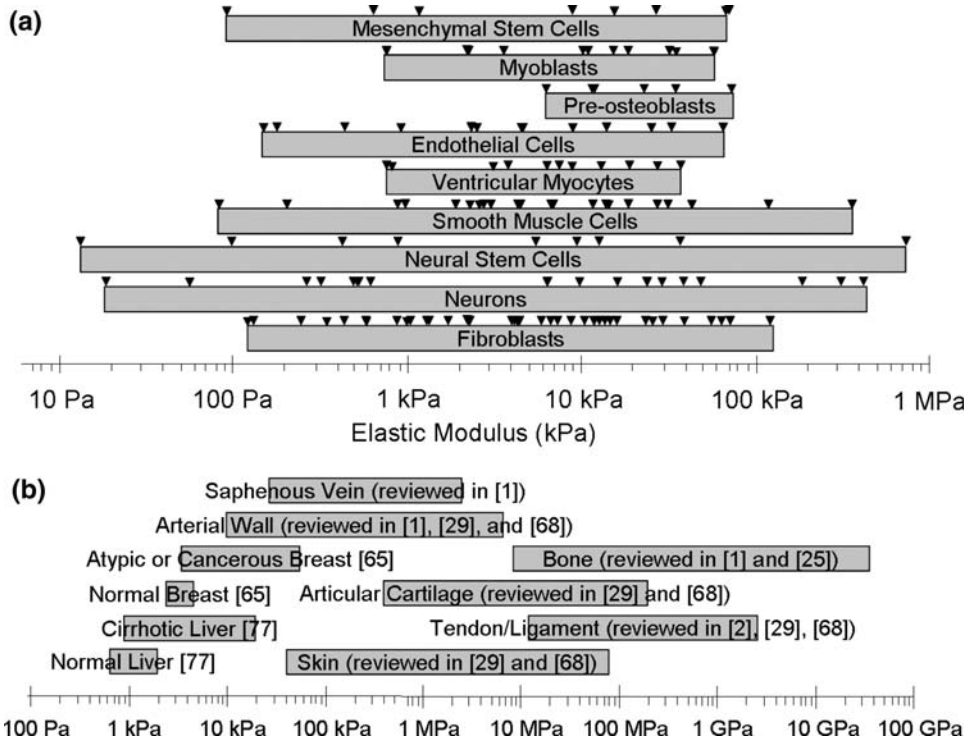

FIGURE 1. (a) Ranges of rigidities studied for various cell types (compiled from Figs. 2, 5, 7, and 9). *Gray bars* indicate range of rigidities studied; *arrowheads* indicate specific rigidities used. (b) Range of rigidities found in selected human tissues.

CELL RESPONSE TO SUBSTRATE RIGIDITY IN TWO DIMENSIONS

Fibroblasts

Much of the earliest work in cell response to substrate rigidity was performed using fibroblasts. These studies almost exclusively used polyacrylamide substrates with type I collagen or fibronectin as the adhesive ligand (Fig. 2). Several groups noted increased fibroblast spreading with increasing substrate rigidity from less than 1 to greater than 100 kPa,[14,28,36,47,54,55,69,75,78] with a corresponding increase in actin fiber formation[14,54,55,69,78] and organization.[24] Stress fibers formed in cells on substrates of modulus of 10 kPa and higher and were undetectable in cells on substrates softer than 5 kPa.[78] Ghosh *et al.*[24] noted that the increase in cytoskeletal organization corresponded to an increase in cell modulus of ~150% between cells on substrates with elastic moduli of 0.28 and 12.7 kPa. Solon *et al.*[69] also found an increase in cell stiffness following substrate stiffness, with cell modulus matching that of the substrate up to a modulus of 5 kPa, then remaining slightly below the stiffness of the adjacent substrate up to 10 kPa. Above this range, cells began to form stress fibers and no longer matched the rigidity of the substrate.[69] This 10 kPa cutoff for cell–substrate modulus matching also promoted the greatest degree of cell spreading, with no further increase in spread area even on glass.[69] Yeung *et al.*[78] found a similar substrate modulus of 8.4 kPa corresponding with maximal spread area, which was within the range of the elastic modulus of a fibroblast spread on a rigid surface (shear modulus 3 kPa, assuming $\upsilon = 0.4$,[17] see Appendix). This suggests that the change in fibroblast spreading behavior near the elastic modulus of the cell may occur as internally generated tractional forces exert a deforming effect not only on the substrate (as would be true on softer gels) but also on the cell itself.[78]

Guo *et al.*[28] hypothesized that the poorly spread morphology seen in cells on softer substrates might correspond to a decrease in cell adhesion to that substrate. Using a centrifugation assay, they showed significantly weaker cell adhesion to softer substrates, with only ~30% of cells remaining on a 2.68 kPa substrate compared to >80% on a 7.69 kPa gel.[28] Paszek *et al.*,[54] using a similar assay, found no difference in either number of adherent cells or shear force required to detach fibroblasts on ~1 and 66 kPa substrates. Both groups noted an increase in focal adhesion size and organization with increasing substrate stiffness (Fig. 3),[28,54] and Paszek *et al.* also noted increased recruitment of vinculin to adhesion sites on their stiffer substrates.[54] Collin *et al.*[14] investigated the formation of transient actin-based adhesive structures called podosomes and found an increase in both the

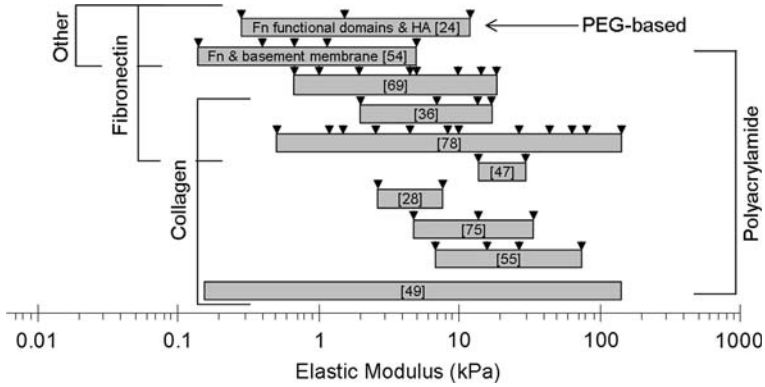

FIGURE 2. Studies of fibroblast response to substrate rigidity. Substrate type is indicated on the right and adhesive ligand on the left. *Gray bars* indicate range of rigidities studied; *arrowheads* indicate specific rigidities used in that study. Fn: fibronectin, HA: hyaluronan.

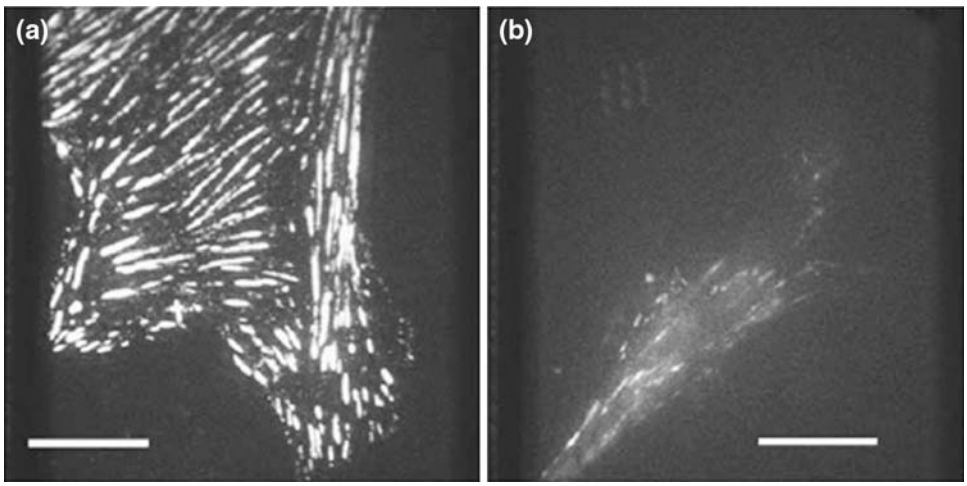

FIGURE 3. Fibroblasts fixed and stained with anti-paxillin monoclonal antibody show well defined, elongated focal adhesions when plated on 7.69 kPa substrates (a) but only small, punctate staining on 2.68 kPa substrates (b). Scale bar 20 microns (reprinted from Guo *et al.*[28] with permission from Elsevier).

frequency of podosome rosette formation and podosome stability with increasing substrate stiffness. Podosome spacing was found to decrease significantly with increasing substrate rigidity, as did the velocity of rosette expansion and contraction.[14] These podosome rosettes occurred alongside focal adhesions and actin stress fibers, and the authors hypothesized that podosomes might act as fast-moving mechanosensors due to their rapid turnover rate, while focal adhesions act as more stable mechanotransmitters.[14]

Cell growth and apoptosis are also influenced by substrate rigidity.[75] 3T3 fibroblasts had ~2- and ~4-fold greater cell proliferation on 14 kPa gels compared to 4.7 kPa gels after 24 and 48 h, respectively.[75] There was also a ~2-fold increase in apoptosis on softer gels compared to stiffer ones after 24 h; at the 48 h timepoint, cells on softer gels maintained a 30–35% apoptosis rate compared to less than 5% on stiffer gels.[75] Thus, increases in cell numbers on substrates of higher elastic moduli could be attributed to both increased cell proliferation and decreased apoptosis.[75] Similar results were later found with human fetal lung fibroblasts as well.[49]

In an attempt to better understand the cellular pathways governing cell response to substrate rigidity, researchers have used a combination of inhibition, knockout cell lines, and exogenous expression for elements believed to play a role in cell–substrate rigidity sensing (Fig. 4). Yeung *et al.*[78] observed an increase in α5-integrin expression with cell area on substrates of different rigidities, but exogenous α5-integrin expression did not affect cell spreading. Jiang *et al.*[36] investigated the spreading behavior of a number of knockout fibroblast lines. While knocking out

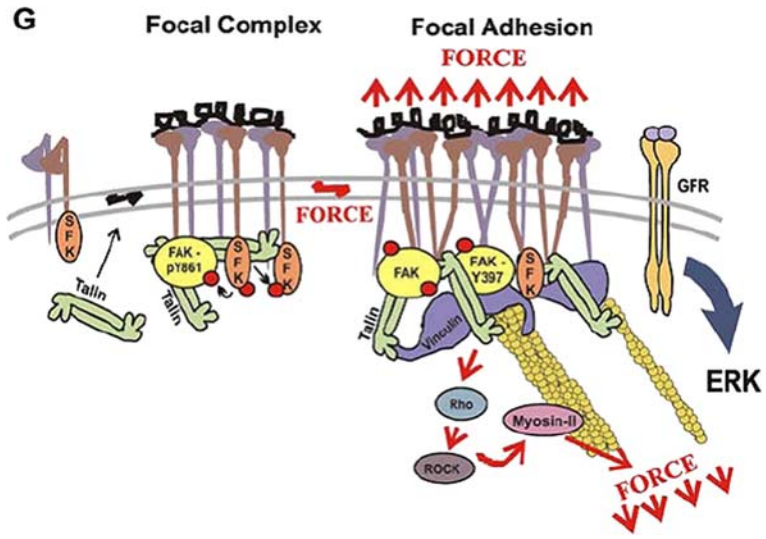

FIGURE 4. Schematic of some focal adhesion and cytoskeletal elements involved in transmitting force information from the extracellular matrix to the cell (reprinted from Paszek *et al.*[54] with permission from Elsevier).

TABLE 1. Fibroblast responses to increasing substrate rigidity.

