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Mechanotransduction in cancer

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Abstract

Tissue stiffness is tightly controlled under normal conditions, but changes with disease. In cancer, tumors often tend to be stiffer than the surrounding uninvolved tissue, yet the cells themselves soften. Within the past decade, and particularly in the last few years, there is increasing evidence that the stiffness of the extracellular matrix modulates cancer and stromal cell mechanics and function, influencing such disease hallmarks as angiogenesis, migration, and metastasis. This review briefly summarizes recent studies that investigate how cancer cells and fibrosis-relevant stromal cells respond to ECM stiffness, the possible sensing appendages and signaling mechanisms involved, and the emergence of novel substrates — including substrates with scar-like fractal heterogeneity — that mimic the *in vivo* mechanical environment of the cancer cell.

Introduction

The fact that tumors are often stiffer than the surrounding uninvolved tissue has been known for as long as the disease has been identified. The rigid nature of tumors is the basis for using palpation as a diagnostic method in soft tissues like breast and abdomen, and more recently, as the basis for high-resolution detection of small lesions by MRI elastography [1•, 2•,3•] or ultrasound [4•]. These clinical observations, together with *in vitro* experiments which demonstrate that stiffness-sensing by cancer and stromal cells influence cell survival and proliferation, opened the door for many investigations that employ novel biocompatible materials with tunable viscoelastic properties. These *in vitro* systems have the potential to elucidate the mechanical and molecular mechanisms by which cells detect changes in their environment and transduce physical signals to the biochemical signals that control their function, biochemistry, and gene expression.

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Mechanotransduction of physical cues to initiate intracellular signaling pathways has recently been documented in many cancer types and a wide range of effects have been observed, ranging from acute changes such as activation of ion channels or protein kinases to long-term changes in cell phenotype that require initiation of gene transcription and protein production. This review summarizes some recent studies that use materials of tunable rigidity to identify the mechanosensing ability of cancer cells, how substrate stiffness affects some of the cancer hallmarks, and the possible mechanisms involved. New materials that mimic the viscoelasticity of normal and cancerous tissues are also highlighted.

Effects of mechanics on proliferation and apoptosis

Several studies demonstrate that mechanotransduction by cancer cells might be significantly blunted compared to normal cells, but others show the opposite. An important pioneering study by Wang *et al.* demonstrates that whereas normal NIH 3T3 fibroblasts are highly dependent on a rigid substrate for DNA synthesis and decreased apoptosis, cells transformed with the H-ras oncogene lose their stiffness-sensing ability [5]. More recently, a number of cancer cell types and Ha-Ras^{V12}-transformed cells (pancreatic, breast, and kidney) exhibit stiffness insensitivity, as measured by DNA synthesis rate and cell stiffness that is unaffected by the underlying substrate rigidity [6••]. This may be associated with decreased caveolin-1 (cav1), which has an inhibitory function in cell proliferation through the extracellular signal-regulated kinase 1/2, phosphoinositide 3-kinase (PI3K) or β-catenin-T-cell factor/lymphoid enhancer factor pathways, as well as a regulatory function in focal adhesion and integrin-mediated actin remodeling. When Cav1 is overexpressed or re-expressed in Ha-Ras^{V12} transformed cells, stiffness sensing is restored; when knocked down, cells soften, their stiffness is not dependent upon substrate rigidity, and they are able to grow on soft substrates. Additionally, tumor-initiating cells or cancer stem cells are largely insensitive to stiffness with respect to spreading, migration, and proliferation, but can regain their stiffness response when myosin-dependent contractility is increased [7••]. However, the generalization that cancer cell proliferation is stiffness-independent cannot be applied to all cancer types. For example, SK-N-DZ neuroblastoma cells preferentially proliferate on softer substrates [8].

