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Mechanobiology of cancer cell responsiveness to chemotherapy and immunotherapy: mechanistic insights and biomaterial platforms

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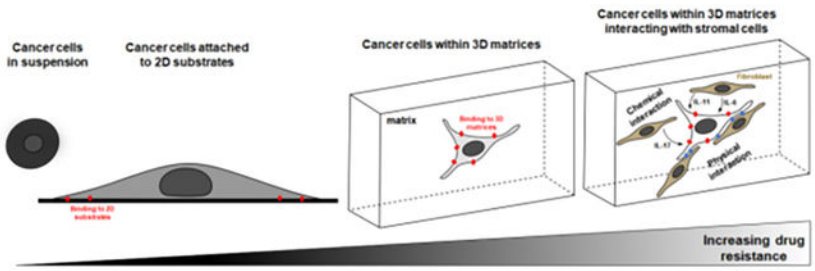
Abstract

Mechanical forces are central to how cancer treatments such as chemotherapeutics and immunotherapies interact with cells and tissues. At the simplest level, electrostatic forces underlie the binding events that are critical to therapeutic function. However, a growing body of literature points to mechanical factors that also affect whether a drug or an immune cell can reach a target, and to interactions between a cell and its environment affecting therapeutic efficacy. These factors affect cell processes ranging from cytoskeletal and extracellular matrix remodeling to transduction of signals by the nucleus to metastasis of cells. This review presents and critiques the state of the art of our understanding of how mechanobiology impacts drug and immunotherapy resistance and responsiveness, and of the *in vitro* systems that have been of value in the discovery of these effects.

Graphical Abstract



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Keywords

hydrogel; stiffness; substrate compliance; drug responsiveness; chemotherapy

1. Introduction

Biological systems react to force through diverse responses including growth, death, differentiation, remodeling, and changes to regulation that can, recursively, change forces and promote further and enhanced force responses [1–3]. Such changes range from adaptive to pathological and are a major focus of the emerging field of mechanobiology [4,5]. Pathologies including certain cancers can accentuate or change the responses of cells to mechanics [6–8], and can thereby alter the stiffness and permeability of a tissue, as well as cell-cell and cell-extracellular matrix (ECM) connections. A side effect of these modulations is that they can change the ability of drug or immunotherapy agents to reach a target site, the ability of cells at the target site to take up these drugs, and the ability of a cell to respond to a drug. A growing body of evidence points to a set of pathologies in which this often self-reinforcing cascade of events can prevent drug treatment from being effective. This review aims to link together these pathologies into a single class of bioengineering challenges in which mechanobiology governs the success of drug treatment. The hope is that by studying key phenomena from this unified perspective, these pathologies of mechanobiology will begin to be recognized as the critical, integrated, cross-scale barriers to healthcare that we believe them to be, and that cross-cutting strategies to address them can begin to emerge.

The review begins with several examples of pathologies in which mechanobiological factors block the delivery or efficacy of drugs that might otherwise be effective. A key theme is that, while it has long been known that force can drive physiology and pathophysiology through mechanobiological factors, the discovery of mechanobiological pathways affecting drug delivery and efficacy has the potential to lead to new mechanobiological approaches to improved treatment. These mechanisms are often multifactorial, and to date linked only loosely in the medical literature. Because of the complexity of these mechanisms, many have been discovered only recently with the advent of simplified *in vitro* systems. The review concludes with a summary of these *in vitro* systems and of the continuing value of these systems for both identification and amelioration of mechanobiological effects on cancer cell responsiveness to drug and immunotherapy.

2. Mechanobiology in drug and immunotherapy resistance and responsiveness

2.1. Roles of cell-cell interactions

2.1.1. Chemotherapy resistance and responsiveness—We begin with observations across a broad range of cancers. Many cancerous cells interact with a diverse range of components of their surrounding microenvironment, including other cells [9]. Those interactions can determine how cancer cells respond to treatment. For example, interactions between cancerous cells and nearby stromal cells have been shown to increase cancer cell survival [7]. Epithelial ovarian cancer cells interacting with stromal cells display

chemoresistance to platin and taxans, which indicates that the presence of stromal cells within a patient's tumor might enhance chemoresistance [10].

One mechanism for drug resistance arising from cell-cell interactions is the activation of anti-apoptotic signaling. Binding of the integrin receptors of cancer cells to ligands in extracellular matrix proteins secreted by surrounding stromal cells can activate such ant-apoptotic signaling [11]. A key example occurs in patients with acute myelogenous leukemia. Here, the survival of even a few cancer cells in the bone marrow can cause minimal residual disease, meaning a relapse of the cancer after chemotherapy. In this case, drug resistance is induced by the ligation of the protein very late antigen 4, a member of the integrin family ($\alpha_4\beta_1$) on leukemic cells to fibronectin associated with bone-marrow stromal cells [12].

Direct cell-cell interactions can similarly lead to drug resistance through a phenomenon known as cell adhesion-mediated drug resistance (CAM-DR). CAM-DR was first described in human myeloma cell lines at the turn of the century [13], and has since been discovered in a variety of other tumor types. Acute lymphoblastic leukemia cells adhere directly to the integrin α4 of bone marrow stromal cells, leading to CAM-DR and implicating α4 as a therapeutic target for drug resistant leukemia [14]. Ovarian cancer cells in physical contact with mesenchymal stromal cells exhibit a pro-metastatic and chemoresistant profile [15–18]. Glioblastoma multiforme (GBM) cells, in the absence of ECM proteins, employ an alternative mode of CAM-DR by forming spheres that undergo CAM-DR via cell–cell interactions, implicating the role of gap junctions in chemoresistance [19]. Note that cell-cell interaction-based drug resistance is found to be more significant in 3D culture models than in 2D models. For example, resistance to cisplatin and sorafenib by cancer spheroids and cancer-associated fibroblasts is higher in 3D than 2D culture models [20].

Chemokines can trigger chemoresistance in cancer cells even in the absence of contact-based chemoresistance. Mesenchymal stromal cells can induce chemoresistance in ovarian cancer cells without contact through interleukin-6 (IL-6) [21]. Other chemokines secreted by cancer-associated fibroblasts that play an important role in cytokine-mediated chemoresistance of cancer cells include interleukin-17 (IL-17), which is overexpressed by colorectal cancer-associated fibroblasts in response to chemotherapy [22]. Similarly, interleukin-11 (IL-11) can promote cancer cell chemoresistance by protecting cancer cells from cisplatin-induced apoptosis [23]. Taken together, these results show that both contact-based and noncontact-based interactions of cancer cells with surrounding cells can confer drug resistance and promote cancer cell survival.

Tumor-stroma mechanical interactions in the form of compressive stresses can reduce drug efficacy [24]. Cell-cell contacts can also affect responsiveness to chemical modulators in human hepatocytes [25]. Specifically, decreased expression and localization of intercellular gap junctions and E-cadherin-mediated cell adhesions correlates with decreased constitutive and rifampicin-induced levels of cytochrome P450 3A4 activity. In general, cell-cell interactions are key to preserving the function of primary hepatocytes in culture, which can then be used to detect hepatotoxicity and drug–drug interactions [26].

2.1.2. Immunotherapy resistance and responsiveness—While there is a rich literature on cell-cell interactions in the context of immunotherapy and immunotherapy resistance [27,28], here we focus the discussion on interactions driven by mechanical stimuli. It is now understood that while cancer tissues are generally stiffer than normal tissues, cancer cells are typically softer compared to normal cells [29]. Cancer cells have been shown to rearrange their cytoskeletal network and soften their membrane to allow them to move through confined spaces [30], which contributes to their malignancy and metastatic potential [31]. The question is whether such changes in cancer cell mechanics can directly influence response to immunotherapy. For example, cytotoxic T lymphocytes need to directly interact with the surface of the target cells to kill them and it is known that T cells can respond to the stiffness of their microenvironment [32]. Recent work has shown that T cells also respond to the stiffness of cancer cells, which the authors termed a "mechanical immune checkpoint" and suggested that it could become a therapeutic target [33]. Specifically, Lei and co-workers showed that T-cell mediated cancer cell killing was less efficient for soft cancer cells, which had cholesterol enriched membranes, compared to stiff cancer cells, which had cholesterol depleted membranes [33]. Interestingly, cancer cell stiffness had no effect on T cell signaling and cytolytic protein production, but it impaired T cell mechanical forces at the immunological synapse (Figure 1) [33]. Immunological synapse is the physically active structure, capable of exerting a mechanical force, that forms between a cytotoxic T lymphocyte and a cancer cell [34]. To kill cancer cells, T cells are known to exert a mechanical force at the immunological synapse, which is associated with enhanced perforin pore formation on the target cancer cell due to increase in target cell tension [34]. Traction force microscopy studies have shown that the increase in mechanical forces correlates with local increases in actin density [35].

Another recent study used melanoma and breast cancer cells to show that myocardin-related transcription factors (MRTFs) A and B, which are essential for cancer cell migration and metastasis, also improve cancer cells responsiveness to immune checkpoint blockade antibodies [36]. The authors showed that cancer cells overexpressing MRTFs induce stronger cytotoxic T lymphocyte activation and cytotoxicity because they have more rigid filamentous actin cytoskeleton [36]. The authors further suggested that this mechanical dimension of the immunosurveillance, which they termed mechanosurveillance, might be relevant for the targeting of metastatic disease. Immunosurveillance, the process by which immune cells detect and eliminate cancer cells, plays a critical role in immunotherapy treatments for cancer [36].

2.2. Roles of cell-extracellular matrix interactions

2.2.1. Chemotherapy resistance and responsiveness—Cells are known to sense the physical cues from their extracellular matrix (ECM), including mechanical forces, dimension, stiffness, viscosity, plasticity, shape, and confinement [1–3,37–41]. More importantly, cells respond to these physical cues by regulating their cytoskeletal and nuclear components [4,42–48], which in turn affect almost every aspect of cellular behavior including migration, differentiation, proliferation, signaling, adhesion, and gene expression [8,49–51]. As cells adapt themselves to the physical properties of their ECM, it is important to understand how these ECM-induced cellular changes impact the resistance and responsiveness of cancer

cells to different drugs. In fact, interactions between cancer cell surface integrins and ECM components have been linked to drug resistance to various agents, from DNA damaging agents to kinase inhibitors, suggesting that integrin antagonists could sensitize tumor cells when used in combination with standard chemotherapy [52]. The chemoresistance conferred by the cancer microenvironment has led to various therapies targeting cell-ECM interactions being investigated as an adjuvant, combination or stand-alone treatments [53].

To sense the physical cues from the ECM, cells first need to be connected to the extracellular environment through focal adhesions [54], which together with the cytoskeleton and the nucleus compose a three-way feedback loop through which physical signals are transmitted from the ECM to the nucleus [55]. Thus, various experimental approaches have been used to study how cell-matrix adhesion impacts the responsiveness of cells to different drugs. Most of these studies showed that cell-matrix adhesion increases the resistance of both normal and cancer cells to drugs [13,56–58]. For example, compared with cells in suspension, human myeloma cells attached to fibronectin exhibit higher resistance to the apoptotic effects of melphalan and doxorubicin [13]. As integrins play an important role in cell-matrix adhesion [59], different studies have addressed the specific contribution of integrins to drug resistance [60]. For example, dependence of cell survival on β1-integrin ligands fibronectin and laminin was tested in human lung cancer, MDA-MB-231 breast cancer cells, and normal fibroblasts upon the Ukrain drug treatment, and it was shown that fibronectin and laminin significantly increase resistance to the cytotoxic drug [61]. Other studies have demonstrated that in MDA-MB-231 and MDA-MB-435 breast cancer cells, β1 integrin signaling inhibits paclitaxel- and vincristine-induced apoptosis by inhibiting the release of cytochrome *c* from the mitochondria, which in turn is dependent on the activation of the PI 3-kinase/Akt pathway [62]. In a clinical study of 249 breast cancer patients with a median follow-up of 8.4 years, high levels of β1 integrin expression and co-expression of fibronectin were linked to more aggressive and invasive breast cancer and it was suggested that such patients could benefit from targeted therapy [63]. For a detailed recent review on the role of integrins in breast cancer on drug resistance and how targeting specific integrins and integrin-binding proteins may lead to new therapies, the readers are referred to the following review [64]. Adhesion through β1 integrins to fibronectin, laminin and collagen IV of small-cell lung cancer cells, has also been linked to chemotherapy resistance due to stimulating protein tyrosine kinase (PTK) signaling downstream of DNA damage [65,66]. In ovarian cancer, drug resistance to chemotherapeutic drugs such as cisplatin as well as poor patient outcomes, have been linked to the upregulation of integrin α-6 (ITGA6) [67]. A clinical study has correlated high expression of Lewis *y* antigen and integrin $\alpha 5\beta 1$ in ovarian carcinoma tissues to chemotherapeutic drug resistance [68]. For a review on tumor targeting via integrin ligands, specifically integrins recognizing the RGD cell adhesive sequence, the readers are referred to the following review [69].