Factor	Response	References
Spread area	↑	55,75,28,78
Migration speed	↓	55,24
Stress fiber formation	↑	55,24,78,69
Focal adhesion formation	↑	55,28,78
Proliferation	↑	75,24,49
Apoptosis	↓	75,49
Adhesion/Traction forces	↑	75,28,36,24
Cell modulus	↑	24,69

↑ indicates increase, ↓ indicates decrease.

elements of the integrin adhesome such as talin1 and integrin $\beta 1$ did not affect spreading trends on fibronectin-coated surfaces, receptor-like protein tyrosine phosphatase-α (RPTPα) knockouts lost sensitivity to substrate stiffness, showing no significant difference in spread area over the range of substrates studied.[36] Blocking $\alpha_V\beta_3$ integrins, which form a complex with RPTPα in the adhesome, also prevented differential cell spreading on soft vs. stiff substrates.[36] This inhibition of spreading was lost when the adhesive ligand coating the gels was changed from fibronectin to collagen IV, however, indicating that RPTPα is not required for rigidity sensing mediated by collagen-IV-binding integrins such as $\alpha_1\beta_1$.[36]

Rho-kinase has been shown to phosphorylate myosin and multiple myosin-related proteins, leading to increased actin stress fiber formation and contractility (reviewed in Pellegrin and Mellor[56]), and has been implicated as playing a role in cell response to substrate rigidity.[28,34,37,54,58] Focal adhesion kinase (FAK) may also play a role through its regulation of focal adhesion assembly and participation in cellular migration pathways.[38,54,66,74] Paszek *et al.* found phosphorylation of FAK^{pY397} on 66 kPa but not ~1 kPa substrates,[54] and Jiang *et al.* found that FAK knockouts had a significantly smaller spread area on stiff gels than soft.[36] This decrease in spread area was attributed to hypercontraction of the cells in response to the stiffer substrate, as inhibition of Rho-kinase or myosin returned spreading to control levels.[36]

Fibroblast responses to changes in substrate rigidity are summarized in Table 1.

Neurons and Related Cells

Studies using cells of the nervous system have been motivated, at least in part, by suggestions that glial scarring following injury to the central nervous system might pose a mechanical barrier to neuronal growth and healing.[70] The substrates used in these studies have covered a wide range of rigidities, with elastic moduli ranging from 10 Pa[64] to over 750 kPa (Fig. 5).[72] These values were chosen to cover the range of stiffness of brain and spinal cord[22] as well as the stiffness levels investigated for other cell types.

In primary neurons, Flanagan *et al.* noted an increase in neurite branching with decreasing substrate rigidity.[22] Similarly, in PC12 cells, a rat adrenal pheochromocytoma line that can be induced into a neuronal phenotype, decreasing substrate rigidity led to increased neurite extension, but only for gels with elastic moduli over 200 kPa.[27] Leach *et al.* found longer, more highly branched neurites on stiffer substrates, but no significant difference in PC12 neurite

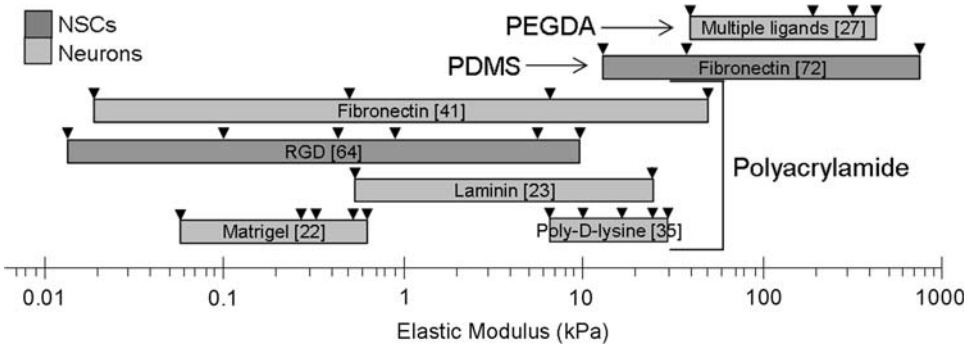

FIGURE 5. Studies of neuron and neural stem cell response to substrate rigidity. Adhesive ligand is specified for each study. *Gray bars* indicate range of rigidities studied; *arrowheads* indicate specific rigidities used in that study.

length, number of neurites expressed per cell, or percentage of cells expressing neurites as a function of substrate rigidity between cells on ~0.5–51 kPa substrates, though all three measures were decreased on ~0.02 kPa gels.[41] Jiang *et al.*[35] found 10% more primary dendrites in spinal cord neurons grown on ~30 kPa gels than those on softer gels, with no significant differences in primary dendrite length per neuron. Axonal length and expression of focal adhesion kinase decreased with increasing stiffness.[35]

One of the most striking findings was a difference in neuron and astrocyte viability between substrates of varying rigidity. Flanagan *et al.*[22] saw no glial growth on gels with shear moduli ranging from 50 to 550 Pa (elastic moduli 140–1540 Pa, assuming $\upsilon = 0.4$[17]) in marked contrast to glass, which became overrun with glial cells after a few weeks of culture. Neurons, by contrast, grew well on even the softest gel.[22] Similarly, Georges *et al.*[23] found reduced astrocyte adhesion on softer gels. Astrocytes on the stiffer gels also exhibited more pronounced organization of the actin cytoskeleton.[23] In co-cultures of astrocytes and neurons, there was a marked difference in cell distribution between gels, with ~80% of cells on the softer gels staining for neuronal marker β3-tubulin compared to less than 45% of cells on the stiffer gels.[23] Since neurons are post-mitotic, this difference in cell number can best be attributed to differential astrocyte behavior, either decreased proliferation on or increased detachment from the softer substrate.[23] On the stiffer substrate, neurons were only observed growing on top of glia, whereas they were able to grow independently on the softer gel, though they developed long neurites on both.[23] Using cells harvested from rat spinal cords, Jiang *et al.*[35] also observed increasing cell density with substrate rigidity. In contrast to other studies, the stiffest gels were reported to support significantly more neural cells than the softest two substrates, with no significant differences in astroglia number between groups.[35] This last study used DNA instead of bisacrylamide to crosslink the polyacrylamide substrates, studied a stiffer range of substrates, and used a different adhesive ligand than the other two studies, any of which could have contributed to the differences in cell behavior seen.[22,23,35]

Differentiation of neural stem cells (NSCs) has also been shown to be significantly influenced by substrate rigidity.[64,72] Rat NSCs attached equally well on substrate ranging from 10 Pa to 750 kPa, with robust proliferation and spreading on all but the softest substrate.[64,72] Cell proliferation peaked slightly in the range of 1–4 kPa, near the stiffness of normal brain tissue.[64] In astrocyte media, cell number increased with increasing substrate stiffness over the range of 10 Pa to 10 kPa, but there was not a significant difference in percent of cells positive for glial fibrillar acidic protein expression (GFAP, an astrocyte marker) with substrate rigidity after 6 days.[64] In neurogenic media, almost all cells on all substrates stained negative for GFAP but positive for β3-tubulin (a neuronal marker), with peak expression on ~500 Pa substrates.[64] When cells were cultured under conditions that allowed for differentiation down either neural or astrocytic lineage, Saha *et al.* observed a striking shift from primarily β3-tubulin+ cells on ~10 and ~100 Pa substrates to primarily GFAP+ cells on 10 kPa substrates (Fig. 6).[64] Teixeira *et al.*, using substrates ranging in stiffness from 750 kPa to 'close to zero,' found the opposite trend, with GFAP expression increasing with decreasing substrate rigidity and no change in β3-tubulin over the range of stiffness studied.[72] They also noted increasing neurite length and synaptotagmin expression with decreasing substrate rigidity.[72] Oligodendrocyte spreading was noted to increase with substrate rigidity.[72]

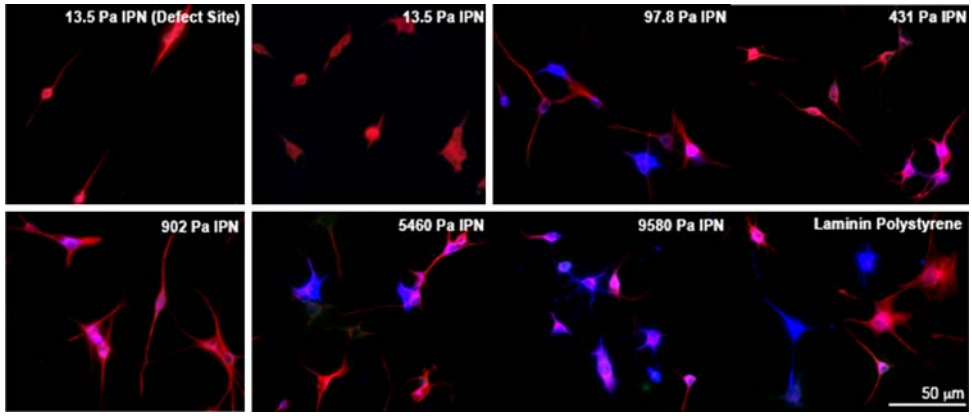

FIGURE 6. Immunostaining of NSCs after six days of culture on polyacrylamide/PEG interpenetrating network (IPN) hydrogels under conditions that allowed for either neuronal or glial differentiation. Cells labeled for nestin (*green*), β3-tubulin (*red*), and GFAP (*blue*) (reprinted from Saha *et al.*[64] with permission from Elsevier).

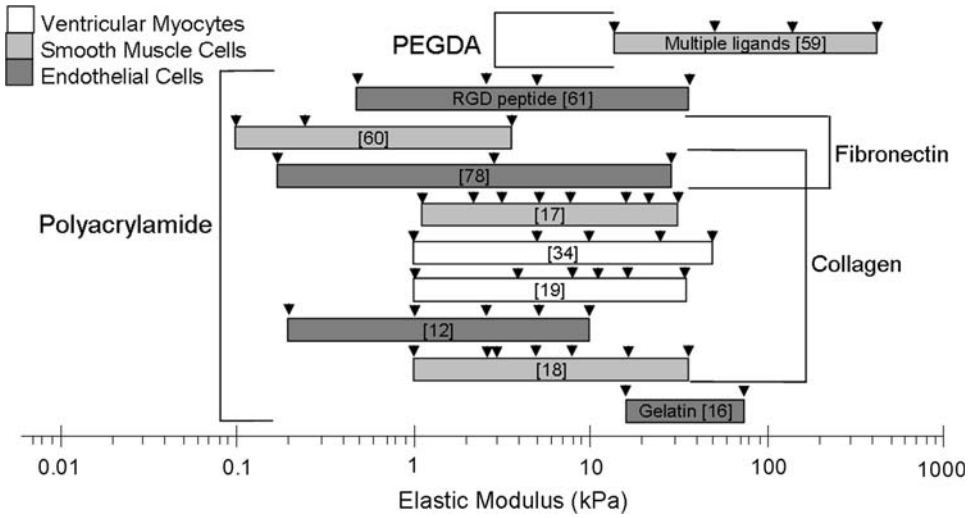

FIGURE 7. Studies of cardiovascular cell response to substrate rigidity. Substrate type is indicated on the left and adhesive ligand is specified for each study. *Bars* indicate range of rigidities studied; *arrowheads* indicate specific rigidities used in that study.