Concomitant with deregulated proliferation, the suppression of apoptosis is also necessary for the expansion and invasion of cancer cells and is influenced by substrate stiffness. As seen in TGF-β1-treated normal murine mammary gland epithelial cells and Madin-Darby canine kidney epithelial cells, soft gels induce transforming growth factor-β1 (TGF-β1) mediated apoptosis, nuclear fragmentation, and caspase activity [9]. In contrast, stiff gels trigger epithelial–mesenchymal transition (EMT) characterized by elongated morphology, delocalization of epithelial junctional markers zonula occludens-1 and E-cadherin, as well as increased N-cadherin, α-smooth muscle actin (α-SMA), and Snai1 (a TGF-β-mediated transcription factor that represses E-cadherin expression and can induce EMT). Interestingly, as substrate stiffness increases, there is a switch around 1–8 kPa where caspase-3 activity decreases and Snai1 expression increases. Various genetic and pharmacologic experiments suggest the involvement of focal adhesion kinase (FAK) and PI3K/ Akt signaling in the stiffness-sensitive apoptotic and EMT switch [9]. Regulation of EMT by substrate stiffness is supported by numerous studies [10–13].

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Effects of mechanics on angiogenesis

Angiogenesis is essential for tumor growth. Since the pioneering studies of Folkman, a number of proteins and factors that promote and inhibit angiogenesis have been identified. However, knowledge of how cellular mechanics influences new vessel growth is limited. Likely, cell-generated contractile forces are needed for capillary sprouting, as shown in a 3D co-culture model with human umbilical vein endothelial cells (HUVEC) and normal human lung fibroblast, where angiogenesis was inhibited by fibrin density and even more so with the addition of myosin inhibitors [14].

Possibly the most well-studied growth factor involved in the initiation and regulation of angiogenesis is vascular endothelial growth factor (VEGF). The biochemical events that stimulate VEGF expression have been well studied, but not mechanical cues, although there exists evidence that environmental cues such as hypoxia and acidosis can drive VEGF expression. When seeded onto stiff collagen I-coated polyacrylamide gels representative of rigid, cirrhotic liver tissue, highly metastatic hepatocellular carcinoma (HCC) MHCC97H cells and lowly metastatic Hep3B cells upregulate their VEGF expression and phosphorylation levels of PI3K and Akt [15•]. When the integrin β1 is blocked in both HCC cell lines using a specific monoclonal antibody, VEGF expression and PI3K and Akt phosphorylation are lower compared to control. This result suggests that integrin β1 has a mechanosensing role in HCC cells and can mediate VEGF expression through the PI3K/Akt pathway.

A splice variant of the ECM ligand fibronectin (FN) that includes the extra domain-B (EDB-FN) is upregulated in tumors and may promote angiogenesis [16••]. When endothelial cells are seeded onto hard polyacrylamide gels, total FN and EDB-FN protein as well as pro-angiogenic PKC βII expression is increased and anti-angiogenic VEGF 165b expression is decreased compared to soft gels. The Rho/Rho-associated kinase (ROCK) pathway appears to mediate expression of EDB-FN, suggesting a mechanism by which matrix stiffness can affect angiogenesis.

Effects of mechanics on metastasis

Studies of breast cancer cells show that a compliant ECM is non-conducive to tumor cell invasion [17]. Paradoxically, some tumor cores are less stiff than the periphery, presumably because they contain mainly cancer cells and little ECM. This feature has been observed in not only breast carcinoma [18], but also prostate cancer tissue (on the micro-scale, but not on the macro-scale) [19]. The seemingly contradictory observation that tumors are stiff grossly, but soft on the micro-scale or in their core, might be explained by the stiffness of the individual cells. Lin *et al.* showed that cancer cells are softer than their normal counterparts across several cell types, including breast, bladder, cervix, pancreas, and transformed cells [6••]. One caveat is that these measurements were made from cells on glass, with the exception of cervical cells (accessible *in situ*). Softer cells are associated with increased motility and tumor invasiveness, and the development from radial growth phase to invasive vertical growth phase to metastasis is characterized by decreased cell stiffness, likely allowing cells to move through gaps within tissues and vessels [18].

The stiffness and integrity of endothelial cells and the vascular wall are also important factors in the context of extravasation. Infiltration of breast cancer cells onto pulmonary artery endothelial cells causes the activation of myosin light chain kinase and myosin II, followed by subsequent contractility of the endothelial cells, as studied in a co-culture model with MDA-MB-231 (breast cancer) and endothelial cells [20]. As a result, the ECM softens and the cytoskeleton is rearranged, leading to degradation of the endothelial cell layer which aids extravasation. Tumor cell contractility is needed for extravasation, as inhibition of ROCK and MLCK-mediated traction forces diminishes invasion through endothelial cells. However, actin polymerization and myosin II-mediated contractility are not required for cancer cell migration, as MDA-MB-231 metastatic cells can still travel toward a chemoattractant through narrow channels.