As cell-matrix adhesion within three-dimensional (3D) environments exhibit a few key differences from the one on two-dimensional (2D) substrates [70], it is expected that cells within 3D matrices show different levels of resistance to chemotherapy. Indeed, experimental studies show that cells cultured in 3D are usually more chemoresistant compared to cells cultured on flat 2D substrates (Figure 2) [71,72]. Note that while not the focus here, matrix composition in addition to matrix stiffness can affect cancer cell

responsiveness to drugs [73]. For example, one study developed a high-throughput ECM microarray to investigate the effect of ECM composition on lung adenocarcinoma cell drug response and identified ECM proteins (e.g. fibronectin) that mediated resistance to cisplatin and sunitinib [74]. In other examples, only in type 1 collagen matrices, cell-matrix interaction is regulated by many different parameters including collagen concentration, degree of nonlinear stiffening of the ECM, matrix pore size, cell density, ECM crosslinking, matrix constraint, ECM degradability, and growth factors [45,75–78]. How each of these parameters changes the resistance of cells to chemotherapy remains a difficult question to answer as most of these parameters are intertwined and cannot be varied independently of others (e.g., collagen pore size decreases with increasing collagen concentration).

In both 2D and 3D, adherent cells can sense the stiffness of their microenvironment through exerting contractile forces and usually adjust the magnitude and the direction of these contractile forces in response to the stiffness of the microenvironment [1,79]. Interestingly, these cellular forces can themselves alter the stiffness of the microenvironment leading to a positive feedback loop between cell contractile forces and ECM stiffness [80]. For solid tumors in particular, a feedback loop between cell contractility and matrix alignment and stiffening has been noted, which enables cells to polarize and become more invasive due to their increased contractility [81]. On the other hand, for many cancers such as glioblastoma, increased invasiveness has been associated with increased stemness and drug resistance [82]. However, the specific role of the positive feedback loop between matrix stiffening and cancer cell contractility and potentially invasiveness, and its effect of drug responsiveness needs further investigation.

In general, it has been shown that cellular drug resistance in both 2D and 3D increases with matrix stiffness [83–86], with a some exceptions such as for osteosarcoma cells for which stemness and drug resistance were enhanced on softer substrates due to miR-29 downregulation [87]. For example, Liu et al., [88] studied the effect of matrix stiffness on cellular drug resistance within a 3D context. In this study, hepatocellular carcinoma cells were cultured in 3D alginate gels with different stiffness (21, 75, and 105 kPa) and treated with paclitaxel, 5-FU, and cisplatin. It was shown that cells within the stiffest matrix show higher resistance to the drugs indicating that an increase in matrix stiffness can decrease the effectiveness of cancer therapy. The fact that matrix stiffness negatively affects the effectiveness of cancer therapy becomes of significant importance in the context of chemotherapy knowing that tumor tissues are significantly stiffer than healthy tissues [6,89]. In another study on the effect of matrix stiffness on cellular drug resistance, MDA-MB-231 breast cancer cells were cultured in 3D alginate gels with different stiffness. The chemoresistance of cells to doxorubicin in the stiff 2000 Pa gel was found to be three-fold higher compared with cells in the soft 200 Pa gel. Interestingly, MCF7 breast carcinoma cells cultured in the same gels did not show stiffness-dependent resistance to the chemotherapeutic doxorubicin [90]. Using 3D alginate-based scaffolds with different stiffness and adhesive ligand, it was shown that both matrix stiffness and cell-matrix adhesions can strongly influence cell responses to toxins [91]. Human breast cancer cell lines (MDA-MB-231, BT549, and SkBr3) showed the same response when they were cultured on substrates with different stiffness [92]. Similar to cells within 3D matrices, cells on stiffer substrates showed more resistance against sorafenib (Raf kinase inhibitor) independent of

ROCK activity [92]. In agreement with these results, hepatocellular carcinoma cells on stiff substrates showed reduced apoptosis upon cisplatin treatment [93]. However, surviving cells from soft substrates exhibited higher clonogenic capacity than surviving cells from stiff substrates [93], indicating a higher metastatic potential (Figure 3).

As noted above, various studies have shown that substrate stiffness significantly affects focal adhesion complexes, cell force generation, cytoskeletal organization, cell stiffness, nuclear morphology, cell spreading, and cell migration. As these substrate-induced changes often involve the Rho-Rho-associated protein kinases (ROCK) pathway, it is important to study whether the Rho-ROCK pathway is also involved in cellular drug resistance [94]. For example, the Rho-ROCK pathway has been implicated in greater malignancy and chemoresistance of metastatic ovarian cancer cells on soft substrates [95], in regulating motility and metastasis in gastric cancer [96], and greater malignancy in breast cancer [97]. Note that Rho-GTPases are known to promote the tumor metastasis by disrupting epithelial-sheet organization, increasing cell motility and promoting ECM degradation [98]. For a review on the role of the Rho-ROCK pathway in cancer and tumor invasion and metastasis, the readers are referred to the following review [99]. Cytoskeletal organization due to Rho-GTPases has been also linked to intrinsic and acquired drug resistance of cancer cells [100]. For example, inhibition of the Rho/ROCK pathway has been shown to enhance responsiveness to cisplatin for ovarian cancer cells by blocking hypoxia-inducible factor-1α signal transduction [101]. Inhibition of ROCK signaling has also been shown to enhance cisplatin resistance in neuroblastoma cells [102]. For a review on the potential of the Rho-ROCK pathway as a target for cancer therapy including immunotherapy, the readers are referred to the following review [103,104].

2.2.2. Immunotherapy resistance and responsiveness—Similarly to chemotherapy and radiotherapy responsiveness, cell-ECM interactions can also affect the effectiveness of cancer immunotherapy [105]. For example, even though therapies such as chimeric antigen receptor (CAR) T-cells and checkpoint inhibitors have been successful in treating cancer [106], many patients show therapy resistance stemming in part from excessive ECM deposition and cancer cell-ECM interactions [107,108]. In some cases, the explanation could be that the dense ECM serves as a physical barrier between immune and tumor cells, preventing immune cells from getting deep into the tumor and in contact with the cancer cells even when they are attracted to the tumor site via chemokine gradients. For example, in vitro studies have shown that ECM presence significantly influenced migration and cytotoxicity of cytotoxic lymphocytes compared to 2D cultures and, hence, their ability to kill cancer cells [109]. Other in vivo studies have shown that cytotoxic lymphocytes can get trapped and accumulate in the dense tumor ECM without being able to reach the tumor cells [110]. In a study of urothelial patients, it was shown that a lack of response to programmed death-ligand 1 (PD-L1) checkpoint inhibition treatment correlated with accumulation (i.e. trapping) of cytotoxic lymphocytes into the tumor ECM [111]. Further, many immunomodulatory drugs are antibodies of large hydrodynamic radius (e.g. ipilimumab and pembrolizumab), whose diffusion into the tumor would also be impeded by the dense ECM, hence reducing their efficacy. The dense ECM and obstructed transport also leads to hypoxia, which in turn is known to upregulate immunosuppressive

factors like IL-10, CCL18, CCL22, TGF-β, and prostaglandin E2, as well as to inhibit T cell proliferation and macrophage phagocytosis [112].

Importantly, denser and highly crosslinked ECM also translates into a matrix of higher compliance or stiffness. A recent in vitro study on the immune escape of melanoma cells showed that a stiffer matrix enhanced immune escape of A375 cells due to overexpression of SNF5 (a core subunit of the SWI/SNF chromatin remodeling complexes), which activated the STAT-3 pathway and elevated the level of tumor-infiltrating CD8+T cells (Figure 4) [113]. Another recent study of HCC827 lung adenocarcinoma cells on polyacrylamide hydrogels of 2 kPa (soft) and 25 kPa (stiff), showed a higher immune escape on stiffer substrates linked to elevated expression of PD-L1 via actin-dependent mechanisms (cell treatment with cytochalasin D, an actin polymerization inhibitor, reduced PD-L1) [114]. Certain cancers, such as the pancreas, prostate, colon and others, are highly fibrotic with dense collagen matrix of high stiffness and high numbers of CAFs, limiting the efficacy of immunotherapy [115]. As discussed above, the dense and stiff fibrotic ECM may act as a physical barrier to cytotoxic T cell infiltration into tumors and impede T cell velocity and migration. Further, in breast cancer, fibrosis has been shown to correlate with tumor-associated macrophages (TAMs) infiltration due to the overexpression of chemoattractants CCL2 and CSF-1 by tumor cells or CAFs in response to stiff collagen-rich ECM [116]. Fibrosis-induced hypoxia can further suppress T cell infiltration and function and lead to constant activation of HIF-1α and increased NF-κB activation [117].

Multiple studies have also shown that the tumor ECM type can affect immune cell motility, myeloid polarization, T-cell phenotype and immune cell metabolism and survival [118]. TAMs, which are abundant in the tumor microenvironment and mediate adaptive immune response in cancer, can be immunosuppressive and pro-tumorigenic (M2 polarization) or anti-tumorigenic (M1 polarization) based on interactions with the ECM. For example, ECM molecules such as hyaluronic acid, collagen Type I and tenascin-C have been shown to drive M2 polarization in TAMs [119–121], while fibronectin has been shown to drive M1 polarization [122]. Overall, tumors with high infiltration of TAMs are associated with poor patient prognosis and resistance to therapies, suggesting that TAMs depletion or re-polarization could be a successful therapeutic strategy [123]. It is also known that collagens, which are functional ligands for the inhibitory immune receptor leukocyte-associated immunoglobulin-like receptor (LAIR)-1, promote immune invasion by interacting with LAIR-1 expressed on immune cells [124] and can also act as reservoirs for TGF-β and other immunosuppressive factors. A recent large-scale analyses found a distinct set of ECM genes upregulated in cancer, which correlated with the activation of TGF-β signaling in CAFs, were linked to immunosuppression in otherwise immunologically active tumors, suggesting that those genes could be targeted using TGF-β blockade to enhance responses to immune-checkpoint blockade [125]. For more details on the immunosuppressive properties of TGF-β, the readers are referred to the following review [126]. Consequently, strategies that target the ECM in order to improve the efficacy of immunotherapy are gaining momentum and represent an exciting direction for the field [127]. For a recent review on mechanical immunoengineering and potential therapeutic applications in the context of T cells, the readers are referred to the following review [128].

2.3. Cell-tissue interactions: the role of vasculature in drug and immunotherapy responsiveness

The integration of cells and extracellular matrix with vasculature defines tissue-level structure. The health and spatial disposition of the vasculature are primary determinants of the success or failure of delivering both drug and immunotherapy to cancerous cells. Interruptions to these have been exploited to enhance drug efficacy and targeting, but also can lead to reduced efficacy. We describe two key examples: diabetes mellitus, and leaky vasculature associated with cancer.

2.3.1. Diabetes mellitus—An emerging example of a pathology that transforms the cell, ECM, transport, and tissue properties that are critical for delivery of therapeutic agents to tumors is diabetes mellitus. Here, cells, cell-cell interactions, ECM and vasculature all change in response to glycation from high blood sugar levels [129]. For diabetes patients, this translates to substantially diminished prognosis.

At the core of these pathologies is the process of glycation, in which ECM proteins undergo glycation that leads to stiffening of tissues. Here, the high blood sugar levels in diabetes cause an oxidative and non-enzymatic reaction between glucose and collagen [130–132], which in turn can affect the physical properties of the extracellular matrix [133,134]. For the critical case of type I collagen, the effects are easily measurable, with glycated type I collagen matrices exhibiting significantly higher stiffness under shear testing [45]. Similarly, ECM stiffness increases with incubation in glucose and ribose [135]. These effects can change myocardial and liver function, and can damage vasculature [130,136]. This damage to the vasculature and these changes to the ECM have broad and well-characterized deleterious effects on the delivery of drugs to tissues, with the archetypal example being reduced access of antibiotics and the patient's own immune system to tissues affected by foot ulcers and infection; the result is the spontaneous foot ulcers, chronic wounds, infections, and ischemic tissue necrosis that are a hallmark of the disease [137,138].