Cells of the Cardiovascular System

Neuronal cells are not the only cell type to thrive on a softer substrate. Work done by several groups has shown that endothelial cells tubule formation is influenced by substrate rigidity (Fig. 7). Similar to fibroblasts, the cells exhibited increased spreading with substrate rigidity,[12,16,78] though the difference was not as pronounced as with fibroblasts.[78] Endothelial cell migration and compaction on substrates of varying rigidity also follows trends seen in fibroblasts, with strong cell–cell interactions on soft (0.5 kPa) substrates and cell dispersion on stiffer (33 kPa) ones.[28,61] Cells on substrates of intermediate stiffness (2.5 and 5.5 kPa) tended to remain near neighboring cells, repeatedly forming and breaking cell–cell contacts, and isolated cells demonstrated significantly higher dispersion than cell–cell pairs.[61]

The formation of organized blood vessels from endothelial cells is a major topic of interest in tissue engineering, as the delivery of nutrients to and removal of waste products from tissue engineered constructs in the absence of a viable microvascular network is a constant issue. Reliable vessel formation from endothelial cells is therefore an important measure. Deroanne *et al.*[16] observed the formation of cordlike structures on 17 kPa gels while cells on 75 kPa gels formed a monolayer. Califano and Reinhart-King[12] observed networks of elongated cells forming on 0.2 and 1 kPa gels with high collagen density, while cells on 2.5, 5, and 10 kPa substrates were evenly

distributed and did not form structures. When ligand density was decreased 100-fold, network formation was seen on 10 kPa gels with some cord development on 2.5 and 5 kPa substrates and only rounded adherent cells on 0.2 and 1 kPa gels, indicating that network formation was governed by a combination of ligand density and substrate modulus.[12] Reinhart-King *et al.*[61] found a significant difference in the type of cell–cell contacts formed on substrates of identical stiffness but varying ligand density, with cells on substrates with lower density demonstrating significantly more attractive connections than those on higher-density gels, which may contribute to the increased structure formation seen on gels of lower ligand density. A similar effect of ligand density or identity may explain the cord formation seen by Deroanne *et al.* on stiffer, 17 kPa substrates.[16] Califano and Reinhart-King[12] hypothesized that network formation by endothelial cells occurs when the combined effects of substrate mechanics and ligand density are optimized. This interplay between substrate biochemistry and mechanics has been noted in many cell types[6,38,59,62,67] and highlights the importance of using substrate systems that allow independent control of the two factors.

Although they reside in close proximity to endothelial cells, smooth muscle cells do not share endothelial cells' preference for very soft substrates. Much like fibroblasts, vascular smooth muscle cells (SMCs) also exhibit an increase in spread area[17,18,59,60] and proliferation[59] and a decrease in migration speed[76] with increasing substrate rigidity. The range of rigidities studied is shown in Fig. 7. Peyton *et al.* found a clear increase in both focal adhesion area and elongation with increased substrate stiffness.[59] Immunostaining revealed increased co-localization of caldesmon and calponin with smooth muscle α-actin on 49.0 kPa gels compared with stiffer ones.[59] Caldesmon expression was highest on 49.0 kPa gels, though no staining data was reported for the softest (13.7 kPa) hydrogel.[59] Polte *et al.*[60] noted an increase in myosin light chain phosphorylation with increased substrate stiffness. By transfecting smooth muscle cells with GFP-actin and GFP-paxillin, Engler *et al.* showed that a 5–10% increase in cellular actin stores could cause cell spreading on gels whose elastic modulus would not support spreading of untransfected cells, demonstrating the intricate interactions between the mechanical environment and the cytoskeleton.[17]

Jacot *et al.*[34] and Engler *et al.*[19] investigated the dependence of cardiomyocyte behavior on substrate modulus. Cells developed more defined striations as substrate rigidity increased from 1 to 10 kPa, then lost sarcomere alignment and developed stress fibers as the stiffness increased further (Fig. 8).[19,34] Interestingly, this value of ~10 kPa is the same stiffness at which fibroblasts begin to develop stress fibers. Engler *et al.* observed increased cardiomyocyte spread area with substrate rigidity,[19] while Jacot *et al.* found no rigidity effect on cell spreading or the ratio between long and short cell axes except on 50 kPa gels, where cells were significantly more circular and less spindle-shaped than those on 10 kPa gels.[34]

Cardiomyocyte contraction also appeared sensitive to substrate rigidity. Twenty to 40% of cells on 1 and 11 kPa substrates were observed to beat, compared to 2–8% on 34 kPa gels.[19] Cells on all substrates began beating at a rate of about 1 Hz, but those on 34 kPa substrates slowed almost to zero after 48 h, while those on 1 and 11 kPa gels maintained frequency over the same time period.[19] Response to electrical stimulation and velocity of shortening decreased with increasing substrate rigidity.[34] Among those cells that contracted, the highest axial force generation was found on 10 kPa gels, with force dropping off for stiffer and softer substrates.[34] This difference in force generation was echoed by calcium transient size and sarcoplasmic/endoplasmic reticular calcium ATPase expression.[34] The authors noted that 10–11 kPa is within the range of stiffness values for normal resting myocardium, which may account for the increased contractile response seen at this level.[19,34]

Interestingly, expression of cardiac α-actin, vimentin, and myosin heavy chain did not differ significantly between substrates, indicating that the difference in contractile response was not a direct result of shifts in contractile protein concentrations.[19,34] There did appear to be conformational differences between myosin heavy chains, filamin, vimentin, and pyruvate kinase M1 in cells grown on 1 vs. 34 kPa substrates, which may have affected the activity of proteins independent of their concentrations.[19] Inhibition of RhoA or RhoA kinase led to significantly greater traction force on 25 and 50 kPa substrates than controls, indicating that the RhoA/ROCK pathway is active in regulation of contraction at higher substrate rigidities.[34] Cells on the stiffest gels also developed well-defined sarcomeres instead of the actin stress fibers seen in uninhibited cells on the same substrate.[34]

Neutrophils

All of the cells discussed so far reside in well-defined tissue niches. Neutrophils, by contrast, must be able to migrate through and function in a variety of tissue environments as part of their role in the innate immune system. Yeung *et al.* found no change in cell size or shape of quiescent or fMLP-stimulated neutrophils on substrates of different stiffnesses.[78] Oakes *et al.*[50] and Stroka *et al.*,[71] by contrast, found a significant increase in activated neutrophil spread area with substrate

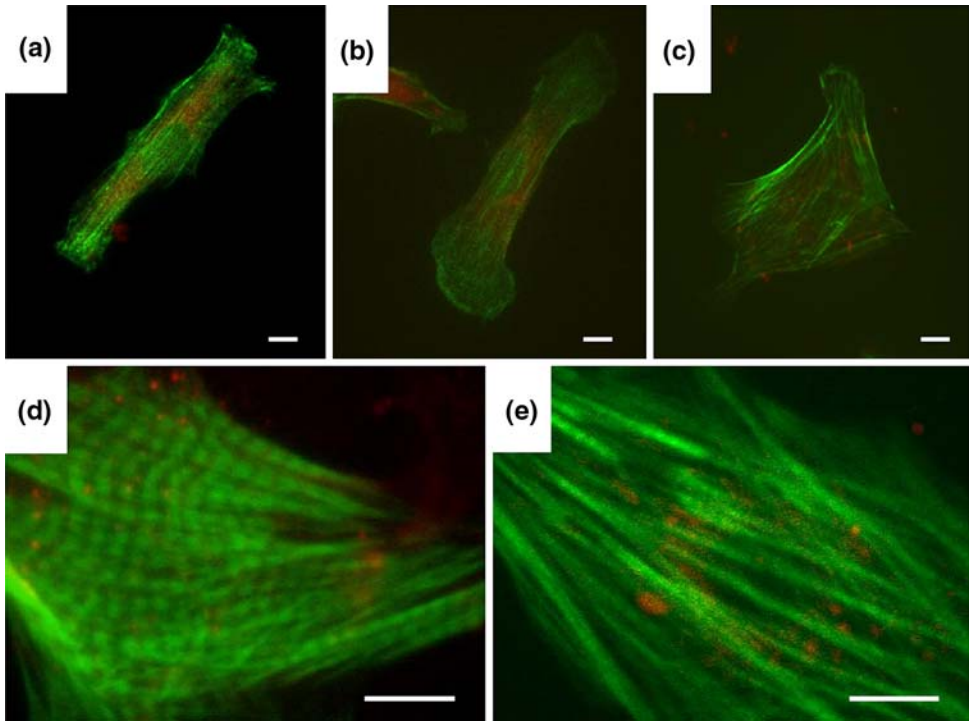

FIGURE 8. Neonatal rat ventricular myocytes labeled with phalloidin (*green*) and DiI (*red*) reveal highly aligned actin fibers on 1 and 10 kPa gels (a and b, respectively) but a loss of alignment on 50 kPa gels (c). Cells on 10 kPa gels develop well-defined sarcomeres (d) which are not seen on 50 kPa gels (e). Scale bars 10 microns (reprinted from Jacot *et al.*[34] with permission from Elsevier).

stiffness. Oakes *et al*. also noted a difference in morphology between cells on 20 and 50 kPa substrates and those on their softer gels, with stiffer surfaces promoting ridges at the leading edge and edge ruffling during migration.[50] Similar to trends seen in fibroblasts and smooth muscle cells, neutrophils showed decreased migration speed on stiffer substrates.[50] Cells on stiffer substrates were less likely to turn during migration, however, giving a greater displacement of cells over time on stiffer substrates despite greater path length on softer ones.[50,71] The trend was consistent in both chemotactic and chemokinetic models.[50] Neutrophils also generated higher traction force on stiffer gels,[50] consistent with what has been observed in other cell types. Overall, neutrophil responses to substrate rigidity were consistent with trends seen in non-migratory cells.

Structural Tissues: Muscle, Bone, and Beyond

Many tissues in the body require that specific cell types work together to form larger structures. Myoblasts, for example, must fuse into myotubes and eventually align to form contractile muscle fibers in order to fulfill their functional role. Such three-dimensional tissues can be challenging to form *in vitro*. Studies examining these cell types have largely used type I collagen-coated polyacrylamide gels, though other ligands have been investigated as well (Fig. 9). Similar to fibroblasts and SMCs, C2C12 skeletal myoblast spread area and adhesion strength increased with increasing substrate modulus.[20] Cells also elongated more on stiffer gels, though by 24 h this difference had largely disappeared.[20] At 24 h, cells tended to align their major axis with those of nearby cells.[20] This alignment decayed over 5–10 cell widths and decayed more quickly on stiffer substrates, perhaps indicating that these substrates did not transmit alignment information as well over longer distances.[20]

Initial cell adhesion was similar on gels ranging from 1 to 45 kPa, though cell fusion into myotubes was absent on the softest, 1 kPa gels.[7] C2C12 cells on all gels exhibited increased levels of muscle creatine kinase (MCK) over four days of culture, though the increase was higher on 13 and 45 kPa gels compared to 1 kPa substrates.[7] When collagen was patterned in 20 μm wide stripes, myoblasts fused into multi-nucleated myotubes on all substrates independent of modulus but only exhibited significant myosin striation on gels of intermediate stiffness.[20] Striations were visible in almost 25% of cells on 11 kPa substrates after two weeks and in almost 50% after four weeks.[20] On glass, myotubes develop abundant actin stress fibers and robust focal adhesions, but no myosin striation.[20]

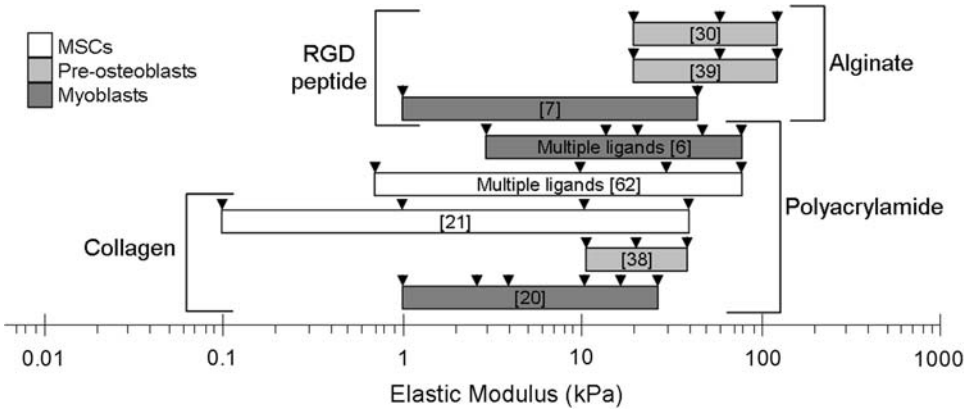

FIGURE 9. Studies of MSC, pre-osteoblast, and myoblast responses to substrate rigidity. Substrate type is indicated on the right and adhesive ligand on the left. *Bars* indicate range of rigidities studied; *arrowheads* indicate specific rigidities used in that study.