In culture, induction of a malignant phenotype does not necessarily require a pathologically stiff ECM. A number of colon and prostate cancer cells undergo a transition from an adhesive epithelial to a rounded dissociated phenotype on soft substrates that is reminiscent of metastasis, and these rounded cells express genes that are characteristic of cancer cell metastasis, migration, and proliferation [21]. Similarly, melanoma tumor-repopulating cells (TRCs) exhibit cell softening, histone 3 lysine residue 9 demethylation, and Sox2 gene expression — all of which are promoted in compliant 3D fibrin matrices, but not stiff ones [22]. Together, these studies suggest that TRCs or metastatic cells may lie dormant in stiff environments, but function optimally in softer ones, and such a switch may serve as a driving force for cancer metastasis.

Stiffened matrices as a model for tumors

The change in mechanical properties during breast cancer is relatively well-studied, and tissue stiffening associated with malignancy has been correlated with increased collagen deposition and the formation of linear patterns of collagen fibers [23•]. Also associated with high-risk breast cancer and metastasis is increased FN expression (reportedly threefold), which in normal tissue is low (~1%) [24–27]. As an arguable model for breast cancer associated stromal cells, 3T3-L1 preadipocytes produce a more rigid FN network with decreased porosity and increased fiber diameter when preconditioned with soluble tumor factors compared to controls [28]. These changes in FN are accompanied by decreased cellular adhesion and increased VEGF levels, suggesting a role for FN in migration, angiogenesis and growth of breast tumor.

A few studies have also shown that the ECM can be stiffened through crosslinking mechanisms, such as the non-enzymatic formation of advanced glycation end-products (AGE) and lysyl oxidase. In a prostate epithelial cell acini 3D model, AGE-dependent crosslinking of two major components, collagen IV and laminin, stiffens the basal lamina matrix and induces malignant transformation characterized by loss of cell polarity, loss of cell–cell junctions, and luminal infiltration [29].

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Mechanisms of mechanosensing by cancer cells

Most studies of mechanosensing in cancer cells focus on the role of integrins, cadherins and other transmembrane protein complexes that link cells to essentially solid material, either other cells or the ECM. Interestingly, primary cilia have been found on the MG63 human osteosarcoma cell line and on HeLa cells [30], suggesting that cells can mechanosense fluid from their dorsal surface. This finding is consistent with the report of a connection between the overexpression of polycystin 1 and 2, proteins present in the plasma membrane and cilia, and negative clinical outcomes and invasiveness in colorectal cancer [31].

The focal adhesion protein vinculin, a membrane-cytoskeletal protein involved in cell spreading and stability of focal adhesions, is upregulated in primary invasive human cancers [32]. Nonmalignant mammary MCF10A spheroids implanted into soft collagen/recombinant basement membrane gels keep their structure, as shown by spherical acini, intact adherens junctions, and tissue polarity, whereas in stiff gels, basal polarity and cell–cell junctions are disrupted. HA-ras MCF10AT premalignant mammary spheroids keep some tissue polarity within soft gels, but the structures are completely compromised in stiff gels. Together, these results show the dependence of invasion and malignancy on matrix stiffness, and furthermore, this is a result of integrin-mediated FAK signaling, changes in vinculin function, and induction of Akt signaling [32].

Transcriptional changes elicited by matrix stiffness

The Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) transcriptional regulators are the major downstream effectors of the Hippo pathway and are recognized as oncogenes. Increased substrate stiffness upregulates YAP in a lung cancer cell line, suggesting a role for YAP/TAZ and the Hippo pathway in lung cancer cell growth [33].

Twist1 is a transcription factor that has been implicated in cell differentiation as well as cancer metastasis. With respect to matrix stiffness, it has been shown that substrate rigidity drives translocation of Twist1 to the nucleus, inducing EMT, tumor invasion, and metastasis, with changes to collagen, MMPs, and lysyl oxidases [34•].