Although the effects of these glycation on treatments for cancer are less well understood, increases in ECM stiffness are, as discussed earlier, associated with cellular drug resistance, and are therefore expected to increase cancer chemoresistance. Hyperglycemia is associated with poor responses to chemotherapy, with high blood glucose a part of the metabolic syndrome that is associated with a poor response to chemotherapy in breast cancer [139]. Similarly, high glucose enhances cell proliferation, migration, and invasion in gastric cancer,[140] and increases gastric cancer chemoresistance both in vivo and in vitro [141]. Hyperglycemia reduces the antiproliferative effect of 5-Fluorouracil (5-FU) on colon cancer cells [142] and inhibits the apoptosis of prostate cancer cells induced by docetaxel [143].

Although the causality is, again, unclear at present, much circumstantial evidence exists for a role of diabetes and its associated mechanical effects on tissues in poor cancer treatment outcomes. Chemotherapy in diabetes mellitus patients results in lower survival and lower reduction in tumor mass following pancreatic cancer [144]. Diabetes and its mechanobiological effects increase complications of adjuvant chemotherapy in certain populations for breast cancer [145]. Because complications such as neutropenia are also higher among cancer patients with diabetes, chemotherapy can be less efficacious due to the need to

attenuate its severity [146]. Overall, the mechanobiological effects of diabetes mellitus appear to affect the delivery, uptake, and dosage of drug and immunotherapies.

2.3.2. Leaky vasculature—In many cancers, tumor growth is accompanied by hastily formed irregular vasculature with endothelial cells that do not connect as well as those in healthy tissues [147–149]. The gaps between endothelial cells give rise to a "leaky vasculature" that leads to the "enhanced permeability and retention" (EPR) effect. This leakiness and the associated retention of nanoparticles and small molecules has long been proposed and exploited as a mechanism for delivering drugs specifically to the tumor site [147,150]. The leaky and aberrant blood vessels also lead to decreased blood supply to tumors causing hypoxia that is associated with drug resistance.

Irregular and leaky tumor vasculature is also responsible for increased interstitial pressure in the tumor microenvironment, which in turn affects the tumor growth and metastasis as well as drug delivery. For example, a recent modeling study of glioma showed that leaky vasculature and elevated interstitial fluid pressure (due also to lack of lymphatic drainage) produced tensile stress within the tumor in opposition to the compressive stress produced by tumor growth, leading to elevated stiffness in the tumor rim [151]. Cancer cells respond to the elevated interstitial pressure in the tumor by altering their proliferation, apoptosis, migration, and metastasis. For a review on the role of the cancer cell cytoskeleton and the nucleus in mediating cancer cell response to elevated interstitial pressure, the readers are referred to the following article [152]. For a review on the role of fluid mechanics in cancer and cancer therapy, the readers are referred to the following article [153].

Besides the leaky vasculature changing tumor tissue mechanics, it is now being understood that it could also be caused by changed mechanics. For example, a study of cancer-associated fibroblasts (CAFs), which have enhanced mechanical activity regulated by the Rho-ROCK pathway (compared to normal fibroblasts), lead to increased vascularization in a 3D gel-based model of vasculogenesis compared to normal fibroblasts, due in part to increased mechanical deformations of the 3D gel (Figure 5) [154]. In another study by the same group, the authors developed a microfluidic device to mimic vascularized tumors and allow for decoupling of interstitial flow and mechanical strain and showed that higher mechanical strain induced by CAFs promoted tumor angiogenesis, even though it prevented diffusion of soluble factors to stimulate the growing vasculature [155]. A recent screening study of a novel drug that induces apoptosis in CAFs, has shown that in addition to decreased cancer cell proliferation and apoptotic resistance, the drug reduced intratumoral collagen and eliminated leaky tumor angiogenic vessels, which consequently reduced tumor hypoxia and improved drug delivery [156]. Such findings imply that the tumor vasculature could affect tumor drug and immunotherapy responsiveness not only by allowing nanoparticle accumulation (leaky vasculature) or hindering efficient chemotherapy delivery (aberrant vasculature), but through mechanical signaling. For a comprehensive review on the how mechanical cues from the tumor microenvironment promote aberrant tumor angiogenesis and its impact on tumor progression and therapeutic treatment, the readers are referred to the following article [157].

3. Biomaterial platforms to study the role of mechanobiology in cancer cell responsiveness to chemotherapy and immunotherapy

It has been broadly recognized that screening cell lines on unnaturally rigid plastic substrates does not properly recapitulate in situ cell responsiveness to therapies [158]. Consequently, more complex platforms have been developed to study cell mechanobiology and associated chemotherapy and immunotherapy responsiveness (Figure 6). The simplest and most heavily used platforms include hydrogels and other biomaterials spanning a wide range of stiffnesses in either a discrete fashion or in the form of a gradient or a pattern. A more recent innovation is developing biomaterial platforms with dynamically switchable stiffness. In either 2D or 3D context, such dynamic materials can be stiffened or softened in the presence of cells. Other platforms that provide for mechanical manipulation of cells and manipulation of cell-ECM interactions include microfluidic devices and custom bioreactors. Added benefit of such biomaterial-based platforms is that they enable cell co-cultures with support or immune cells. Here we give a brief description of these major platforms and focus on the ways in which they have enabled the study of drug and immunotherapy resistance and responsiveness.

3.1. Two-dimensional hydrogel platforms

Perhaps the most heavily utilized and arguably simplest platform to study cell mechanobiology has been the polyacrylamide gel, which has led to many seminal discoveries [3,159]. Polyacrylamide gels are formed by free radical polymerization between an acrylate monomer (Ac) and a bisacrylate (Bis) crosslinker, where stiffness can be modulated by varying the concentration of each component as well the ratio between the two [85,160]. Once polymerized, polyacrylamide gels are non-cytotoxic and span a Young's modulus of ~0.1 – 300 kPa, which encompasses the stiffness of most biological tissues [161]. Polyacrylamide gels can also be fabricated via photopolymerization, where stiffness gradients can be created by simply adjusting light exposure time for different parts of the same gel [162]. Further, to facilitate the high-throughput requirement for drug screening and the study of stiffness-dependent cell biology, we and others have developed multi-well polyacrylamide gel platforms (Figure 7) [83,163]. Despite its broad use, polyacrylamide gels have some limitations. For example, because of the toxicity of the individual monomers and the nanoporosity of the resulting gel, polyacrylamide gels cannot be used as 3D substrates. Further, polyacrylamide gels are synthetic materials and adhesive ligands need to be added to elicit cell attachment. While a limitation, the gel inertness allows for decoupling biomechanical and biochemical contributions to cell behaviors, which is of particular importance in the study of cell mechanobiology.

Not surprisingly, polyacrylamide gels have been used extensively to study cell responsiveness to drugs. For example, we have previously shown that drug responsiveness is cell-type dependent [83]. In another study, fibronectin-coated polyacrylamide gels of increasing stiffness were used to study chemotherapeutic responses of primary and immortalized breast cancer cells [158]. The authors demonstrated that primary cells underwent phenotypic changes when cultured on stiff rigid substrates, which further led to high susceptibility to the chemotherapeutic drugs paclitaxel and doxorubicin. On the other hand,

when the same cells were cultured on soft substrates, they had similar gene expression profiles to in situ tumor cells and low susceptibility to paclitaxel and doxorubicin. In another study collagen I-coated polyacrylamide gels (0.4 – 40 kPa) were used to study HER2-amplified breast cancer cells response to the a HER2 receptor tyrosine kinase inhibitor lapatinib [164]. Yes-associated protein (YAP) and WW-domain-containing transcription regulator 1 (WWTR1; also known as TAZ) activation correlated with resistance to lapatinib, and when YAP was knocked out in orthotopically implanted tumors grown in mice, tumor growth slowed, and they became more sensitive to lapatinib.

Another simple 2D hydrogel platform for studying the role of mechanosensing in cellular responses to drugs is the synthetic polydimethylsiloxane (PDMS) gel. PDMS gels offer orders-of-magnitude modulus tunability from kPa to MPa, controlled independently of other material properties, which is not achievable by most any other hydrogel system [165]. PDMS gradient gels can be prepared with high fidelity by means of a temperature gradient during curing [166]. Methods are also being developed to produce high quality 2D and 3D PDMS substrates by additive manufacturing techniques such as electrohydrodynamic inkjet printing [167]. In one study PDMS substrates with different stiffness (mimicking articular cartilage, collagenous bone and mammary tumor, respectively) were used to study the responses of breast cancer MCF-7 cells to the antitumor drugs, cisplatin and paclitaxel [168]. The authors showed that cell sensitivity to the drugs was highly enhanced on the stiff compared to the soft substrates, which was attributed to increased cell cycle progression on stiff substrates.

3.2. Three-dimensional hydrogel matrices

Multiple studies have shown that cells cultured in 3D environments, which are more physiologically relevant, are more resistant to drugs then their monolayer counterparts [71,72,169]. On the other hand, studies have shown that a fully confluent cell monolayer can show increased drug resistance similar to a 3D culture due to both decreased drug penetration and higher intrinsic resistance of confluent cells [170,171]. Note that cells are typically seeded at sparse density (typically 4×10^4 cells/mL on a 2D substrate) for drug screening. Despite that, 3D hydrogel cultures provide conditions not available in 2D monolayers, such as cell-ECM interactions, tethering of growth factors and other biomolecules directly to the gel to guide cell fates, and the ability to form co-cultures with precise spatial patterns. Multiple hydrogels with tunable stiffness, including gelatin methacrylamide, polyethylene glycol (PEG), alginate, silk, and hyaluronic acid (HA) have been developed [172] and could be adapted for use in drug screening platforms. However, hydrogels are intrinsically soft and excessive crosslinking, which is typically used to achieve higher stiffness, could also lead to diminished nutrient and oxygen diffusion.

While it has been established that 3D cell culture technologies can improve precision in drug discovery, there are multiple challenges in applying 3D cultures for high-throughput screening (HTS) of drugs and immunotherapies. Those include labor intensiveness and material cost, scalability to 384- and 1,536-well plates, reproducibility, incorporation into an automated screening setup (e.g. liquid handlers), compatibility with currently available assay and detection methods, and visualization of 3D structures with automated imaging systems [173]. Development of new 3D platforms for drug discovery should take into

account HTS compatibility, while also being versatile and tunable to emulate the in vivo microenvironment [174]. Synthetic hydrogels are typically preferred for drug screening applications due to their reproducibility and their wide range of properties, which can be tuned with high precision. Biochemical cues can be selectively added to synthetic hydrogels to support cell adhesion and other desirable cell behaviors and to emulate the in vivo environment with more fidelity. While some natural hydrogels can be fabricated with reproducible properties (e.g. agarose, alginate), most cell-adhesive natural hydrogels (e.g. Matrigel, collagen) suffer from batch-to-batch variability and cannot be tuned to cover a wide range of mechanical properties. Hence, their use in high-throughput drug screening applications is limited. Lastly, the number of high-throughput methods applicable to stiffness appear to be limited because the range of stiffness is often inadequate due to the choice of polymer and crosslinking methodology. Here is a recent review on high-throughput fabrication of 3D cell laden biomaterials [175].

For many nanoporous hydrogels, cells need to be added to the hydrogel precursor solution and encapsulated during gelation. While this assures immediate and homogeneous cell distribution within the hydrogel, it does not allow the preparation of hydrogels in multiwell plates and other high-throughput formats in advance. To decouple hydrogel production from cell seeding, Zang *et al.* [176] developed a 96-well plate containing pre-cast, MMP-degradable PEG hydrogels with in-depth density gradient at the surface to promote the infiltration of cells deposited on top of it. The one drawback of the system was the time required to accomplish cell infiltration: it took 3 days for the cells to reach a depth of 200 μm and 10 days to reach a depth of 500 μm. Despite the above limitations, 3D platforms have been invaluable in understanding the role of mechanosensing on cell drug responsiveness [84]. For example Shin *et al.* [177] used alginate hydrogels to demonstrate in vitro and in vivo that matrix softening accelerated cancer growth kinetics and caused resistance to standard chemotherapy in myeloid leukemia cells (Figure 8). In another study, a methoxypolyethylene glycol (mPEG)-modified chitosan hydrogel was used to show that increased hydrogel stiffness promoted increased resistance of breast cancer cells to doxorubicin [178].