These results suggest that there is a critical range of substrate rigidity on which myotubes will form myosin striations, with little striation on either softer or stiffer substrates.[20]

Primary myoblast behavior had a greater dependence on substrate stiffness, as primary cells on 1 kPa gels failed to adhere, spread, or proliferate to the same degree as those grown on 12 and 45 kPa gels.[7] Proliferation was greatest on 21 kPa gels, with decreases on both softer and stiffer substrates.[6] Primary cells exhibited increased levels of MCK with time when cultured on 45 kPa gels but not on 1 and 13 kPa gels.[7] Myotubes were able to form on all substrates, but striation and spontaneous contraction were highly dependent on both substrate stiffness and ligand identity, with no striation or contraction on 3 kPa gels regardless of ligand and with highest striation on 21 kPa gels coated with poly-D-lysine or laminin (compared to 3 and 80 kPa gels).[6] Gels coated with Matrigel or collagen IV supported no striation on the stiffnesses investigated.[6] Clearly cell response to substrate rigidity is highly dependent on the adhesive ligand used.

Noting that the rigidity showing the greatest myotube striation is very near the stiffness of C2C12 myotubes themselves, Engler *et al.*[20] examined myoblasts seeded in two layers on glass substrates patterned with collagen in stripes. They hypothesized that the lower layer of myotubes would provide the top layer with appropriate mechanical signals for myosin striation.[20] Their results seemed to support this theory; the top layer of myotubes was found to form striations over time (68% of cells at 1 week and 85% at 4 weeks), while the lower layer remained unstriated.[20] The percent of cells forming striations was significantly higher in cells grown in this setting compared to polyacrylamide gels of similar stiffness, indicating that striation in the top layer cannot be attributed to mechanical signals alone but perhaps to some combination of mechanical signaling and cell–cell interactions.[20] The lack of striation in the lower myotubes, however, seems to indicate that cell–cell interactions alone cannot override the inhibitory effects of the stiff substrate beneath them.[20] Engler *et al.* repeated these experiments using a lower layer of fibroblasts; myotubes grown on top of this fibroblast layer did not striate after two weeks.[20]

Another cell type that must organize at the tissue level to meet its functional role is the osteoblast. Khatiwala *et al.*[38] found greatest mineralization of pre-osteoblastic MC3T3-E1 cells on polystyrene controls followed by ~40 kPa and then 20 kPa gels. A second study found an opposite trend in differentiation: decreased gel stiffness was found to correspond to a significant increase in secretion of osteocalcin, a marker of osteoblast differentiation, as well as a 20-fold increase in mineralization on 20 vs. 110 kPa gels.[39] The difference in differentiation between these studies highlights the complexity of cell responses to substrate rigidity; changes in culture conditions unrelated to stiffness, such as adhesive ligand identity or media used, may profoundly alter trends in cellular behavior with stiffness.

Other results obtained using pre-osteoblasts more closely matched those seen with other cell types. Proliferation, for example, was found to increase with substrate rigidity.[38,39] This increase in proliferation was accompanied by a decrease in the fraction of apoptotic cells as substrate stiffness increased.[39] While cells did not appear to show the same strong influence of substrate rigidity on spread area or cytoskeletal organization as had been seen in other cell types, there was a significant shift in intracellular vinculin between the soluble and insoluble forms with changes in substrate stiffness, indicating increased focal adhesion formation on stiffer substrates, especially at lower

ligand density.[38,39] Depolymerization of microtubules caused an increase in focal adhesion-associated vinculin on soft substrates but not on the stiffest gel and polystyrene control.[38] Phosphorylation of FAK increased with increased substrate stiffness; this response appeared less sensitive to microtubule depolymerization.[38]

To evaluate whether differentiation stage impacted sensitivity to substrate rigidity, Hsiong *et al.*[30] compared D1 cells, a clonally derived murine stem cell line, and MC3T3-E1 preosteoblasts on RGD-coated alginate hydrogels. D1 cells did not show any of the stiffness-dependent change in proliferation seen with more differentiated MC3T3-El cells.[30] When D1 cells were cultured in osteoblast differentiation media for two weeks prior to seeding, however, they exhibited a strong increase in proliferation with increased substrate stiffness.[30] A similar increase in proliferation with substrate stiffness was found using human bone marrow stromal cells (MSCs).[30,62] MSCs also showed increased spreading with substrate rigidity and a maximum in attachment on 25 kPa substrates, though the stiffness associated with the highest attachment or spreading differed between ligands used.[62]

Studies of MSC differentiation found clear differences in protein and gene expression between cells cultured on substrates of different stiffnesses.[21,62] When cultured in basic, non-differentiating media, cells on the softest (0.1–1 kPa) gels exhibited branching and filopodia formation similar to that of primary neurons as well as upregulation of neurogenic markers such as nestin, β3 tubulin, and neurofilament light chain after one week in culture, while cells on their stiffest gels (25–40 kPa) took on a more osteoblastic, polygonal morphology with upregulation of numerous osteogenic markers (Fig. 10a).[21] Cells on gels of intermediate stiffness (8–17 kPa) became elongated, with a spindle-factor approaching that of myoblasts and upregulation of myogenic markers (Fig. 10a).[21] Cells on 11 kPa substrates developed ''premyofibrillar'' myosin striations with the same spacing as age-matched C2C12 myocytes, while those on stiffer and softer substrates did not.[21] Rowlands *et al.* found slightly different stiffnesses promoting maximum marker expression, with the highest expression of Runx2 (an osteogenic marker) on 80 kPa gels and highest expression of MyoD1 (a myogenic marker) on 25 kPa substrates, though expression profiles were also found to depend upon the ligand used.[62] Interestingly, marker expression did not appear closely linked to cell spread area,[62] despite previous findings that cell spreading could independently influence MSC differentiation.[48] F-actin and focal adhesion staining followed trends previously seen in other cell types, with diffuse staining on the softest gels and well-developed structures on the stiffest gels and glass.[21] Of note, maximum marker expression in hMSCs is only ~50% of that observed in differentiated cells at optimal substrate rigidity, indicating that substrate rigidity alone is unable to guide full differentiation of these cells (Fig. 10b).[21]

Role of Substrate Stiffness in Cancer

The changes in proliferation and apoptosis seen with changes in substrate rigidity may have profound implications in cancer research. Cancer is primarily a disease of abnormally high cell proliferation, and the changes in tissue mechanics that often accompany malignant transformation may provide an additional growth signal to transformed cells. Alternatively, cell response to substrate rigidity may have implications for oncogenesis in the absence of mechanical changes. Transformed cells may lose sensitivity to substrate mechanics, demonstrating increased proliferation and decreased apoptosis on substrates that would not normally permit such changes. Altered response to substrate mechanics may also affect metastatic potential, as cells with an abnormal rigidity response could be more likely to venture out of their tissue niche.[53]

Wang *et al.*[75] were first to apply cell response to substrate rigidity to the study of the pathogenesis of disease. Comparing normal and H-*ras*-transformed 3T3 fibroblasts, they were able to show a reduced sensitivity to substrate rigidity in transformed cells.[75] Transformed cells on 4.7 kPa gels but not 14 kPa gels had a ~2-fold higher growth rate after 48 h than non-transformed cells.[75] Transformed cells also exhibited a loss of rigidity-dependence in apoptosis, maintaining a 15–20% apoptotic rate on both 14 and 4.7 kPa gels.[75] The differences in proliferation and apoptosis rates in response to substrate rigidity between normal and transformed cells suggest a possible role for substrate rigidity in maintaining appropriate cellular growth *in vivo*.

In addition to changes in proliferation and apoptosis, transformed cells did not show any difference in spread area with increased substrate stiffness above 10 kPa, though a significant difference had been observed in non-transformed cells in this range.[75] This finding was echoed by researchers comparing normal fibroblasts with SaI/N fibrosarcoma cells, who found no change in spread area between SaI/N cells on 500 and 2000 kPa substrates despite significant differences in normal fibroblasts.[73] Percent of SaI/N cells reaching a polarized morphology (defined as a ratio of short to long cell axes $\leq$0.75) increased with substrate rigidity to ~55% of cells on the stiffest, 2000 kPa substrates but stopped

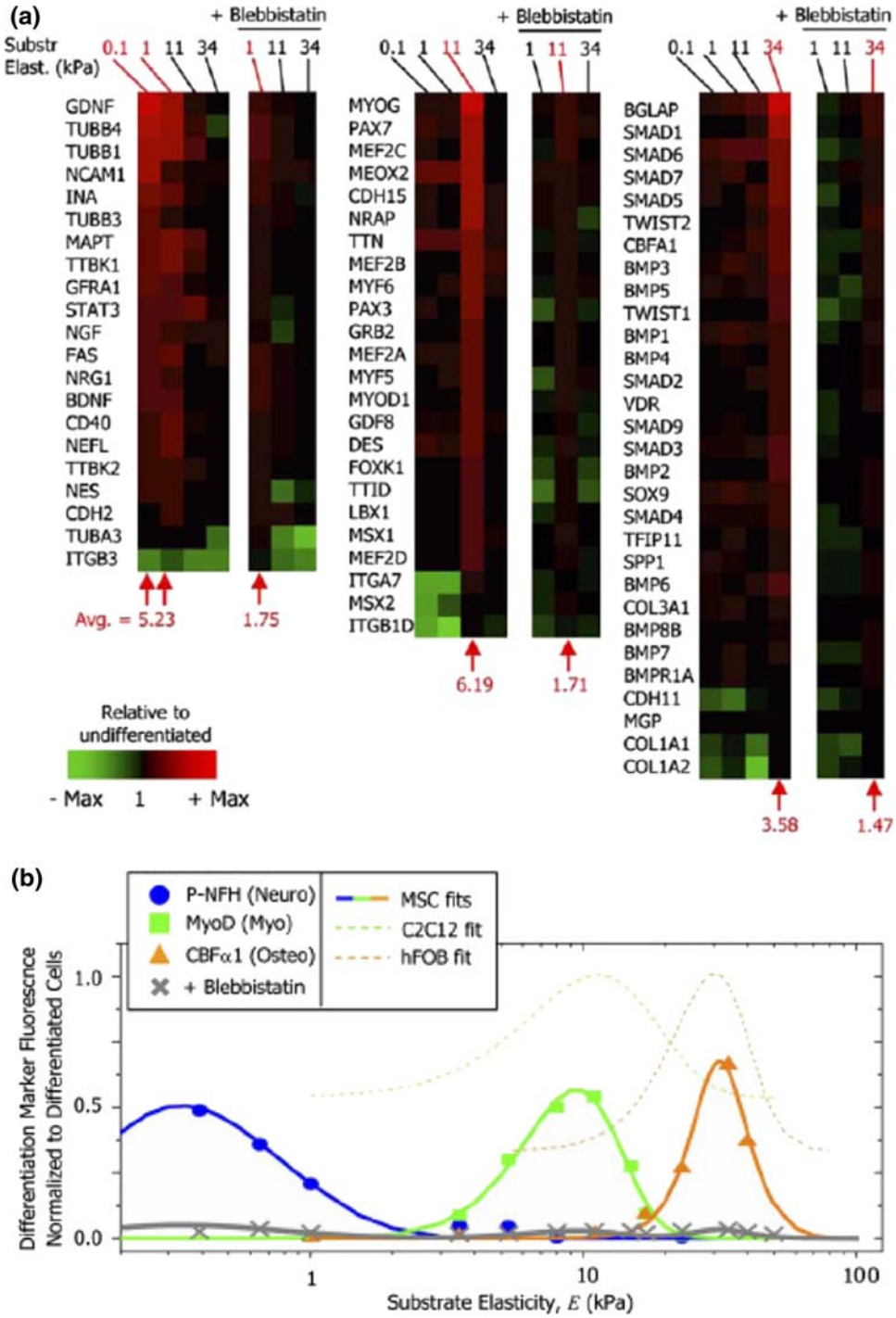

FIGURE 10. (a) Microarray profiles of MSCs grown on polyacrylamide gels of different elasticity, normalized to actin levels and expression in naïve MSCs. Neurogenic markers are shown in left column, myogenic in center, and osteogenic in right. Red numbers below indicate average fold-increase in expression. Blebbistatin blocks this response. (b) Fluorescence analysis of differentiation marker expression in MSCs grown on substrates of different elasticity (*solid lines*), normalized to expression in differentiated cells (*dashed lines*). Again, blebbistatin blocks the response (adapted from Engler *et al.*,[21] reprinted with permission from Elsevier).