New materials and approaches for mechanobiology research

Recently, an area of intense interest is the development and characterization of 3D matrices with tunable physical properties as models for cell biology and also as test platforms for drugs and toxins. Soft and stiff alginate scaffolds with different RGD concentrations have been used as platforms to test the cytotoxic response of glioblastoma cells to various compounds. Both substrate rigidity and cell–matrix adhesions have an effect on cellular toxicity; cells are more sensitive to toxins when seeded onto soft substrates with stiffness similar to brain and these effects are lost when integrin binding is pharmacologically inhibited [35].

Polyethylene glycol diacrylate (PEGDA) hydrogels with compressive moduli between 2 and 70 kPa are used to encapsulate cancer stem cells and evaluate their optimal matrix stiffness

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for growth without any confounding environmental factors [36]. The optimal stiffness for cell survival and proliferation as well as YAP/TAZ expression is dependent upon the tissue of origin, that is, 5 kPa for breast, 25 kPa for colorectal and gastric, and 50 kPa for bone.

Instead of using chemically inert and non-physiological polyacrylamide gels, hydrogels made from crosslinked networks of biopolymers such as hyaluronic acid (HA) have been used to study cancer cell behavior to stiffness. HA, a component of the ECM is increased in many cancers [37••,38], including ovarian [39], non-small-cell lung adenocarcinomas [40], prostate cancer [41,42], gastric and colorectal [43,44], bladder [45], breast [46], and head and neck [47]. In many cases, *in vitro* HA is methylated to allow crosslinking, with the consequence of losing the ability to activate HA receptors such as CD44 that have been implicated in cancer [48]. Nonetheless, HA hydrogels elicit cellular behavior different from polyacrylamide or tissue culture plastic. For example, HT1080 fibrosarcoma cells encapsulated in HA recovered from hypoxic stress, but cells cultured on tissue culture plastic did not [37••]. Networks of HA that more closely resemble the native glycosaminoglycan can be made when the chain is sparsely modified with sulfhydryl groups and then crosslinked by oxidation or PEGDA. When HA gels are coupled to FN, proliferation of a number of cell types — including neonatal ventricular rat myocytes, human mesenchymal stem cells (hMSC), 3T3 fibroblasts and HUVECs — can be strongly enhanced even on soft substrates (200 Pa), which, if made of polyacrylamide, would halt proliferation [49].

In addition to variations of 2D hydrogel substrates, numerous methods are being developed to study how physical signals affect cancer cells *in vitro*. Such methods include production of pillar arrays that impede cell migration by trapping nuclei [50], simplified microfluidic methods that apply pressure to single cells [51–53], patterned type I collagen micro-tracks that mimic the paths by which cancer cells move *in vivo* [54], and optical tracking microrheology to measure the very soft pericellular matrix which changes in the tumor environment [55]. These more sophisticated techniques will hopefully provide greater insight into how cancer cells sense their mechanical environment.

Fibrosis models for cell culture: heterogeneous structure with homogeneous ligand

Although HA modifications for covalent crosslinking can at least sometimes inhibit normal binding to cell receptors (Figure 1a) [43], soft gels of crosslinked HA can also uniquely allow reorganization of some matrix macromolecules such as fibronectin into fiber-like regions (Figure 1b) [30]. Such observations of matrix heterogeneity are often anecdotal, but certainly raise questions about the effects of non-homogeneous gels on cells. Decoupling the effects of ligand density, which can certainly be non-homogeneous and lead to haptotaxis, from the effects of non-homogeneous compliance are also key to elucidating mechanosensing processes used by adherent cells. A heterogeneous matrix is a particularly distinctive feature of fibrosis, which is frequently associated with solid tumors [13]. Fibrosis also results from acute injury, such as a heart attack [56], as well as chronic diseases such as liver cirrhosis or muscular dystrophy [57], and it is often referred to as a scar. A scar forms

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locally in most or all tissues of higher animals and is compositionally characterized by an abundance of crosslinked collagen-I fibers heterogeneously distributed within a fibrotic tissue. A scar tends to be locally stiff and long-lasting [56,57]. Focusing on the cancer context, cancer cells respond to matrix stiffness, which results from increased collagen and crosslinking (fibrosis). However, where does increased collagen come from?