3.3. Electrospun scaffolds and cryogels

Electrospun matrices and cryogels are characterized by microporosity to macroporosity and high permeability, which makes them excellent cell scaffolds. Due to their large pores, cells can move, proliferate, and infiltrate the scaffold and experience minimal gradients of nutrients and oxygen. It could be argued that macroporous scaffolds represent a bridge between 2D and 3D materials as a typical cell would only “see” the surface of the pore and might not experience a true third dimension. To control stiffness of such materials, in addition to manipulation of polymer concentration or crosslinking, one can control the fiber diameter or wall thickness with thicker walls and fibers leading to higher stiffness. Another general rule is that higher porosity or larger pores (void spaces) typically correlate with lower modulus materials. For electrospun scaffolds, polymeric composition, followed by fiber orientation and fiber diameter (in that order) are the main factors that determine their elastic modulus [179]. Additionally, processing parameters such as polymer weight ratio (for multipolymeric scaffolds), mandrel speed and orientation can have an indirect impact

on the modulus by affecting fiber diameter or orientation. Other techniques to control the mechanical properties of electrospun scaffolds are sintering (increases modulus), salt leaching (decreases modulus) [180], or ice crystal formation (decreases modulus) [181]. For cryogels, mechanical properties are modulated through controlling the rate of freezing, ice crystal formation, and polymer concentration, where smaller pores and highly concentrated polymer phase, correspond to higher modulus [182,183].

One study developed elecrospun scaffolds to mimic the native environment of prostate cancer bone metastatic cells [184]. The authors showed that cells on electrospun substrates were more resistant to docetaxel and camptothecin compared to cells grown on collagen-coated tissue culture polystyrene. Another study used coaxial electrospinning of gelatin and polycaprolactone (the most widely used material combination for electrospun scaffolds) with tunable mechanical properties (modulus ranged between ~2 and 60 MPa) as 3D osteosarcoma models [185]. Osteosarcoma cells responded to decrease in substrate stiffness by increasing nuclear localization of YAP and TAZ (Hippo pathway effectors), while downregulating total YAP and increasing resistance to combination chemotherapy compared to monolayer controls. In another study, a cryogel made of PEG-diacrylate and gelatin methacrylamide was shown to support the formation of breast cancer spheroids in the absence of additional growth factors, but only when a low concentration of gelatin methacrylamide (1% w/v) was used [186]. The cryogel-grown spheroids exhibited more resistance to paclitaxel compared to 2D grown cells, which the authors attributed partially to the epithelial to mesenchymal transition observed in spheroids. Another group used cryogel scaffolds to develop high-throughput platforms for drug screening applications and again showed significantly higher drug resistance in the cryogels compared to cells seeded on 2D plastic dishes [187]. In another interesting approach, cryogels of micro sizes (microcryogels) were fabricated, loaded with cells to create microtissues and assembled on a chip into 3D microtissue arrays for high-throughput drug screening [188].

3.4. Hydrogels with stiffness gradients

A variety of gradient stiffness hydrogels have been developed and have brought excellent insights into cell mechanosensing [189]. For recent detailed reviews on gradient stiffness hydrogels, the readers are referred to the following reviews [190,191]. Stiffness gradients in hydrogels can be made by controlling the degree of crosslinking [162] or polymer concentration, by controlling gel thickness [192], by layering hydrogels of different stiffness [193], by blending different polymers [194], or by using a syringe pump in combination with photocrosslinking to flow more polymer solution during active polymerization [195]. Stiffness gradient hydrogels can be coupled with microfluidic devices [196] and be presented as both 2D substrates [162] and 3D matrices [197]. Stiffness gradients could also be coupled with biochemical and other physical gradients such as porosity [198] or even with gradients of soluble biomolecular cues [199] or oxygen [200] using microfluidic approaches. They can also be made compatible with high-throughput screening technologies [197].

While stiffness gradient gels have been used extensively in the study of cell mechanosensing, they have rarely been used in drug screening, where single stiffness gels are preferred. This could be due to the fact that most cell viability assays (e.g. colorimetric

assays) used to determine drug efficacy (e.g. 50% effective concentration or IC_{50}), give results on all cells in a particular area, making it hard to pinpoint the role of stiffness in a gradient gel. On the other hand, viability assays that allow for probing specific cells in defined gel areas (e.g. live/dead staining) are usually time consuming and more costly to perform. Also, since cells are known to migrate along the gradient, viability data in relation to gel stiffness might be hard to interpret. Still, several groups have performed drug screening on cells seeded on gradient gels. In one example, Lam *et al.* [201] seeded MDA-MB-231 breast cancer spheroids at the interface of dual stiffness collagen gels, namely 0.3–1.2 kPa and 0.3–6.0 kPa. The spheroids infiltrated the softer matrix more significantly than the stiffer matrix. They also suffered from apoptosis earlier when treated with paclitaxel compared to cells in stiffer matrices, suggesting that reduced invasion in the stiffer matrix could be linked to reduced drug sensitivity. Our lab has shown that when U87 GBM spheroids were seeded at the stiffness interface in dual-stiffness PEG hydrogels, spheroid cell invasion was observed away from the interface with only individual cells migrating along the stiffness interface [202]. Spheroids also showed similar responses to TMZ treatment in the soft and stiff gels, but cell viability was higher in the spheroid periphery than the core for stiff gels and in the core for soft gels [202]. In another study, glioblastoma xenograft cells were seeded in gradient PEG hydrogels with 5 stiffness zones spanning a range from ~150 to 1300 Pa (Figure 9) [195]. Cells were cultured for 21 days and either formed spheroids in the stiffer regions or interconnected networks in the softer regions and showed higher susceptibility to temozolomide in the softer compared to the stiffer gel regions. The study did not address cells at the stiffness interfaces. Lastly, Wang *et al.* [200] developed a microfluidic device to generate a stiffness gradient over an oxygen gradient created by using oxygen scavengers. The authors treated lung cancer A549 cells with the hypoxia sensitive anti-cancer drug triapazamine and demonstrated matrix stiffness-dependent cell drug resistance and hypoxia-induced cytotoxicity of triapazamine.

3.5. Patterning and topography

Substrates with topographical features, such as microposts, micropillars or microridges have also been used extensively to study cell mechanotransduction; various fabrication techniques and uses in cell mechanics and mechanobiological studies are reviewed in the following articles [203,204]. It should be noted that micropillar arrays affect both cell and nuclear shape and both need to be considered in studies of cells on topological surfaces [205]. Micropillar arrays are usually made of PDMS or silicone [206] and offer reproducible and well-defined cell microenvironments. Substrate stiffness can be modulated by changing parameters such as micropillar height, width and spacing [206] as well as polymer concentration or crosslink density [207]. The micropillar technique can also be adapted to develop stiffness gradient gels by using gradients in micropillar height [208] or spacing [209]. A gradient gel has also been achieved by embedding magnetic beads in a micropillar array and then applying a magnetic field gradient in its vicinity [210]. Also, multiple approaches have been developed to mimic the hierarchical structure of the natural extracellular matrix by combining both nanoscale and microscale topographies in the same substrate to influence cell behaviors [211]. Lastly, micro- and nanopillar arrays can be fabricated in multi-well plates amenable to semiautomated acquisition, detection, and quantification, and hence, adapted for high-throughput screening of therapeutics [212].

It should be noted that surface topography alone can modulate cell stiffness and mechanical forces. For example, studies have shown that microtopography-induced cell shape changes lead to differential single cell stiffness [213]. It has also been shown that nanotopography alters integrin clustering and focal adhesion assembly, which in turn leads to changes in cytoskeletal organization and single cell stiffness [214]. Other exciting studies have used micropatterning to examine the interplay between substrate stiffness and geometric confinement and have shown that interfacial cues guide CAFs migration and direct cancer cell assembly [215]. In another study, substrate curvature and confinement have been shown to lead to a higher probability of cancer cells expressing stemness markers [216]. While micro- and nanopillar arrays are mostly used to study cells on 2D environments, patterning could be used to provide spatiotemporal control of stiffness and viscoelasticity in 3D matrices [217]. A common approach to pattern 3D materials and enable stiffening of defined regions is by photoillumination [218] or click chemistry [219].

Micropillar arrays assembled in multi-well plates offer a great potential to be used in high-throughput anti-cancer drug screening. For example, a recent study has shown that micropillar arrays, through modulation of micropillar rigidity and topography, could induce epithelial-to-mesenchymal transition (EMT) without the use of exogenous cytokines, highlighting the utility of such arrays as drug screening platform (Figure 10) [220]. In yet another study it was shown that pillar-based mechanical stimuli can be used to induce enhanced ameboid-like migration in A549 cells and more aggressive tumorigenic cancer cell models in general [221]. In another study A549 cells incubated on micropillars showed EMT-like behavior and FAK activation - a hallmark of cancer cell adhesion and migration, typically induced by TGF-β [222]. The authors then used this platform to screen a drug candidate with activity against TGF-β-induced cancer cell metastasis with favorable results. In another interesting application, PDMS micropillars of high aspect ratio were used as peripheral flexible force sensors by trapping tumor spheroids within a micropillar circle, showing potential for use in drug screening [223].

3.6. Hydrogels for in situ dynamic stiffness modulation

Many groups have developed both 2D and 3D hydrogel systems that enable dynamic modulation of substrate modulus in the presence of cells. We give a brief summary of several such systems and refer the readers to some excellent detailed reviews on dynamic hydrogels to emulate ECM complexity [224–227]. It is important to note that such dynamic systems are yet to be used in drug screening applications and could represent an exciting new frontier.

As mentioned earlier, single stiffness PDMS gels have already been used in drug screening applications and some new developments have led to dynamically switchable PDMS gels. Yeh *et al.* [228] developed a 2D PDMS substrate with tunable mechanical stiffness spanning from 3 to 200 kPa. This was achieved by a two-step reaction, where PDMS was first gelled by platinum-catalyzed crosslinking and then a thiol-ene click photopolymerization reaction was used to increase the crosslinking and stiffen the substrate. The photopolymerization reaction was carried out in the presence of cells and increased the modulus up to 10-fold within minutes. Some limitations of this system include the irreversibility of the stiffening

reaction and the fact that this process could only be done once. A PDMS with reversible dynamic stiffening and softening was also developed by incorporating magnetic particles in the substrate [229]. The soft PDMS was stiffened and then softened nearly instantaneously in the presence of cells by applying a magnetic gradient, where the magnetic field was manipulated by the distance of the magnet from the substrate. This allowed an incremental increase in modulus from 10 to 60 kPa, which was completely reversible.

A 3D thermoresponsive hydrogel where reversible stiffness has been incorporated consisted of gelatin methacrylamide hydrogel network interpenetrated by a poly(N-isopropylacrylamide-co-2-hydroxyethyl methacrylate) (NIPAM-HEMA) nanogel [230]. The stiffness of this material was then regulated in situ in the presence of cells by reversibly stiffening soft niches via multicyclic temperature changes from 25 to 37 °C. Further, based on the initial concentration of gelatin methacrylamide, the stiffness ranges achieved by this system ranged from 80–120 Pa in G' (for 1.5 w/v% gelatin methacrylamide) to 800–4000 Pa in G' (for 3.5 w/v% gelatin methacrylamide). A similar thermoresponsive gelatin-based hydrogel with dynamic modulus regulation has been developed by another group [231]. Here the authors used norbornene substituted gelatin (GelNB) photocrosslinked with the thermoresponsive poly(N-isopropylacrylamide-s-2-hydroxypropyl methacrylate-s-mercaptoethyl acrylate) using thiol–norbornene reactions. A storage modulus of ~5–19 kPa was achieved by varying the thiol to ene stoichiometric ratios and the hydrogels could be softened by 2.7–3.5 kPa using thiol–disulfide exchange reactions in the presence of cells.