well short of the 80–90% polarization seen with 3T3 cells.[73] Time to morphological steady state increased for 3T3 cells as substrate rigidity increased; SaI/N cells were unaffected. Transformed cells also failed to show an increase in traction forces[75] or decreased persistence of motion during migration[73] with increasing stiffness, unlike normal cells.[24,47,73,75] In general, the transformed cells appeared to be relatively insensitive to substrate rigidity when compared with untransformed fibroblasts.[75]

Paszek *et al.*[54] and Kostic *et al.*[40] found similar changes in rigidity response in transformed mammary epithelial cells. Normal mammary epithelial cells showed increased spreading,[40,54] proliferation,[40] FAK^{pY397} phosphorylation[54] and recruitment of vinculin to adhesion sites[54] with increased substrate rigidity. When compared to their untransformed counterparts, transformed cells had greater spreading and generated more force on soft substrates.[54] Both responses could be decreased with Rho or myosin inhibition.[54] Using single cell populations (SCPs) of transformed cells that preferentially metastasized *in vivo* to different organs, Kostic *et al.* investigated whether altered rigidity response correlated with each line's preferred metastatic site.[40] Nonmetastatic SCPs and SCPs metastasizing to the lungs proliferated preferentially on soft (0.6 kPa) matrices, as did SCPs with targeting to both lungs and bone.[40] SCPs that preferentially metastasized to bone had higher proliferation on stiffer (3 kPa) substrates.[40] These results suggest that altered sensitivity to substrate rigidity may be an important step in both oncogenesis and metastatic potential for some cell types.

CELL RESPONSE TO SUBSTRATE RIGIDITY IN THREE DIMENSIONS

Cukierman *et al.*[15] are widely credited with highlighting the differences in cellular behavior, particularly in cell–matrix adhesions, when cells are grown on 2D vs. 3D scaffolds. This work, among others, prompted researchers studying cell response to substrate rigidity to look for ways to study rigidity effects in 3D. The cytotoxic nature of the acrylamide monomer forced those wishing to study response to substrate rigidity with encapsulated cells to find alternative substrates. The study of encapsulated cells comes with its own inherent difficulties, among these the challenge of separating stiffness effects from effects of altered transport that often accompanies changes in substrate rigidity. The studies described in this section have largely ignored mesh size or transport properties when analyzing cell response to substrate rigidity in 3D, but these effects are likely not inconsequential and will need to be controlled in order for future 3D studies to be truly insightful.

Balgude *et al.*[3] used agarose gels of varying stiffness to examine dorsal root ganglion (DRG) neurite extension. The rate of neurite extension decreased with increased gel elastic modulus. Yu and Bellamkonda[79] looked at DRG neurite extension across mechanical barriers in 3D using agarose gels. Cells were seeded in a gel with a shear modulus of 12.8 Pa ($E = 38.3$ Pa, assuming $\upsilon = 0.495$[13]), and analyzed for neurite extension into an adjacent gel layer of equal or higher modulus.[79] There was no significant difference in the percentage of neurites crossing into gels with elastic modulus up to ~210 Pa, but the percentage of neurites crossing into stiffer gels decreased significantly.[79] As this experiment was not repeated with cells initially residing in a layer of different elastic modulus, it is unclear whether the difference in neurite crossing is primarily driven by the magnitude of the modulus mismatch or by the absolute modulus of the ingrowth layer.[79] Balgude *et al.*[3] postulated that the change in neurite growth rate could be explained by the resistive force applied by the matrix to neurite extension—that stiffer gels resisted neurite ingrowth to a greater degree than softer gels.

Boontheekul *et al.*[7] encapsulated myoblasts within alginate gels of tunable rigidity and degradation. Although the changing modulus associated with degradation complicates analysis, they did note decreased proliferation in their stiffest, most slowly degrading gel compared to softer ones.[7] This trend in proliferation was opposite that seen when myoblasts were cultured on top of alginate gels, highlighting the differences between cells cultured in two and three dimensions.[7]

Bryant *et al.*[8–10] and Park *et al.*[52] investigated chondrocyte response to substrate rigidity in 3D PEG-based gels. Like myoblasts,[7] chondrocytes demonstrated decreased cell proliferation with increasing rigidity in 3D.[10,52] Stiffer constructs also had significantly less glycosaminoglycan (GAG) synthesis after 72 h,[10] though the difference was not seen in a similar study at 2 and 4 week timepoints.[8] Collagen production was highest in the softest gels after two and four weeks.[8,52] Aggrecan and matrix metalloproteinase 13 expression were also higher in softer gels after four weeks.[52] After six weeks of culture in degradable gels of varying stiffness, GAG and collagen content was highest in gels of intermediate stiffness.[9] Again, analysis of this data is complicated by the change in modulus over time as the substrates degrade.

Peyton *et al.*[57] used PEG-based gels to study SMCs in 3D. They found increased F-actin assembly with increasing substrate rigidity, as had previously been shown in 2D.[57] Proliferation, however, appeared to be independent of substrate stiffness in this model.[57] When cells were transduced to produce RhoA constitutively, vinculin expression levels began to increase with increasing substrate stiffness.[57] Expression of a-actin and calponin (smooth muscle markers) increased with substrate rigidity only in transduced cells.[57] Transduction of cells also decreased spreading and proliferation, especially on the stiffest gels studied.[57]

Some groups have studied cells sandwiched between gels to mimic 3D culture.[5,54] Paszek *et al.* studied

mammary epithelial cells placed between basement membrane-crosslinked polyacrylamide gels of controlled elasticity and a layer of basement membrane (BM).[54] While this system only controlled rigidity on one side of the cells, it can be assumed that differences in nutrient and waste diffusion through the upper BM layer were minimal between samples. The authors noted development of well-differentiated, growth-arrested acini on the most compliant (0.150 kPa) gels, with loss of tissue polarity and lumen formation and increased cell colony size as rigidity increased to 5 kPa.[54] Stress fibers began to form only on their stiffest, 5 kPa substrates, which they noted as similar in stiffness to breast tumors.[54]

CELL RESPONSE TO SUBSTRATES WITH PATTERNED RIGIDITY

The vast majority of work investigating cell response to substrate rigidity has been performed using uniformly compliant substrates. There are, however, limits to what this type of study can reveal. All of the studies reviewed so far have used substrates of several discrete moduli, chosen over the range of moduli of interest. However, it is entirely possible that the moduli chosen for study do not represent the full range of cellular behavior over that stiffness range. To address this issue, some researchers have investigated cell behavior on substrates with gradient rigidity. These substrates also form a model system for the study of durotaxis (cell migration in response to changes in substrate rigidity). Much as cells show differential behavior in response to chemotactic signals vs. chemokinetic ones, surfaces that promote durotaxis may reveal behavior that could not be captured on uniform-modulus surfaces.

Lo *et al.*[47] created polyacrylamide gels with "soft" and "stiff" regions with elastic moduli of 14 and 30 kPa, respectively, and a 50–100 μm wide transition region. While it is difficult to judge whether this technique created a step or more gradual gradient, as microscale elastic moduli within the transition region were not well characterized, the authors were able to observe differential 3T3 fibroblast behavior as the cells approached the transition region from either side.[47] Cells crossing the transition region from the softer side of the gel exhibited a transient increase in migration rate (0.44–0.54 μm min^{-1}), whereas cells approaching the transition region from the stiffer side of the gel were observed to turn away from the rigidity boundary and reorient themselves to avoid crossing onto the softer substrate (Fig. 11).[47] Migration rates at locations distant to the transition regions followed the same trend noted previously,[55] with an average rate of 0.44 μm min^{-1} on the softer substrate and 0.26 μm min^{-1} on the stiffer side.[47]

Differential cell behavior near the transition region was only observed when cells were seeded at very low density with no other cells nearby.[47] At higher cell densities, cells were observed moving freely across the transition region.[47] The authors postulated that cell–cell and cell–substrate interactions at high cell densities provided sufficient mechanical stimulation to override signals given by substrate stiffness.[47] This theory was supported by their demonstration that micromanipulation of the substrate could be used to guide cell migration by altering local tension.[47] Cells were shown to migrate toward areas of high tension (such as those created by pulling on the substrate with a microneedle) and away from areas of low tension (such as those created by pushing the substrate toward the cell), even when this required a change in the direction of migration.[47]

Wong *et al.* studied the response of vascular SMCs to substrate rigidity using gradient compliant polyacrylamide gels.[76,80] Cell morphology varied with compliance; cells assumed a well-spread morphology on the stiffer end of the substrate but remained rounded on softer regions, and F-actin appeared well-organized only in stiffer regions.[80] BALB/c 3T3 fibroblasts yielded similar results.[80] Interestingly, the authors noted a "threshold" value for vascular SMC spreading located at a modulus of about 30 kPa; below this value cells exhibited limited spreading.[80] Of note, this threshold was not seen by groups using substrates of uniform rigidity within this modulus range. Cells consistently moved toward stiffer regions of the gels, in contrast to the random cell movement observed on uniformly compliant gels.[76] Cell location 18 h after seeding revealed increased density at the stiffer end of the gel[80]; however, it is not clear whether this is primarily due to a difference in initial cell adhesion between the regions, differential proliferation, or a net migration of cells from softer areas to stiffer ones. Based on results seen in other studies, it is reasonable to believe that a combination of these effects might occur.