Scar matrix seems to be made largely *de novo*, and a major role in the development of organ fibrosis has recently been ascribed to ubiquitous MSCs, which reside in perivascular niches of many organs including heart, liver, kidney, lung, and bone marrow [58]. MSCs have been well-known for decades to proliferate and to differentiate toward multiple tissue lineages (e.g. fat, bone), but genetic lineage tracing recently demonstrated that tissue-resident MSCs (specifically the Gli1+ MSCs), rather than circulating MSCs, proliferate after organ injury to generate myofibroblast-like cells typical in scars. In mouse models, genetic ablation of these cells ameliorates fibrosis (Figure 1c), and after induced heart failure, the heart also maintains ejection fraction. To better understand and perhaps control the sensitivity of human MSCs and other cell types to matrix heterogeneity and fibrosis, new reductionist culture models with scar-like heterogeneity are thus needed.

To clarify the effects of non-homogeneous matrix stiffness on cells, one recently developed approach for making minimal matrix models of scars (MMMS) entails mixing soluble collagen-I subunits with acrylamide monomers plus bis-acrylamide crosslinker and then polymerizing the mix into a gel [57]. Upon initiation of polymerization, collagen-I fibers phase separate from pre-gelation clusters of polyacrylamide, leading to highly branched fractal fiber bundles that segregate as islands heterogeneously entrapped at the subsurface of the hydrogel (Figure 1d). Importantly, collagen in the subsurface fiber bundles is not accessible for cell adhesion. A uniform over-coating of matrix ligand is therefore provided for cell attachment. Formation of this type of model scar could be viewed as a diffusion limited cluster aggregation process, with fractal sizes that could be easily controlled by varying the concentration of collagen-I. With the proper mixing ratios, a surface coverage of collagen fiber bundles of ~30% approximates the extent of fibrosis seen, for example, in muscle cross sections [59,60].

Differences in mechanoresponses have been observed when MSCs are cultured in parallel on homogeneous gels and MMMS. A key marker of fibrosis and scarring is the stress fiber associated protein α-SMA, and although α-SMA is not unique to scarring, its expression increases with contractility [61]. α-SMA increases *in vivo* in hepatic stellate cells (i.e. liver MSCs) in parallel with stiffening of toxin-injured liver, but preceding the detection of fibrotic collagen [62]. Despite the soft-stiff heterogeneity of MMMS gels, MSCs greatly increase expression of α-SMA compared to homogeneously low expression in MSCs on polyacrylamide gels that lack the fiber islands (Figure 1d). Interestingly, α-SMA expression was more homogeneous between cells on MMMS than seen for cells on homogeneously stiff gels. This has been explained by identification of a transcription factor, NKX2.5, that is, a strongly cooperative repressor of α-SMA, which exits the nucleus on stiff substrates (Figure 1d). While many applications might be considered to clarify scarring responses of cells, the effect of other cell types especially cancer cells could be especially interesting. As 80% of hepatocellular carcinomas occurs against a background of cirrhosis and draws

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increasing attention [63], MMMS could be a promising model system to study the mechanotransduction of HCCs in cirrhotic liver.

Conclusions

Whereas the biochemical signaling events involved in cancer progression have been largely investigated, there is still much to be learned about the mechanical cues provided by the ECM, its stiffness, other environmental factors, and their subsequent effects on cell function and behavior. Tumors are generally stiff, but can have soft cores consisting of cells that are softer than their normal counterparts. At first glance, this may seem contradictory, but it is hypothesized that cancer cells need to generate traction forces on stiff substrates to migrate, but also need to be soft to weave through tight spaces and extravasate or metastasize.

Generalizations made about cancer and mechanics cannot be applied to every cell type or to every cancer hallmark. Broadly, however, cancer cells often appear to have reduced mechanosensitivity, and substrate stiffness has less of an effect on spreading, migration, and proliferation. Apoptosis is preferentially promoted on soft matrices, but EMT and angiogenesis occur on hard matrices. Through pharmacological and genetic manipulations, many studies have implicated the involvement of Ras, FAK and PI3K/ Akt signaling.

A variety of platforms, such as polyacrylamide or biopolymer gels with tunable stiffness, have been routinely used to study how cancer cells respond to their underlying stiffness as measured by, but not limited to, cell spread area, proliferation, migration, invasion, and apoptosis. While these 2D substrates provide a simple method to elucidate the effects of stiffness on cellular behavior, new approaches consisting of 3D scaffolds, micropillars, microfluidic devices, and heterogeneous matrices which mimic the tumor environment will likely provide greater insight into the role of mechanotransduction in cancer.