Polyethylene glycol (PEG)-based hydrogels have also been developed as both 2D and 3D materials with dynamic compliance modulation. Kloxin *et al.* developed a 2D photodegradable PEG hydrogel which could be degraded in the presence of cells via UV irradiation generating a range of 32 to 7 kPa in Young's modulus [232,233]. Another type of a 3D in situ softening PEG hydrogels were developed by crosslinking PEG with heparin-based polymers via Michael-type addition and then degrading those by externally applied light [234]. Dynamically stiffening 3D PEG hydrogels (from 0.2 to 13 kPa) have also been developed by using MMP-degradable 8-arm PEG-norbornene hydrogels and then stiffening them in situ via a second, photoinitiated thiol-ene polymerization with 8-arm PEG-thiol [235]. More details on hydrogels with photoswitchable stiffness in particular can be found in the following book chapter [236]. Stowers et *al.* [237] developed a different hydrogel system where light was used to temporally soften or stiffen the material in situ. The authors used calcium-crosslinked alginate where stiffness was modulated by calcium concentration. The dynamic nature of the modulation was achieved by embedding temperature-sensitive liposomes loaded with gold nanorods and either $CaCl_2$ (calcium crosslinker; stiffening) or DTPA (calcium chelator; softening) and irradiating the gel with near-infrared light to trigger the release of $CaCl_2$ or DTPA. That led to a reversible 3D gel stiffening in the presence of cells achieving storage moduli from 91 Pa to 1,179 Pa after 180 s of irradiation.

Another interesting chemistry for developing 3D hydrogel matrices with on-demand tunable stiffness is by using host-guest interactions. Shih et *al.* developed hydrogels formed by thiol–allylether photo-click reaction between thiolated poly(vinyl alcohol) (TPVA), 4-arm poly(ethylene glycol)-allylether (PEG4AE), and mono-functional β-cyclodextrin-allylether (βCDAE) [238]. Hydrogels were stiffened by soaking in adamantane-functionalized 4-arm

PEG (PEG4AD) and softened by soaking in unmodified βCD. The process was fully reversible and cytocompatible and resulted in a moduli range of 0.03 to 6 kPa. Another team developed coumarin-functionalized hydrogels formed via host–guest mediated self-assembly with cucurbituril that could photo-switch to covalent gels and reversibly toggle between the two states spanning a storage modulus range of 0.074 kPa to 4.1 kPa [239].

Other approaches for dynamically tuning hydrogel stiffness include DNA crosslinking of the polymer chains. In one system two DNA strands were covalently attached to polyacrylamide polymer chains and crosslinker DNA strand base-paired with two other strands, forming a crosslink (similar to a zip) [240]. The gelation was reversed by introducing a complement to one of the DNA strands attached to the polymer. Lastly, while the above described systems span a moduli range from Pa to kPa, dynamic modulation in the MPa range has been described in polycaprolactone (PCL) polymers [241]. The authors achieved a modulus of ~1.4 to 61.1 MPa at 37 °C by heating and then cooling PCL in a narrow temperature range of 30–43 °C allowing a phase change between crystalline and amorphous domains.

3.7. Platforms to study immunotherapies

Immunotherapies, such as heat shock protein-based therapies, immune checkpoint inhibitors, poly [ADP-ribose] polymerase inhibitors, dendritic cell vaccines, (CAR) T cell therapy, targeted delivery of stimulatory cytokines, adoptive transfer methods and combination therapies [242–245], are gaining momentum for the treatment of cancer. Unlike chemotherapy treatments which target tumor cells, immunotherapies generally work by restoring the immune system's ability to eliminate the tumors. At the same time, it has been established that mechanical forces play an important role in regulating the interaction and function of immunoreceptor-ligand pairs for a variety of immune cells [246]. Further, activated B cells, which secrete antigen-specific antibodies and cytokines that exert regulatory stimuli on other immune cells, can play an active role in the treatment of cancers and are known to respond to substrate topology and stiffness [247]. Overall, immunotherapy has been less successful against solid tumors, partly because of its focus on the biological and chemical mechanisms and less on the physical and mechanical mechanisms involved in combating cancer. Mechanoimmunology studies have recently emerged to fill this gap and deepen our understanding of the role of mechanosignaling, mechanosensing, and mechanotransduction in immunotherapy efficacy [248].

The correlation between cell mechanosensing and cancer response to immunotherapy could be further investigated trough the development of biomaterial-based platforms that include components of the immune system, such as cells and/or biomolecules. Such platforms could resemble the ones already discussed in this review with the added complexity of immune system component incorporation. For example, microfluidic platforms have shown useful for evaluating cancer-immune cell interactions with the goal of assessing the efficacy of emerging immunotherapies [249]. Microfluidic platforms allow versatile set-ups, where immune cells could be perfused above adherent or matrix embedded cancer cells, immune and cancer cells could be localized in adjacent chambers, immune cells could be "recruited" through chemotactic gradients, etc., to answer different mechanistic questions [249]. In another study, an injection molded plastic array culture device was integrated within

a 96-well plate to allow for high-throughput screening (Figure 11) [109]. Here cancer cells were embedded in collagen and exposed to cytotoxic lymphocytes in the culture media, to mimic the physical barrier presented by the extracellular matrix which hinders the migration, access to cancer cells and therapeutic efficacy of cytotoxic lymphocytes in vivo [109]. Further, ex vivo models of fresh tumor biopsies and surgical excisions that preserve the cellular and microenvironment heterogeneity including the immune compartment, have been used to predict the clinical efficacy of anti-cancer and immuno-oncology drugs, such as immune checkpoint inhibitors [250,251]. Other ex-vivo models include tumor organoids from patient derived cells that could facilitate screening of immunotherapies including cytokines, checkpoint inhibitors, or CAR T therapies on an individual basis [252]. Note that for ex vivo models, timing is important because initially preserved immune cells and microenvironmental characteristics can be lost and diluted over as cells adapt to in vitro culture conditions. For a more detailed review on the engineering approaches and 3D models for screening immunomodulatory drugs, the readers are referred to the following reviews [253,254].

4. Animal models to study the role of mechanobiology in cancer chemotherapy and immunotherapy

Although the in vitro models are far more effective and less expensive for screening chemo- and immunotherapeutic agents, in vivo models are essential prior to translation of candidate drugs to patients. At present, the use of animal models for evaluating the effects of mechanobiology on cancer cell responsiveness to chemotherapy and immunotherapy is very limited. However, these efforts are developing and we provide a review here of challenges and opportunities associated with the using for this purpose two of the most common small animal models in cancer research: mice and zebra fish.

Small animal models are generally cheaper, less time consuming, easier to maintain than large animal models. They reproduce more often, allowing for higher throughout studies. Because of this, they are desirable in chemotherapy and immunotherapy screening applications. Tumor models using these small animals can be xenografted (including patient-derived xenografts), syngeneic, transgenic, carcinogen-induced or spontaneous. Each of these offers different levels of complexity, physiological relevance, and predictability of the human response. For recent comprehensive reviews on animal models in cancer research, readers are referred to the following [255,256]. Our focus here is the state of efforts to identify roles of mechanobiology in treatments using these models.

4.1. Mouse models

Mouse models are the most common models used in cancer research and have proven useful in understanding the molecular and cellular mechanisms of tumor initiation and growth as well as serving as pre-clinical models for therapy testing [257]. Some limitations of mouse models include inability to fully reflect the complex human tumor, low-throughput, and limited modalities for in vivo imaging and data analysis. Different mouse models include syngeneic or xenograft cancer cells implanted subcutaneously or orthotopically in mice, which are widely used due to their low cost and availability. Transgenic models

can be developed by constitutively or conditionally expressing oncogenes, silencing tumor-suppressor genes, or through CRISPR/Cas9 genome editing [258] and offer a designer approach to mimic various aspects of human cancers but are more costly. Various mouse models have also been developed or could be adapted for cancer immunotherapy research [259]. Specifically, refined and humanized genetically engineered mouse models could be invaluable in anticancer drug development, including target validation, assessment of tumor response or resistance to therapy and investigation of drug pharmacokinetics and pharmacodynamics [260].

Mouse models have been used to highlight the effect of mechanobiology on cancer treatment, where tissue mechanics could be modulated through methods such as external mechanical loading or targeted softening of the tumor microenvironment. For example, it has been shown that increased mechanical stimuli from exercise and controlled mechanical loading can have antitumorigenic effect and can mitigate metastatic tumor-induced bone disease [261]. In a different example, a breast cancer xenograft mouse model was treated with free paclitaxel or reactive oxygen species-activatable nanoenzyme (SP-NE) developed by the group to show that matrix softening sensitized the tumor to chemotherapy [262]. These dual-action nanoenzymes were disassociated in the presence of reactive oxygen species, leading to collagenase release and generation of paclitaxel prodrug. The authors demonstrated an enhanced chemotherapeutic efficacy of SP-NE (compared to paclitaxel alone) due to downregulation of integrin-FAK-RhoA and integrin-FAK-pERK 1/2 signaling [262]. Another study used a similar approach of matrix softening to sensitize liver-metastasized colorectal cancer cells to the drug bevacizumab, based on the observation that in liver metastasized tumor, matrix stiffness is higher compared to the primary colorectal tumor [263].

Matrix stiffness can be used to improve drug delivery to cancer tissues. For example, a patient-derived orthotopic xenograft mouse model of glioblastoma has been used to implant mechanically matched (between the implant and the brain) hyaluronic acid hydrogels for chemotherapeutic delivery [264]. Doxorubicin and gemcitabine-releasing hydrogels with mechanical properties tailored to lie within the range associated with brain parenchyma yield improved drug bioavailability and increased survival rate of up to 45% [264].

A very promising advance in the application of mechanobiology to cancer treatment has been enabled by the study of mouse models. Mouse models reveal a correlation between the ECM stiffening associated with recovery from cancer resection surgery and the subsequent increase in cancer metastasis to the lungs [265]. Here, both mice that underwent surgery and control mice that were pre-conditioned with plasma from mice that underwent surgery had lower survival rates following injection with EMT/6-GFP+ breast cancer cells that metastasize to the lungs. This was attributed to increased lysyl oxidase activity and expression (due to hypoxia at the surgical site), which in turn leads to collagen crosslinking, focal adhesion signaling, and finally matrix stiffening and increased cancer cell metastasis to the lungs. The effect could be reversed when the matrix was softened via treatment with a collagen crosslinking inhibitor, showing a potential mechanobiological pathway for chemotherapeutic treatment.

An additional example is that lysyl oxidase and matrix stiffening promote metastasis of mouse mammary carcinomas deficient in transforming growth factor-β.[266] This implies that the rigidity of the matrix of a potential metastatic site can influence cancer cell homing and secondary tumor formation, and could be a treatment target.

4.2. Zebra fish models

Zebra fish models are useful alternative models as they can support multiplexed or high throughput studies and are associated with lower costs and time investment compared to other animal models. They are amenable to pharmacological testing, and they have transparent bodies which allow for real-time live imaging of cancer progression. In addition, the majority of human genes have at least one zebrafish orthologue [267]. Not surprisingly, multiple zebra fish cancer models have been developed, including tumors in various organ sites that resemble human tumors histologically and genetically.[268,269] They have been used as drug discovery platforms [270], and transgenic and xenograft models exist for the potential development of personalized chemotherapy treatment regimens [271,272].

In the context of mechanobiology, mechanotransduction pathways can be replicated and studied in zebra fish. For example, using a transgenic model, Chew et al. have shown that signaling crosstalk between Kras and RhoA regulate liver overgrowth and tumorigenesis and that Rho activation could suppress Kras-induced liver malignancies [273]. Zebra fish can also be used to study the effect of the tumor microenvironment on cancer cells. For example, cancer cells have been implanted in fish tissues to study a range of mechanical and biochemical characteristics, and transgenic models exist to silenced or overexpress specific genes or to tune the microenvironment [274]. The optical transparency of zebrafish has enabled the study of the tumor cell-vascular interface [275], the extravasation of tumor cells across the vasculature [276], and the contribution of biomechanics to the extravasation of circulating tumor cells showing a direct link between hemodynamic forces and metastasis [277].

In another example, Paul et al. showed that several human brain- and bone-homing breast cancer subclones colonize analogous tissues in zebra fish larvae [278]. They then showed that bone marrow homing was related to high integrin expression and focal adhesions associated with mechanosensing, while brain homing was guided by vessel topography during extravasation [278]. In addition, transgenic zebra fish models can be created where host immune cells endogenously express fluorescent proteins to enable the studies of host cell-tumor interactions [279]. For example, Roh-Johnson et al. showed that macrophages transfer cytoplasm to tumor cells, which correlated with melanoma cell motility and dissemination [280].