Other groups have used substrates with micropatterned rigidity to examine effects of anisotropy and spatial differences in substrate modulus. Gray *et al.*[26] created both acrylamide and poly(dimethylsiloxane) (PDMS) substrates with micron-scale stiff and flexible regions using modified soft lithographic techniques. Fibroblasts on patterned acrylamide substrates attached equivalently to both 1.8 and 34 kPa areas 3 h after seeding but had greater spreading on stiffer regions.[26] After 24 h, cells were found to be preferentially located on or near stiffer regions; this difference was enhanced after 48 h.[26]

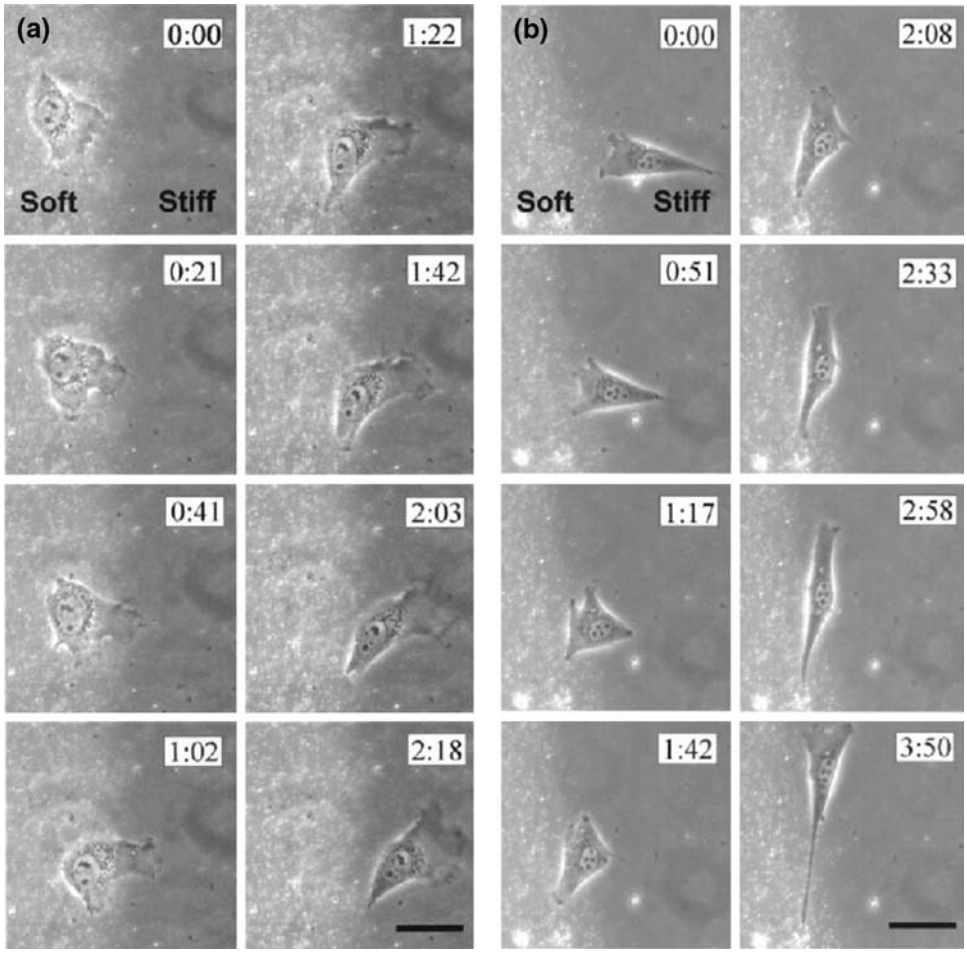

FIGURE 11. Fibroblasts approaching transition region from soft side of gel cross the transition freely (a) while those approaching from the stiff side change direction to avoid crossing onto the softer substrate (b). Transition in rigidity is visualized using fluorescent beads. Scale bars 40 microns (reprinted from Lo *et al.*[47] with permission from Elsevier).

On patterned PDMS substrates, cells were found to attach and spread on both 12 kPa and 2.5 MPa regions of the surface, though there were slightly more cells located on stiff regions than soft 3 h after seeding.[26] Again, cell density on stiff vs. soft regions was increased after 24 h, and more so after 48 h.[26] Interestingly, cell attachment on patterned PDMS substrates was decreased in softer areas close to stiff regions when compared to more distant soft areas, perhaps indicating that the ability of cells to detect an adjacent stiffer region was attenuated with distance.[26] The higher cell density noted adjacent to stiff regions of acrylamide gels when compared with distant soft regions was hypothesized to arise in response to a gradient of elastic moduli caused by diffusion of crosslinker out of stiff regions during fabrication.[26] Inhibition of cell division with mitomycin C prior to seeding slowed or delayed cell accumulation, but accumulation of cells on stiff areas was clear after 5 days.[26] The delay in cell accumulation on these substrates was hypothesized to be due to mitomycin C-induced toxicity.[26] Thus, while both differences in proliferation and migration might contribute to the differential cell accumulation observed, it seems likely that migration played the larger role.[26] Of note, some PDMS samples failed to produce a difference in cell accumulation between regions; these samples were considered to be manufacturing failures and were excluded from further analysis.[26]

Fibronectin concentration on stiff and compliant areas, while relatively even prior to cell culture, began to show evidence of cellular remodeling after 48 h in culture.[26] Specifically, staining on softer regions appeared increased near cells and decreased elsewhere, whereas staining on stiffer regions was increased only at the location of the cells.[26] The authors suggested that the ECM changes could have been either a cause or result of cellular migration.[26] Differences in cell traction forces between different regions of the substrate might have differentially remodeled ECM,

causing effective ECM concentration differences between soft and stiff regions which may have in turn affected cell migration.[26] Alternatively, differences in motility between soft and stiff areas might have affected the degree of remodeling seen.[26] There may also have been minor differences in surface chemistry between stiff and soft regions, which might have affected fibronectin conformation or protein adsorption onto the surfaces.[26]

Saez *et al.*[63] created anisotropic surfaces for cell culture using arrays of micropillars with oval cross-sections such that the pillars' stiffness in bending was greater in the direction of the long axis of the oval surface than that of the short axis. The surfaces were then seeded with Madin-Darby canine kidney (MDCK) epithelial cells, and resulting cell islands were analyzed for alignment with pillar axes.[63] Forty-five percent of the cell islands were found to align within 15° of the pillar major axes; this alignment did not change with island size (Fig. 12).[63] Preferential alignment along that axis was also observed in individual cells within the islands.[63] Of note, there was no preferential direction of mitosis in cells on the micropatterned substrates, indicating that island alignment could not be attributed to differential cell mitosis along that axis.[63] Control surfaces exhibited no preferential island alignment.[63] Alignment of actin stress fibers and focal adhesions in cells on the micropatterned substrates was also observed along the axis of highest rigidity, with no preferential alignment on control surfaces.[63]

The study was repeated using pillars of different heights to determine whether the differential stiffness between the axes or the absolute stiffness of the substrate was more important in impacting cellular behavior.[63] No significant differences in behavior were seen between the substrates, suggesting that differential stiffness between the axes is the predominant factor impacting cell alignment.[63] Using pillar deflection to measure traction forces within cell islands, the researchers found significantly higher forces in the direction of greater stiffness than in the perpendicular direction,[63] which is consistent with results of force mapping on non-patterned substrates.[24,28,36,75] Traction forces were also greater in cells on the edges of cell islands than those within the islands, which corresponded with a greater degree of elongation of edge cells.[63]

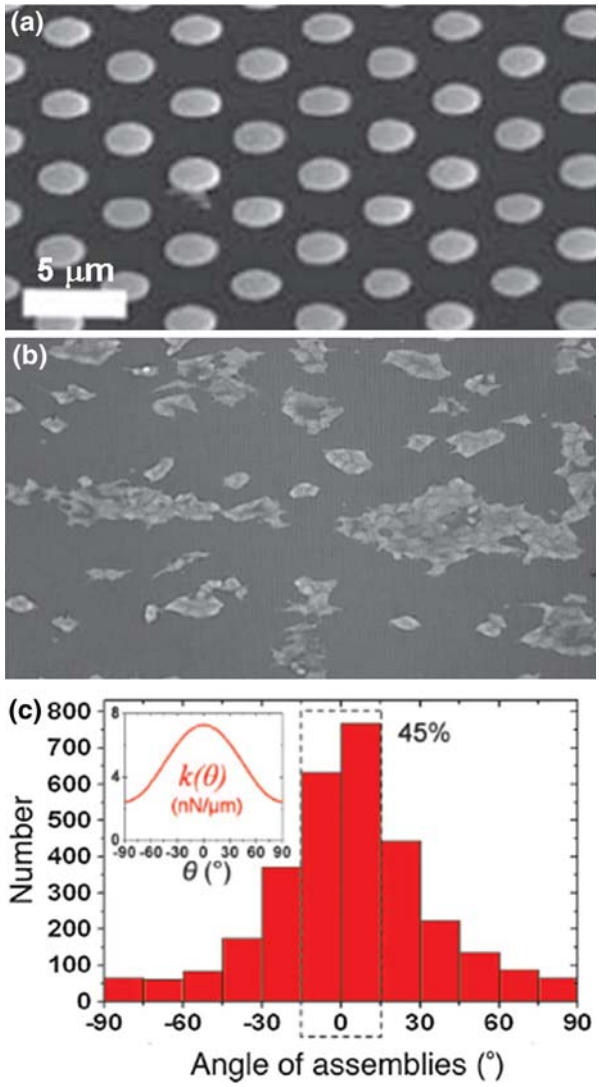

FIGURE 12. Epithelial cell growth on micropillared substrates with anisotropic rigidity. (a) Scanning electron micrograph of an array of oval PDMS pillars. (b) MDCK cell islands grown on these substrates and visualized by optical microscopy. (Image dimensions: 877 × 512 μm.) (c) Angular distribution of cell assemblies with respect to the stiffest direction (θ = 0°, direction of long axis of oval micropillars). The *dashed rectangle* indicates that 45% of the islands are elongated in a 30°-wide sector centered on θ = 0°. (*Inset*) Profile plot of the stiffness $k(\theta)$ for this experiment (adapted from Saez *et al.*,[63] copyright 2007 National Academy of Sciences).

CONCLUSIONS AND FUTURE DIRECTIONS

In reviewing the range of responses to substrate rigidity within and between cell types, certain trends become apparent. Most cell types studied to date exhibit increased spreading as substrate rigidity increases (observed in oligodendrocytes,[72] myoblasts,[20] MSCs,[62] neutrophils,[50,71] endothelial cells,[12,16,78] vascular smooth muscle cells,[17,18,57,58] and fibroblasts[14,28,36,47,54,55,69,75,78]). For many cell types, increased substrate rigidity also leads to increased stress fiber organization (astrocytes,[23] ventricular myocytes,[19,34] and fibroblasts[14,24,54,55,69,78]) and focal adhesion formation (MSCs,[21] vascular smooth muscle cells,[59] osteoblasts,[38,39] and fibroblasts[28,54]), with

increased adhesion strength observed in astrocytes,[23] myoblasts,[20] and fibroblasts.[28] Increased proliferation with rise in substrate rigidity is common across many cell types (neurons,[35] osteoblasts,[30,38,39] vascular smooth muscle cells,[59] fibroblasts,[49,75] MSCs,[30,62] and NSCs in astrocytic media[64]), and decreased apoptosis has also been noted in some (osteoblasts,[39] fibroblasts[75]).

Perhaps more interestingly, some cell types have exhibited a peak for a particular measure at an intermediate stiffness, rather than increasing or decreasing uniformly over the range of stiffnesses studied. As discussed earlier, some groups have found a maximum in both fibroblast spreading and cell–substrate modulus matching on rigidities near 10 kPa, above which cells began to produce stress fibers.[69,78] The ~10 kPa modulus appears to be important for other cell types as well. Ventricular myocytes and C2C12 myoblasts both demonstrated maximum striation around 10 kPa, which was noted in both cases to be near the stiffness of native cells or tissue.[19,34] Ventricular myocytes also showed highest axial force generation, calcium transients, and sarcoplasmic/endoplasmic reticular calcium ATPase expression on 10 kPa substrates.[34] Myoblast proliferation[6] and striation[6,20] were highest on substrates of intermediate stiffness, and MSCs showed peak expression of myogenic markers on substrates in this range as well.[21,62]

Cell responses to substrate rigidity are also dependent upon the identity[6,59,62] and concentration[38,67] of adhesive ligand used. Boonen *et al.*,[6] using Matrigel, ECL gel, collagen IV, poly-D-lysine, and laminin, found changes in myotube formation, striation, and contraction between different ligands. Rowlands *et al.*[62] observed subtle differences in the substrate stiffnesses that maximized MSC attachment, spreading, morphology, and expression of markers of differentiation between cells on gels coated with fibronectin, laminin 1, collagen I, or collagen IV. Peyton *et al.*[59] noted a significant increase in smooth muscle cell focal adhesion size on their stiffest substrate when coated with fibronectin but not collagen. Clearly the choice of adhesive ligand used in a given study may affect the outcomes seen, and this may go a long way in explaining differences in findings between groups using different ligands.