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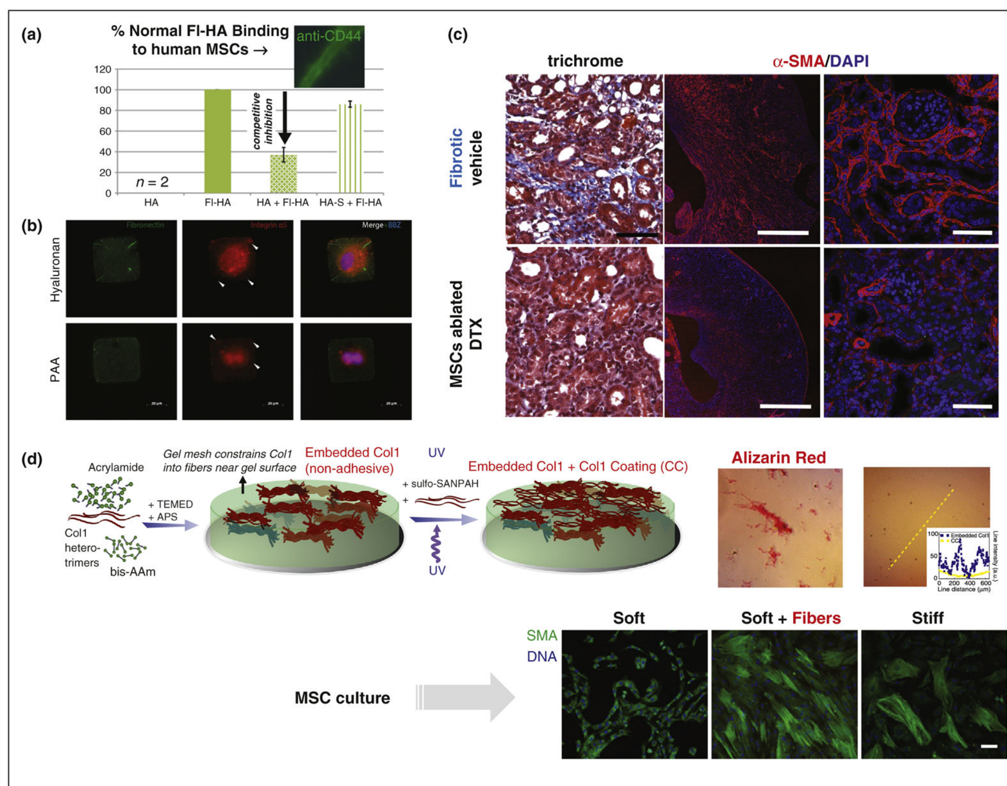

Figure 1.

(a) Modifications of hyaluronic acid (HA) can affect its activity. Thiol-modified HA (HA-S) is not biologically active compared to native HA [64]. Bar graph shows extent of binding of fluorescently labeled HA (Fl-HA) to human MSCs, which is significantly reduced by adding HA as a competing partner, but not upon adding HA-S with its chemical modification. **(b)** Fibronectin (FN) streaks seen on soft HA substrates. Myocytes were cultured on (1000 μm^2) square FN micro-patterned 300 Pa HA and 30 kPa PAA substrates for a period of 72 hours. Myocytes were unable to migrate out of the micropatterns even after attachment to HA substrates for a 3 day period. Arrowheads indicate formation of integrin α5 clusters. FN streaks seen on soft HA substrates are due to micro-contact printing on soft substrates and existed before cell plating [49]. **(c)** Mesenchymal stem cells (MSCs) are perivascular cells in nearly all tissues and have a major role in fibrosis. Ablation of Gli1+ MSCs by diphtheria toxin, DTX, reduced severity of kidney fibrosis as demonstrated by trichrome staining and immunostaining for α-SMA and quantification of interstitial fibrosis. Scale bars, left two panels, 500 μm; others, 50 μm [58]. **(d)** Scar in a dish reveals key role of matrix rigidity, even if heterogeneous. Scar-like islands of collagen-I are heterogeneously entrapped at the subsurface of the soft hydrogel. The heterogeneity of the MMMS was confirmed by both immunofluorescence and staining with the histochemical dye, Sirius Red. Human MSCs cultured on conventional homogeneous gels and separately on MMMS [57].