Strengths of zebra fish models thus include their modest expense, short intergenerational time, genetic control, and optical transparency. These have been used to discover and modulate aspects of mechanobiology in the zebra fish, and to control the spread and growth of cancer. These models seem to hold potential that has not been tapped for study of aspects of mechanobiology on resistance to chemo- and immunotherapy.

5. Modulation of mechanical factors to improve chemotherapy and immunotherapy

Mechanosensing happens between cells and between cells and their microenvironment. Understanding the mechanisms underlying the differential responsiveness could lead to the development of targeted, stand-alone or adjuvant therapies for the treatment of cancer. Many of the attempts fall under the heading of “mechano-medicine,” a phrase coined by Ning Wang to encompass the application of mechanobiology to intelligently manipulate mechanical factors for positive therapeutic outcomes [55].

A key example that has been attempted in the literature for decades is the usage of ultrasound to target cells or manipulate the cell microenvironment to selectively kill cancerous cells (e.g. [281]). However, these direct mechanical modulation efforts have yet to provide their first clinical treatment of a cancer, with the key challenge being delivering energy to cancer cells without injuring so much of the surrounding tissue as to cause substantial side effects. At the core of these difficulties is the challenge that the key factor in energy absorption – the mismatch of acoustic impedance between the components of a cancerous cell and its surrounding environment – is not sufficiently strong to enable targeted ablation [282].

Further along the pathway to potential application are efforts to manipulate the mechanical properties of tissues for therapeutic effect. Although here, too, no cures for cancer have reached the clinic, several promising results can be found. Many of these are in the category of the aforementioned demonstration improved cancer prognosis in a mouse model following drugs administered to modulate ECM properties as part of a treatment regime,[265] in which simply changing mechanobiological factors can alter the progression of pathology. However, our focus here is examples in which mechanical modulations enable

A key example is the heat shock protein (Hsp) 47, a collagen-binding glycoprotein essential in the maturation of collagens, but that leads to fibrosis in overabundance and to pathologies including osteogenesis imperfecta when mutated [283]. Hsp47 has been explored as a target for tumors with dense ECM, such as pancreatic ductal adenocarcinoma, where activated pancreatic stellate cells (PSCs) are responsible for excessive ECM production [284]. In this study, a dual-action therapy of PEGylated polyethylenimine-coated gold nanoparticles that delivered both all-trans retinoic acid (to induce PSC quiescence) and siRNA to target Hsp47 led to decreased ECM density, which in turn supported drug delivery and enhanced chemotherapy efficacy.

Similarly, another study used proteases against collagen and other ECM proteins found in brain tumor tissue but not in normal brain parenchyma, and showed that direct protease pre-treatment enhanced the efficacy of adenovirus-mediated glioblastoma gene therapy by facilitating virus transfection [285]. Although more work is needed to understand the mechanisms underlying these desirable effects, possible explanations include mechanobiological factors. Mechanical factors in the ECM that resist diffusion could possibly be affected by protease pre-treatment, and favorable cell mechanobiological responses might be triggered by proteolytic changes in ECM structure and mechanics.

Another therapeutic target for matrix softening has been lysyl oxidase (LOX), which catalyzes the conversion of lysine moieties into aldehydes that then crosslink collagen and elastin, resulting in ECM stiffening. Since LOX is upregulated by tumor cells and promotes metastasis and malignancy, LOX inhibitors (e.g. β-aminopropionitrile, aminomethylenethiophene, Simtuzumab) have been tested alone or in combination with chemotherapy and have shown antitumor effects against various cancers such as breast, colorectal and pancreatic cancer [286]. In cases where ECM degradation does indeed prove efficacious in enhancing the function of chemo- and immunotherapies, LOX inhibition may hold potential as an adjuvant.

In addition to ECM softening, mechanotransducers such as integrins, present alternative therapeutic targets. One of the most sought after targets is integrin α_v, because it is generally not expressed in epithelial cells, but is involved in tumor angiogenesis and metastasis. For example, the monoclonal antibody Intetumumab (CNTO 95) has been shown to interrupt α_v-activated pathways associated with focal adhesions and cell motility, reducing breast cancer growth and metastasis in mouse models [287] and has been tested alone or in combination with chemotherapy for melanoma treatment in humans [288]. Abituzumab, another monoclonal antibody that targets all α_v integrins, has shown overall limited clinical efficacy in patients with metastatic colorectal cancer when administered in combination with EGFR inhibitor cetuximab and chemotherapeutic irinotecan [289]. However, patients with high $\alpha_v\beta_6$ expression did benefit from the antibody, suggesting that a priori stratification for $\alpha_v\beta_6$ levels might be needed. Volociximab, an $\alpha_5\beta_1$-inhibiting antibody, has also been shown to block angiogenesis and tumor growth in xenograft models, but has shown less efficacy in clinical trials for ovarian and peritoneal cancers [290]. Small molecule integrin inhibitors, such as the RGD mimetic cilengitide, have also been tested alone or in combination with chemotherapy for prostate cancer, non-small cell lung carcinoma, and glioblastoma, but no clear positive outcomes have been noted [291]. An exciting new frontier is the multiple natural agents currently being tested alone or in combination with other therapies as cancer treatments. For example, curcumin has been reported to regulate various integrins such as β_1 and $\alpha_6\beta_4$ in different cancers and has entered clinical trials [292]. The chemo- and immunotherapeutic targeting of integrins, key mediators cell mechanobiological responses, suggests promise for targeting other mechanobiological pathways in cancer treatment. For a detailed review on integrins as therapeutic targets in cancer treatments, the readers are referred to the following review, reference [293].

A final mechanobiological target we will mention is the YAP and TAZ co-effectors of the Hippo pathway. These localize to the nucleus in response to mechanical stimuli to trigger downstream signaling [294–296] associated with matrix stiffness and tumorigenesis, and are thus an important therapeutic target. For example, the YAP inhibitor and photosensitizer verteporfin has been tested in patients with recurrent glioblastoma or advanced pancreatic carcinoma [297]. YAP-TAZ signaling could also be achieved by reducing YAP/TAZ protein levels via proteolysis targeting chimeras (PROTACs) where some small molecule PROTACs have been evaluated in phase I clinical trials [298]. Activation of YAP/TAZ and actin remodeling has been associated with chemotherapy resistance [299,300], and downregulation of YAP/TAZ with therapeutic molecules such as curcumin [301] could improve susceptibility of resistant cells to treatments.

6. Conclusions and future perspectives

In summary, mechanical forces play an important role in the cancer cell responsiveness to therapies, such as chemotherapy or immunotherapy, and mechanobiological factors serve as important therapeutic targets. Overall, matrix stiffening, activation of mechano-induced transcriptional regulators, and leaky vasculature have been linked to therapy resistance, suggesting that mechanosensing molecules and pathways could be viable therapeutic targets [302]. Particularly compelling examples include softening of the tumor matrix via pharmaceuticals to reduce metastasis or sensitize cancer cells to chemotherapy.[262,263] Repair of the tumor "leaky" vasculature via oxygen micorbubbles released via ultrasound [303] or aspirin [304] could reduce interstitial pressure and enhance perfusion to improve drug delivery efficiency. This may also suppress tumor cell extravasation and metastasis.

An important question to resolve is how ECM modulation affects the delivery of chemotherapeutic and immunotherapeutic agents. Certainly, clues are evident from the ways that ECM stiffening caused by other diseases impacts cancer treatments. In diabetes mellitus, high blood sugar levels alter the physical properties of the ECM and cause cellular drug resistance. However, further studies are still needed to determine whether the observed drug resistance is at least partially due to the alteration in the physical properties of the ECM. Alternative explanations are that this drug resistance arises from biochemical sources such as fatty acid synthase that has been reported to be the main cause of the hyperglycemic drug resistance in breast cancer cells [305]. Overall, the mechanobiological effects of diabetes mellitus can affect the delivery, uptake, and dosage of drug and immunotherapies. Understanding and ameliorating these effects represent important frontiers.

Much progress in the field of mechanobiology owes its genesis to the development of biomaterial platforms that enable cells and tissue constructs to be studied under conditions of prescribed stiffness, surface chemistry, and mechanical loadings. Such platforms have been of broad utility in drug discovery [306] and are promising for discovery of drugs and immunotherapies that target cancer cells in different physical environments. However, increased complexity typically comes with higher screening costs, lower throughput and generally less assays and technology available for analysis. For example, most high-throughput systems and liquid handlers have been adapted to work with multiwell plates and most assays to study cell responses depend on dissociated cells or colorimetry. Image-analysis algorithms and in general microscopy technologies are limited for 3D systems. Simpler 2D assays that mimic the tumor ECM mechanically and compositionally, could be more easily adapted to work with the technologies currently developed to conduct large scale drug screening and are generally inexpensive and easy to use.

Further, many of the studies described here were performed with immortalized cell lines, which are well-characterized, cost-effective and allow for comparison of results between labs. However, cell lines are typically expanded on rigid polystyrene dishes and, thus, adapted to such mechanical environments. For example, recent work on breast cancer cells has shown that this adaptation leads to profound changes in cell growth, metastatic potential, and chemotherapeutic response, generally making them more susceptible to some chemotherapeutics [158]. The use of primary cells or re-adaptation of immortalized

cell lines to soft substrates [307] is recommended when testing the interplay between cell mechanosensing and drug responsiveness. Further, routine sequencing could be performed periodically to confirm that the cells are still matching the genetic profile of the original tumor.

Many of the challenges associated with tests on idealized cell lines are overcome by use of animal models. However, the physiological realism of animal models comes at the cost of reduced ability to control mechanobiological factors. In animal models, mechanobiology studies could be further facilitated by development of techniques capable of non-invasively measuring tissue stiffness and strain in vivo, and in real time. Some examples include techniques that actuate magnetics droplets to infer the viscoelastic properties of tissues [308,309], magnetic resonance elastography for estimating breast cancer or brain tissue stiffness [310,311], time resolved 3D ultrasound [312], and in situ calibration of optical tweezers for measuring hemodynamic forces in vivo [313]. As the field of *in vivo* imaging for characterizing biomechanics and diffusion progresses [314], animal models may become increasing relevant to overcoming mechanobiological hurdles to chemotherapy and immunotherapy. In the meantime, innovation *in vitro* systems with prescribed mechanical properties appears poised to continue to lead the way.

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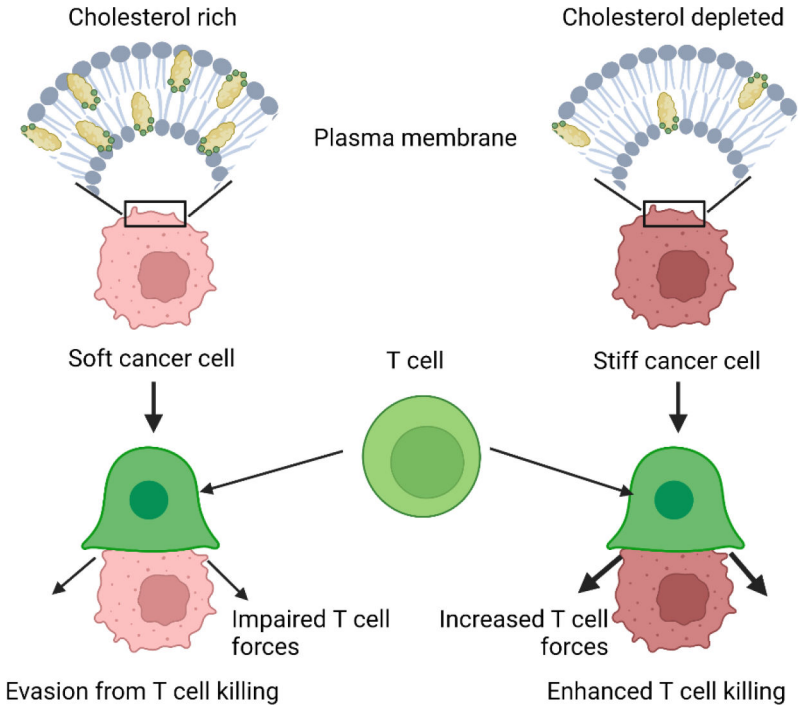

Figure 1:
Stiffening of cancer cells due to membrane cholesterol depletion enhances cancer cell killing by T-cells. Adapted from Lei et *al.* [33].