The concentration of ligand used can also affect the specific results seen. Khatiwala *et al.*[38] observed monotonically increasing pre-osteoblast migration speed with increasing rigidity on low collagen densities, while at higher collagen densities migration reached a maximum on 21.5 kPa substrates, then decreased as rigidity increased further. Higher collagen densities also corresponded to increased cytoskeletal organization and focal adhesion formation, especially on softer substrates.[38] Semler *et al.*[67] saw an increase in cell area and proliferation and a decrease in albumin secretion and cytochrome p450 expression with increases in either ligand density or substrate stiffness. These shifts in cellular behavior with ligand density highlight the importance of carefully controlling substrate biochemistry, especially when comparing between distinct surfaces. They also highlight the difficulty in comparing results among groups using different concentrations of ligands.

While knowledge of cells' ability to sense and respond to the mechanical properties of their substrates is important to our understanding of basic cellular biology, the potential diagnostic and therapeutic value of that knowledge lends the subject particular importance. Improved understanding of how cells respond to changes in substrate stiffness may affect the study or treatment of diseases such as cancer or liver fibrosis, in which tissue mechanics are altered.[44] It is possible that measurements of tissue elasticity may aid in diagnosis or inform prognosis. Further studies of cell responses to substrate rigidity in health and disease may even reveal potential targets for therapeutics.

One of the major challenges in the study of cell response to substrate rigidity is the lack of suitable substrates for studies in 3D. While PEG-based hydrogels overcome many of the limitations imposed by other substrate systems, such as the confounding effects of ligand density in biological gels or the cytotoxicity of acrylamide, uncoupling effects of substrate rigidity and nutrient diffusion remains a challenge. A material that would allow investigation of cell response to substrate mechanics independently of both biochemical and transport properties would prove a major step forward in the field. One major advantage of polyacrylamide-based systems is the combination of excellent optical transparency and elasticity, which allow the use of traction-force microscopy to quantify cells' ability to distort their substrate. An ideal material for 3D studies would maintain these properties as well.

In the field of tissue engineering, substrate elasticity could be utilized to promote desired cellular behavior. The elasticity could be even temporally controlled, starting out at a modulus likely to promote cell proliferation and then softening (perhaps in response to cellular enzymes) to promote a more quiescent phenotype. The ability of matrix stiffness to direct stem cell differentiation is also very exciting, as it provides an additional variable that may be used to engineer three dimensional tissue constructs. Few tissues in the body are composed of a single cell type, and the controlled patterning and incorporation of multiple cell types into a single construct is a major challenge in the engineering of complex tissues. Utilizing patterned substrate stiffness along with traditional tissue

engineering techniques, it may be possible to engineer functional replacements for diseased or injured tissues.

It is clear that we have only begun to understand how cells sense and respond to substrate mechanical properties. While some cell types have been well-studied over a wide range of rigidities, the field has almost limitless potential for growth. As this research progresses, it is likely to profoundly affect the way cell-based research is conducted.

APPENDIX

Conversion of shear modulus to elastic modulus:

$$E = 2(1 + \upsilon)G,$$

where $G =$ shear modulus, $E =$ elastic or Young's modulus, and $\upsilon =$ Poisson's ratio (material-dependent).

REFERENCES

[1]Abe, H., K. Hayashi, and M. Sato. Data book on mechanical properties of living cells, tissues, and organs. Tokyo: Springer-Verlag, p. 436, 1996.

[2]An, K. N., Y. L. Sun, and Z. P. Luo. Flexibility of type I collagen and mechanical property of connective tissue. *Biorheology* 41:239–246, 2004.

[3]Balgude, A. P., X. Yu, A. Szymanski, and R. V. Bellamkonda. Agarose gel stiffness determines rate of DRG neurite extension in 3D cultures. *Biomaterials* 22: 1077–1084, 2001.

[4]Ben-Ze'ev, A., G. S. Robinson, N. L. Bucher, and S. R. Farmer. Cell–cell and cell–matrix interactions differentially regulate the expression of hepatic and cytoskeletal genes in primary cultures of rat hepatocytes. *Proc. Natl Acad. Sci. USA* 85:2161–2165, 1988.

[5]Beningo, K. A., M. Dembo, and Y. L. Wang. Responses of fibroblasts to anchorage of dorsal extracellular matrix receptors. *Proc. Natl Acad. Sci. USA* 101:18024–18029, 2004.

[6]Boonen, K. J., K. Y. Rosaria-Chak, F. P. Baaijens, D. W. van der Schaft, and M. J. Post. Essential environmental cues from the satellite cell niche: optimizing proliferation and differentiation. *Am. J. Physiol. Cell. Physiol.* 296:C1338–C1345, 2009.

[7]Boontheekul, T., E. E. Hill, H. J. Kong, and D. J. Mooney. Regulating myoblast phenotype through controlled gel stiffness and degradation. *Tissue Eng*. 13:1431–1442, 2007.

[8]Bryant, S. J., and K. S. Anseth. Hydrogel properties influence ECM production by chondrocytes photoencapsulated in poly(ethylene glycol) hydrogels. *J. Biomed. Mater. Res*. 59:63–72, 2002.

[9]Bryant, S. J., R. J. Bender, K. L. Durand, and K. S. Anseth. Encapsulating chondrocytes in degrading PEG hydrogels with high modulus: engineering gel structural changes to facilitate cartilaginous tissue production. *Biotechnol. Bioeng*. 86:747–755, 2004.

[10]Bryant, S. J., T. T. Chowdhury, D. A. Lee, D. L. Bader, and K. S. Anseth. Crosslinking density influences chondrocyte metabolism in dynamically loaded photocrosslinked poly(ethylene glycol) hydrogels. *Ann. Biomed. Eng*. 32:407–417, 2004.

[11]Burton, K., and D. L. Taylor. Traction forces of cytokinesis measured with optically modified elastic substrata. *Nature* 385:450–454, 1997.

[12]Califano, J. P., and C. A. Reinhart-King. A balance of substrate mechanics and matrix chemistry regulates endothelial cell network assembly. *Cell. Mol. Bioeng*. 1:122–132, 2008.

[13]Chen, Q., S. I. Ringleb, A. Manduca, R. L. Ehman, and K. N. An. A finite element model for analyzing shear wave propagation observed in magnetic resonance elastography. *J. Biomech*. 38:2198–2203, 2005.

[14]Collin, O., P. Tracqui, A. Stephanou, Y. Usson, J. Clement-Lacroix, and E. Planus. Spatiotemporal dynamics of actin-rich adhesion microdomains: influence of substrate flexibility. *J. Cell Sci*. 119:1914–1925, 2006.

[15]Cukierman, E., R. Pankov, D. R. Stevens, and K. M. Yamada. Taking cell–matrix adhesions to the third dimension. *Science* 294:1708–1712, 2001.

[16]Deroanne, C. F., C. M. Lapiere, and B. V. Nusgens. In vitro tubulogenesis of endothelial cells by relaxation of the coupling extracellular matrix-cytoskeleton. *Cardiovasc. Res*. 49:647–658, 2001.

[17]Engler, A., L. Bacakova, C. Newman, A. Hategan, M. Griffin, and D. Discher. Substrate compliance versus ligand density in cell on gel responses. *Biophys. J*. 86:617–628, 2004.

[18]Engler, A., L. Richert, J. Y. Wong, C. Picart, and D. Discher. Surface probe measurements of the elasticity of sectioned tissue, thin gels and polyelectrolyte multilayer films: correlations between substrate stiffness and cell adhesion. *Surf. Sci*. 570:142–154, 2004.

[19]Engler, A. J., C. Carag-Krieger, C. P. Johnson, M. Raab, H. Y. Tang, D. W. Speicher, J. W. Sanger, J. M. Sanger, and D. E. Discher. Embryonic cardiomyocytes beat best on a matrix with heart-like elasticity: scar-like rigidity inhibits beating. *J. Cell Sci*. 121:3794–3802, 2008.

[20]Engler, A. J., M. A. Griffin, S. Sen, C. G. Bonnemann, H. L. Sweeney, and D. E. Discher. Myotubes differentiate optimally on substrates with tissue-like stiffness: pathological implications for soft or stiff microenvironments. *J. Cell Biol*. 166:877–887, 2004.

[21]Engler, A. J., S. Sen, H. L. Sweeney, and D. E. Discher. Matrix elasticity directs stem cell lineage specification. *Cell* 126:677–689, 2006.

[22]Flanagan, L. A., Y. E. Ju, B. Marg, M. Osterfield, and P. A. Janmey. Neurite branching on deformable substrates. *Neuroreport* 13:2411–2415, 2002.

[23]Georges, P. C., W. J. Miller, D. F. Meaney, E. S. Sawyer, and P. A. Janmey. Matrices with compliance comparable to that of brain tissue select neuronal over glial growth in mixed cortical cultures. *Biophys. J*. 90:3012–3018, 2006.

[24]Ghosh, K., Z. Pan, E. Guan, S. Ge, Y. Liu, T. Nakamura, X. D. Ren, M. Rafailovich, and R. A. Clark. Cell adaptation to a physiologically relevant ECM mimic with different viscoelastic properties. *Biomaterials* 28:671–679, 2007.

[25]Gomez, M. A. and A. M. Nahum. Biomechanics of bone. In: Accidental Injury: Biomechanics and Prevention, edited by A. M. Nahum and J. W. Melvin. New York: Springer-Verlag, 2002, pp. 206–227.

[26]Gray, D. S., J. Tien, and C. S. Chen. Repositioning of cells by mechanotaxis on surfaces with micropatterned Young's modulus. *J. Biomed. Mater. Res. A* 66:605–614, 2003.

[27]Gunn, J. W., S. D. Turner, and B. K. Mann. Adhesive and mechanical properties of hydrogels influence neurite extension. *J. Biomed. Mater. Res. A* 72:91–97, 2005.
[28]Guo, W. H., M. T. Frey, N. A. Burnham, and Y. L. Wang. Substrate rigidity regulates the formation and maintenance of tissues. *Biophys. J.* 90:2213–2220, 2006.
[29]Haut, R. C. Biomechanics of soft tissue. In: Accidental Injury: Biomechanics and Prevention, edited by A. M. Nahum and J. W. Melvin. New York: Springer-Verlag, 2002, pp. 228–253.
[30]Hsiong, S. X., P. Carampin, H. J. Kong, K. Y. Lee, and D. J. Mooney. Differentiation stage alters matrix control of stem cells. *J. Biomed. Mater. Res. A* 85:145–156, 2008.
[31]Ingber, D. E. Tensegrity II. How structural networks influence cellular information processing networks. *J. Cell Sci.* 116:1397–1408, 2003.
[32]Ingber, D. E. Mechanical control of tissue morphogenesis during embryological development. *Int. J. Dev. Biol.* 50:255–266, 2006.
[33]Ingber, D. E., and J. Folkman. Mechanochemical switching between growth and differentiation during fibroblast growth factor-stimulated angiogenesis in vitro: role of extracellular matrix. *J. Cell Biol.* 109:317–330, 1989.
[34]Jacot, J. G., A. D. McCulloch, and J. H. Omens. Substrate stiffness affects the functional maturation of neonatal rat ventricular myocytes. *Biophys. J.* 95:3479–3487, 2008.
[35]Jiang, F. X., B. Yurke, B. L. Firestein, and N. A. Langrana. Neurite outgrowth on a DNA crosslinked hydrogel with tunable stiffnesses. *Ann. Biomed. Eng.* 36:1565–1579, 2008.
[36]Jiang, G., A. H. Huang, Y. Cai, M. Tanase, and M. P. Sheetz. Rigidity sensing at the leading edge through alphavbeta3 integrins and RPTPalpha. *Biophys. J.* 90:1804–1809, 2006.
[37]Khatiwala, C. B., P. D. Kim, S. R. Peyton, and A. J. Putnam. ECM compliance regulates osteogenesis by influencing MAPK signaling downstream of RhoA and ROCK. *J. Bone Miner. Res.* 24:886–898, 2009.
[38]Khatiwala, C. B., S. R. Peyton, and A. J. Putnam. Intrinsic mechanical properties of the extracellular matrix affect the behavior of pre-osteoblastic MC3T3-E1 cells. *Am. J. Physiol. Cell. Physiol.* 290:C1640–C1650, 2006.
[39]Kong, H. J., T. R. Polte, E. Alsberg, and D. J. Mooney. FRET measurements of cell-traction forces and nano-scale clustering of adhesion ligands varied by substrate stiffness. *Proc. Natl Acad. Sci. USA* 102:4300–4305, 2005.
[40]Kostic, A., C. D. Lynch, and M. P. Sheetz. Differential matrix rigidity response in breast cancer cell lines correlates with the tissue tropism. *PLoS One* 4:e6361, 2009.
[41]Leach, J. B., X. Q. Brown, J. G. Jacot, P. A. Dimilla, and J. Y. Wong. Neurite outgrowth and branching of PC12 cells on very soft substrates sharply decreases below a threshold of substrate rigidity. *J. Neural Eng.* 4:26–34, 2007.
[42]Lee, E. Y., G. Parry, and M. J. Bissell. Modulation of secreted proteins of mouse mammary epithelial cells by the collagenous substrata. *J. Cell Biol.* 98:146–155, 1984.
[43]Li, M. L., J. Aggeler, D. A. Farson, C. Hatier, J. Hassell, and M. J. Bissell. Influence of a reconstituted basement membrane and its components on casein gene expression and secretion in mouse mammary epithelial cells. *Proc. Natl Acad. Sci. USA* 84:136–140, 1987.
[44]Li, Z., J. A. Dranoff, E. P. Chan, M. Uemura, J. Sevigny, and R. G. Wells. Transforming growth factor-beta and substrate stiffness regulate portal fibroblast activation in culture. *Hepatology* 46:1246–1256, 2007.
[45]Lin, Y. C., and F. Grinnell. Decreased level of PDGF-stimulated receptor autophosphorylation by fibroblasts in mechanically relaxed collagen matrices. *J. Cell Biol.* 122:663–672, 1993.
[46]Lindblad, W. J., E. G. Schuetz, K. S. Redford, and P. S. Guzelian. Hepatocellular phenotype in vitro is influenced by biophysical features of the collagenous substratum. *Hepatology* 13:282–288, 1991.
[47]Lo, C. M., H. B. Wang, M. Dembo, and Y. L. Wang. Cell movement is guided by the rigidity of the substrate. *Biophys. J.* 79:144–152, 2000.
[48]McBeath, R., D. M. Pirone, C. M. Nelson, K. Bhadriraju, and C. S. Chen. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. *Dev. Cell* 6:483–495, 2004.
[49]Mih, J. D., and D. J. Tschumperlin. Lung fibroblast behavior is tuned by substrate stiffness. *Proc. Am. Thorac. Soc.* 5:364–365, 2008.
[50]Oakes, P. W., D. C. Patel, N. A. Morin, D. P. Zitterbart, B. Fabry, J. S. Reichner, and J. X. Tang. Neutrophil morphology and migration are affected by substrate elasticity. *Blood* 114:1387–1395, 2009.
[51]Oster, G. F., J. D. Murray, and A. K. Harris. Mechanical aspects of mesenchymal morphogenesis. *J. Embryol. Exp. Morphol.* 78:83–125, 1983.
[52]Park, Y., M. P. Lutolf, J. A. Hubbell, E. B. Hunziker, and M. Wong. Bovine primary chondrocyte culture in synthetic matrix metalloproteinase-sensitive poly(ethylene glycol)-based hydrogels as a scaffold for cartilage repair. *Tissue Eng.* 10:515–522, 2004.
[53]Paszek, M. J., and V. M. Weaver. The tension mounts: mechanics meets morphogenesis and malignancy. *J. Mammary Gland Biol. Neoplasia* 9:325–342, 2004.
[54]Paszek, M. J., N. Zahir, K. R. Johnson, J. N. Lakins, G. I. Rozenberg, A. Gefen, C. A. Reinhart-King, S. S. Margulies, M. Dembo, D. Boettiger, D. A. Hammer, and V. M. Weaver. Tensional homeostasis and the malignant phenotype. *Cancer Cell* 8:241–254, 2005.
[55]Pelham, Jr., R. J., and Y. Wang. Cell locomotion and focal adhesions are regulated by substrate flexibility. *Proc. Natl Acad. Sci. USA* 94:13661–13665, 1997.
[56]Pellegrin, S., and H. Mellor. Actin stress fibres. *J. Cell Sci.* 120:3491–3499, 2007.
[57]Peyton, S. R., P. D. Kim, C. M. Ghajar, D. Seliktar, and A. J. Putnam. The effects of matrix stiffness and RhoA on the phenotypic plasticity of smooth muscle cells in a 3-D biosynthetic hydrogel system. *Biomaterials* 29:2597–2607, 2008.
[58]Peyton, S. R., and A. J. Putnam. Extracellular matrix rigidity governs smooth muscle cell motility in a biphasic fashion. *J. Cell. Physiol.* 204:198–209, 2005.
[59]Peyton, S. R., C. B. Raub, V. P. Keschrumrus, and A. J. Putnam. The use of poly(ethylene glycol) hydrogels to investigate the impact of ECM chemistry and mechanics on smooth muscle cells. *Biomaterials* 27:4881–4893, 2006.
[60]Polte, T. R., G. S. Eichler, N. Wang, and D. E. Ingber. Extracellular matrix controls myosin light chain phosphorylation and cell contractility through modulation of cell shape and cytoskeletal prestress. *Am. J. Physiol. Cell. Physiol.* 286:C518–C528, 2004.
[61]Reinhart-King, C. A., M. Dembo, and D. A. Hammer. Cell–cell mechanical communication through compliant substrates. *Biophys. J.* 95:6044–6051, 2008.
[62]Rowlands, A. S., P. A. George, and J. J. Cooper-White. Directing osteogenic and myogenic differentiation of

MSCs: interplay of stiffness and adhesive ligand presentation. *Am. J. Physiol. Cell. Physiol.* 295:C1037–C1044, 2008.
[63]Saez, A., M. Ghibaudo, A. Buguin, P. Silberzan, and B. Ladoux. Rigidity-driven growth and migration of epithelial cells on microstructured anisotropic substrates. *Proc. Natl Acad. Sci. USA* 104:8281–8286, 2007.
[64]Saha, K., A. Keung, E. Irwin, Y. Li, L. Little, D. Schaffer, and K. E. Healy. Substrate modulus directs neural stem cell behavior. *Biophys. J.* 95:4426–4438, 2008.
[65]Samani, A., J. Zubovits, and D. Plewes. Elastic moduli of normal and pathological human breast tissues: an inversion-technique-based investigation of 169 samples. *Phys. Med. Biol.* 52:1565–1576, 2007.
[66]Schlaepfer, D. D., S. K. Mitra, and D. Ilic. Control of motile and invasive cell phenotypes by focal adhesion kinase. *Biochim. Biophys. Acta* 1692:77–102, 2004.
[67]Semler, E. J., P. A. Lancin, A. Dasgupta, and P. V. Moghe. Engineering hepatocellular morphogenesis and function via ligand-presenting hydrogels with graded mechanical compliance. *Biotechnol. Bioeng.* 89:296–307, 2005.
[68]Silver, F. H., Y. P. Kato, M. Ohno, and A. J. Wasserman. Analysis of mammalian connective tissue: relationship between hierarchical structures and mechanical properties. *J. Long Term Eff. Med. Implants* 2:165–198, 1992.
[69]Solon, J., I. Levental, K. Sengupta, P. C. Georges, and P. A. Janmey. Fibroblast adaptation and stiffness matching to soft elastic substrates. *Biophys. J.* 93:4453–4461, 2007.
[70]Stichel, C. C., and H. W. Muller. The CNS lesion scar: new vistas on an old regeneration barrier. *Cell Tissue Res.* 294:1–9, 1998.
[71]Stroka, K. M., and H. Aranda-Espinoza. Neutrophils display biphasic relationship between migration and substrate stiffness. *Cell Motil. Cytoskeleton* 66:328–341, 2009.
[72]Teixeira, A. I., S. Ilkhanizadeh, J. A. Wigenius, J. K. Duckworth, O. Inganas, and O. Hermanson. The promotion of neuronal maturation on soft substrates. *Biomaterials* 30:4567–4572, 2009.
[73]Tzvetkova-Chevolleau, T., A. Stephanou, D. Fuard, J. Ohayon, P. Schiavone, and P. Tracqui. The motility of normal and cancer cells in response to the combined influence of the substrate rigidity and anisotropic microstructure. *Biomaterials* 29:1541–1551, 2008.
[74]Wang, H. B., M. Dembo, S. K. Hanks, and Y. Wang. Focal adhesion kinase is involved in mechanosensing during fibroblast migration. *Proc. Natl Acad. Sci. USA* 98:11295–11300, 2001.
[75]Wang, H. B., M. Dembo, and Y. L. Wang. Substrate flexibility regulates growth and apoptosis of normal but not transformed cells. *Am. J. Physiol. Cell. Physiol.* 279:C1345–C1350, 2000.
[76]Wong, J. Y., A. Velasco, P. Rajagopalan, and Q. Pham. Directed movement of vascular smooth muscle cells on gradient-compliant hydrogels. *Langmuir* 19:1908–1913, 2003.
[77]Yeh, W. C., P. C. Li, Y. M. Jeng, H. C. Hsu, P. L. Kuo, M. L. Li, P. M. Yang, and P. H. Lee. Elastic modulus measurements of human liver and correlation with pathology. *Ultrasound Med. Biol.* 28:467–474, 2002.
[78]Yeung, T., P. C. Georges, L. A. Flanagan, B. Marg, M. Ortiz, M. Funaki, N. Zahir, W. Ming, V. Weaver, and P. A. Janmey. Effects of substrate stiffness on cell morphology, cytoskeletal structure, and adhesion. *Cell Motil. Cytoskeleton* 60:24–34, 2005.
[79]Yu, X., and R. V. Bellamkonda. Dorsal root ganglia neurite extension is inhibited by mechanical and chondroitin sulfate-rich interfaces. *J. Neurosci. Res.* 66:303–310, 2001.
[80]Zaari, N., P. Rajagopalan, S. K. Kim, A. J. Engler, and J. Y. Wong. Photopolymerization in microfluidic gradient generators: microscale control of substrate compliance to manipulate cell response. *Adv. Mater.* 16:2133–2137, 2004.