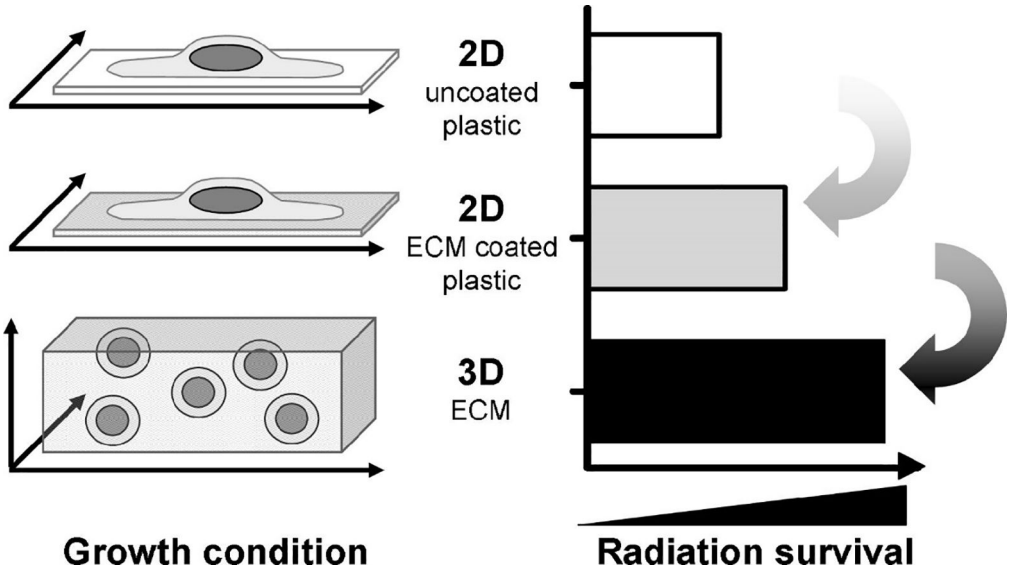

Figure 2:

Growth conditions modulate cellular radiation survival. Comparison of the survival of irradiated cells grown on cell culture plastic, on ECM-coated plastic or in 3D ECM. ECM, extracellular matrix. Illustration adapted with permission from Eke et *at.* [71].

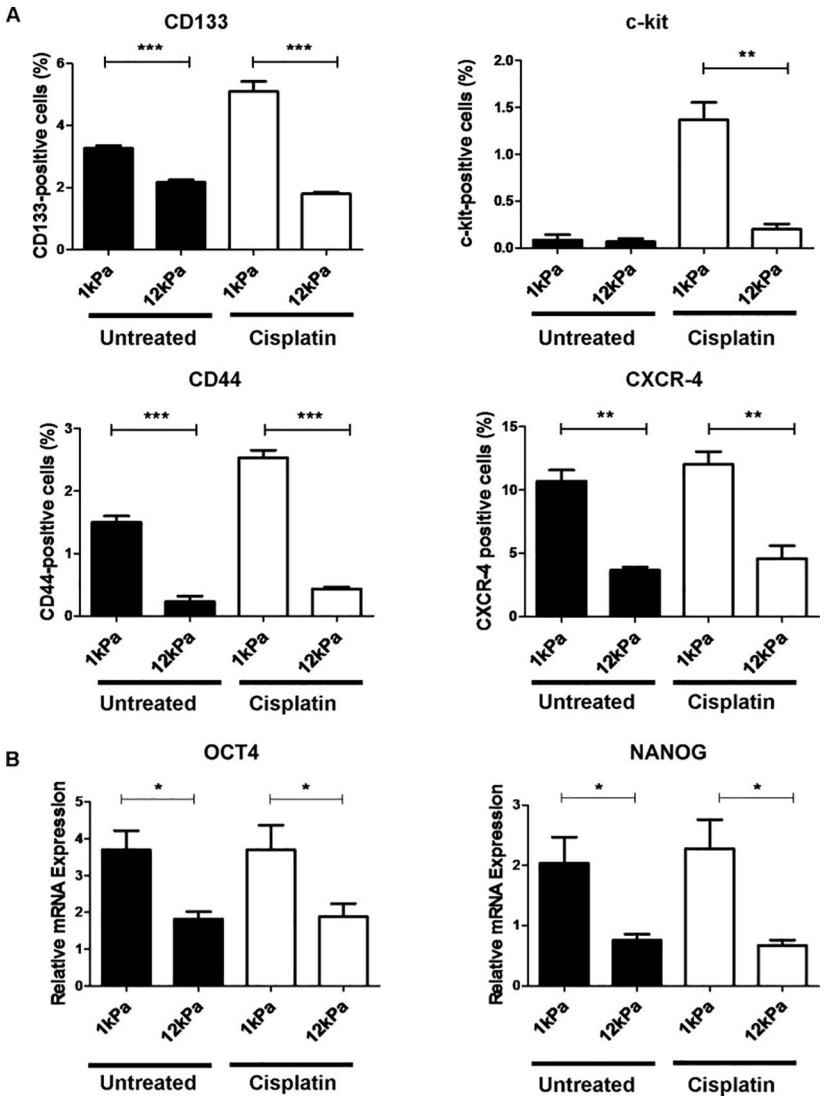

Figure 3:

Matrix stiffness and chemotherapy regulate stem cell marker expression in HepG2 cells. (A) Quantification by flow cytometric analysis of putative cancer stem cell markers CD133, c-kit, CD44 and CXCR-4 in HepG2 cells cultured for 5 days on soft (1 kPa) or stiff (12 kPa) supports. Cells were either left untreated (black) or treated for 24 hours with cisplatin (white). Results are representative of three independent experiments. (B) Real-time quantitative PCR analysis of octamer-4 (OCT4) (left panel) and NANOG (right panel) expression in HepG2 cells cultured for 5-days on soft (1 kPa) or stiff (12 kPa) supports. Cells were either left untreated (black) or treated for 24 hours with cisplatin (white). Expression is relative to the 18*S* housekeeping gene. In each case, error bars represent SEM, *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. Adapted with permission from Schrader et *al.* [93].

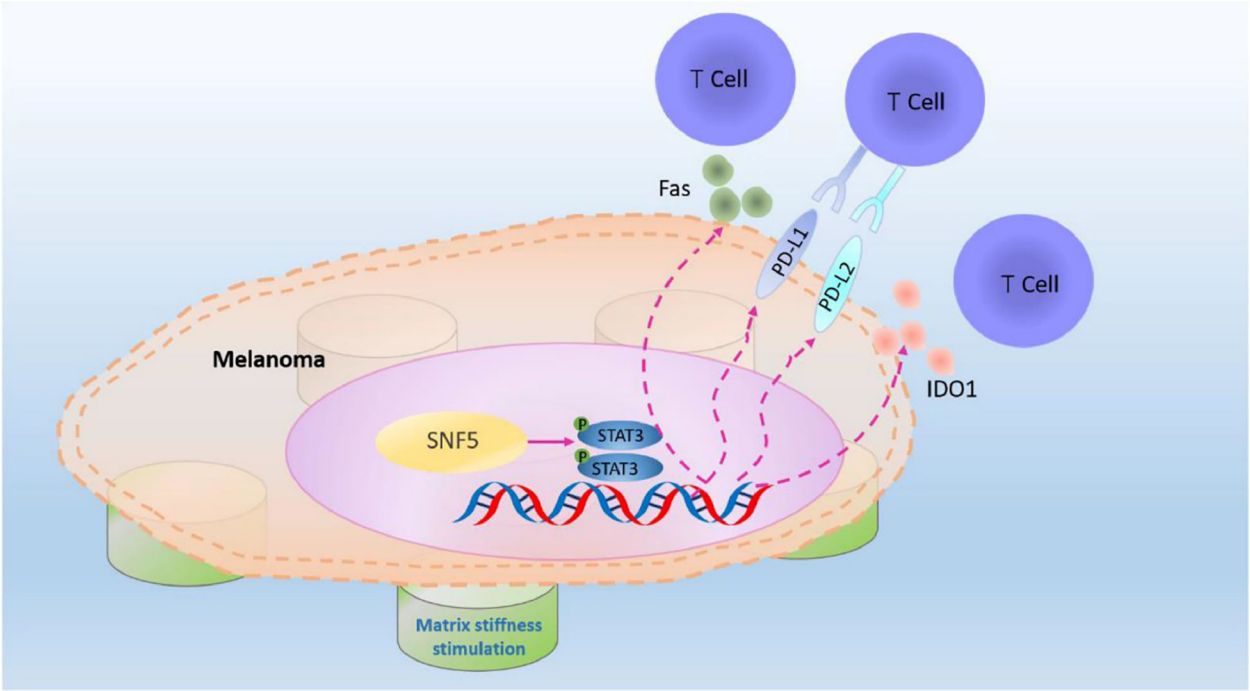

Figure 4:

Proposed model of the role of SNF5 in regulating immune escape upon mechanical stimulation in melanoma. The upregulated expression of SNF5 on the stiffer matrix activates the expression of immune escape genes by activating the phosphorylation of STAT3, thereby inhibiting T cells recognition and infiltration. Illustration adapted with permission from Chen et *al.* [113].

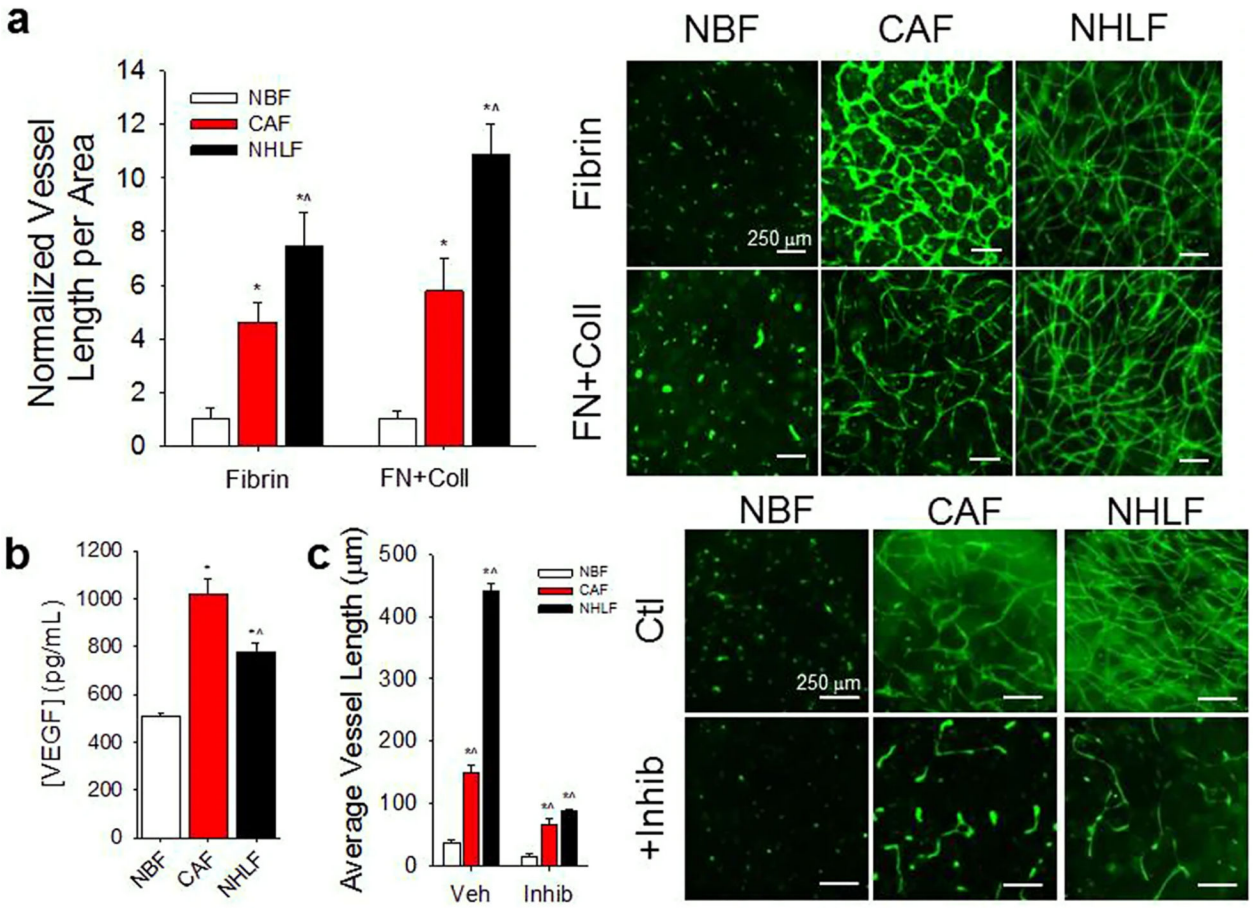

Figure 5:

CAFs support vascularization in 3D microtissues. (**a**) When co-cultured with ECs in Fibrin or combination Fibrin-Collagen (FN + Coll) gels, CAFs support significantly more vascular growth compared to NBFs. Normal human lung fibroblasts (NHLF) also demonstrate significantly higher vascularization potential compared to normal breast fibroblasts (NBFs). Data are presented as total vessel length per unit area, normalized to NBF in Fibrin: 0.0014 ± 0.0002 μm^{-1}; or NBF in FN + Coll: 0.0018 ± 0.0006 μm^{-1}. *p < 0.01 vs. NBF; ^p < 0.01 vs. CAF for same gel type. (Right) Immunofluorescent images of CD31 staining of 3D vessel systems show interconnected vascular networks in CAF & NHLF samples, but not in NBF samples. (**b**) CAFs in co-culture with ECs (CAFs/ECs) demonstrate higher steady state levels of soluble VEGF than NBFs/ECs. NHLF/EC co-cultures exhibit significantly lower levels of VEGF compared to CAF/EC samples. *p < 0.01 vs. NBF; ^p < 0.01 vs. CAF. (**c**) Inhibition of VEGFRs suppresses CAF- and NHLF-supported vascular growth compared to vehicle treated controls but shows significantly larger average vessel growth compared to NBF vehicle controls. Data are presented as average vessel length in μm. *p < 0.01 vs. NBF vehicle; ^p < 0.01 vs. NBF + inhibitor (Right) Immunofluorescent images of CD31 staining show vascular fragments of >100 μm in length present in CAF samples with inhibited VEGFR. Scale bars = 250 μm. Figure adapted with permission from Sewell-Loftin et *al.* [154].

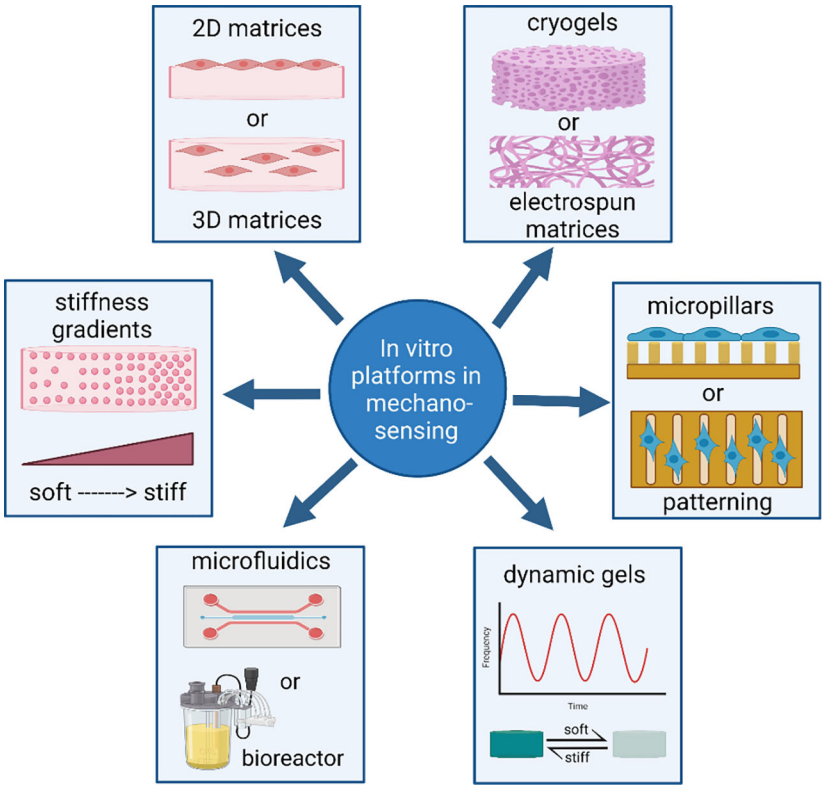

Figure 6:

Schematic representation summarizing the diversity of biomaterial-based platforms that have proven beneficial in mechanosensing and mechanobiology studies.

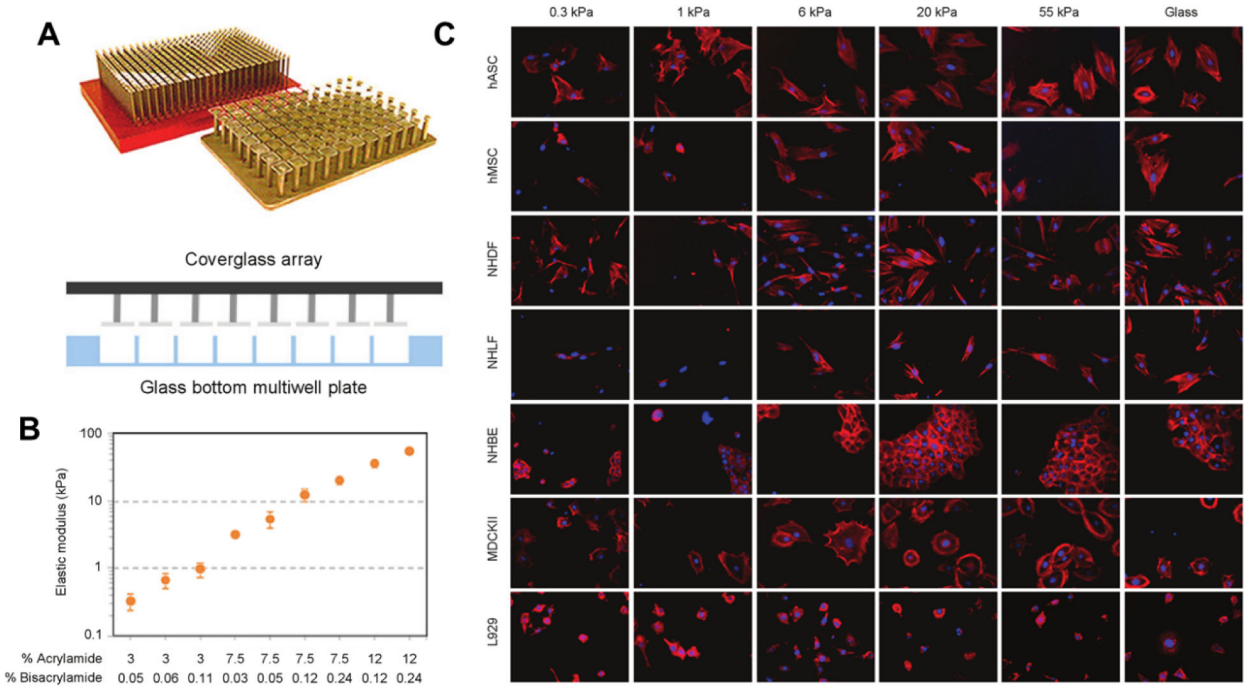

Figure 7:

A) Schematic of polyacrylamide gel incorporation into a multiwell plate. PA gels are cast using an array of coverglass to sandwich polymerization solutions within a multiwell plate, followed by ligand conjugation and sterilization. **B)** Measurement of substrate elastic modulus. Acrylamide: bisacrylamide content was chosen to target a broad physiologically relevant stiffness range. Young's modulus was determined by AFM microindentation of gels cast within three separate 96 well plates. Data are mean ± SD (n = 3). **C)** Automated imaging of cell morphology in a 384 well plate. Seven cell types were cultured across increasing substrate stiffness, stained for F-actin (red) and nuclei (blue). Images were obtained at 200X magnification. Figure adapted with permission from Mih et *al.* [163].

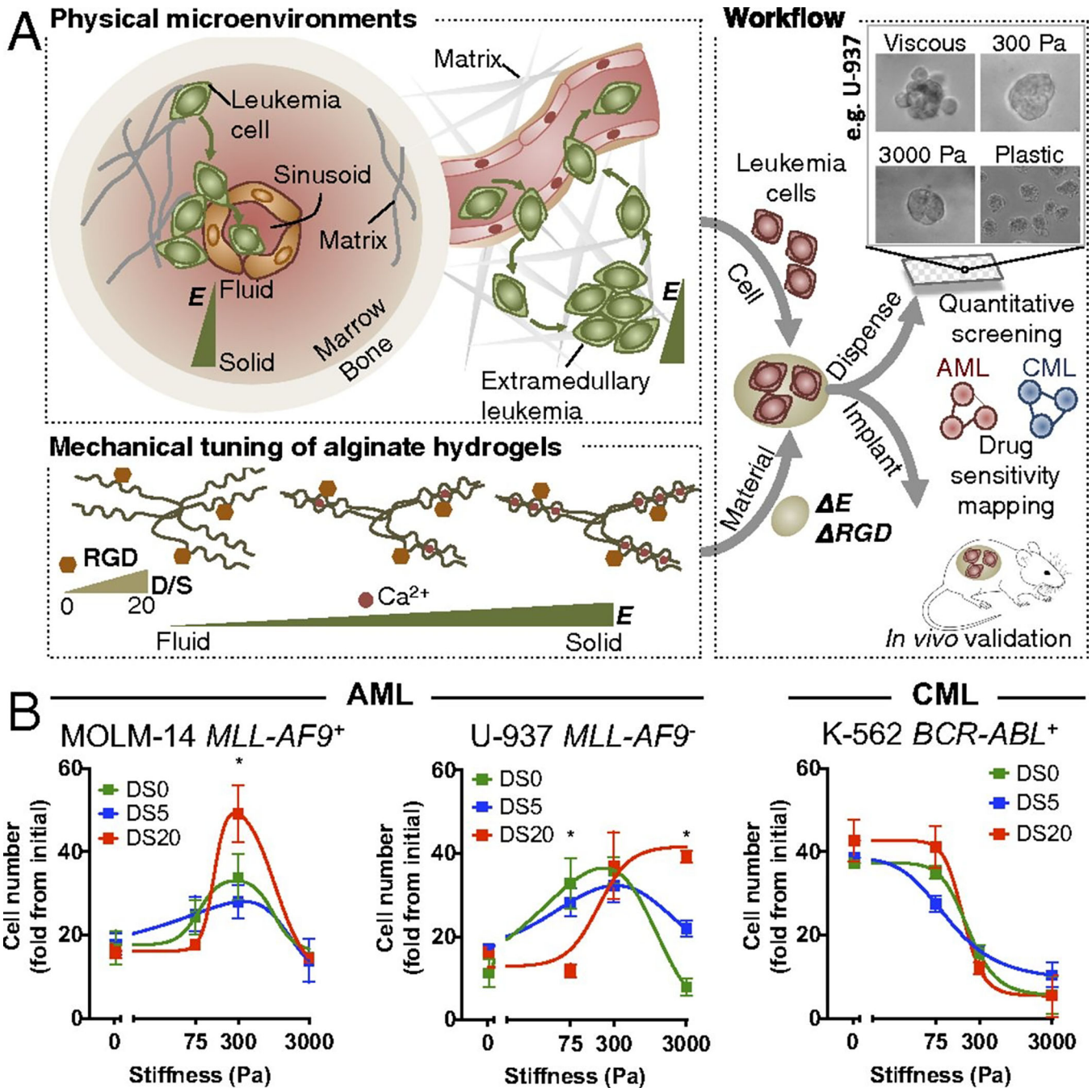

Figure 8:

Development of an integrative approach to systematically investigate the role of matrix mechanics in myeloid leukemias. (A) Schematic showing recapitulation of mechanical properties relevant to the hematopoietic system by ionic cross-linking of alginate hydrogels, followed by adaptation of the 3D hydrogels into quantitative screening and animal validation. (B) Different myeloid leukemia subtypes show distinct proliferative responses against matrix mechanics and ligand density. Ligand density is controlled by “degree of substitution” (DS), which indicates the number of RGD peptides conjugated per alginate

molecule (0~20). The whole cell population was used for viability analysis. The data were fit to biphasic dose–response curves for AML cells and standard dose–response inhibition curves for CML cells. *P < 0.05 from one-way ANOVA with Tukey's honestly significant difference (HSD) test. Figure adapted with permission from Shin et *al.* [177].

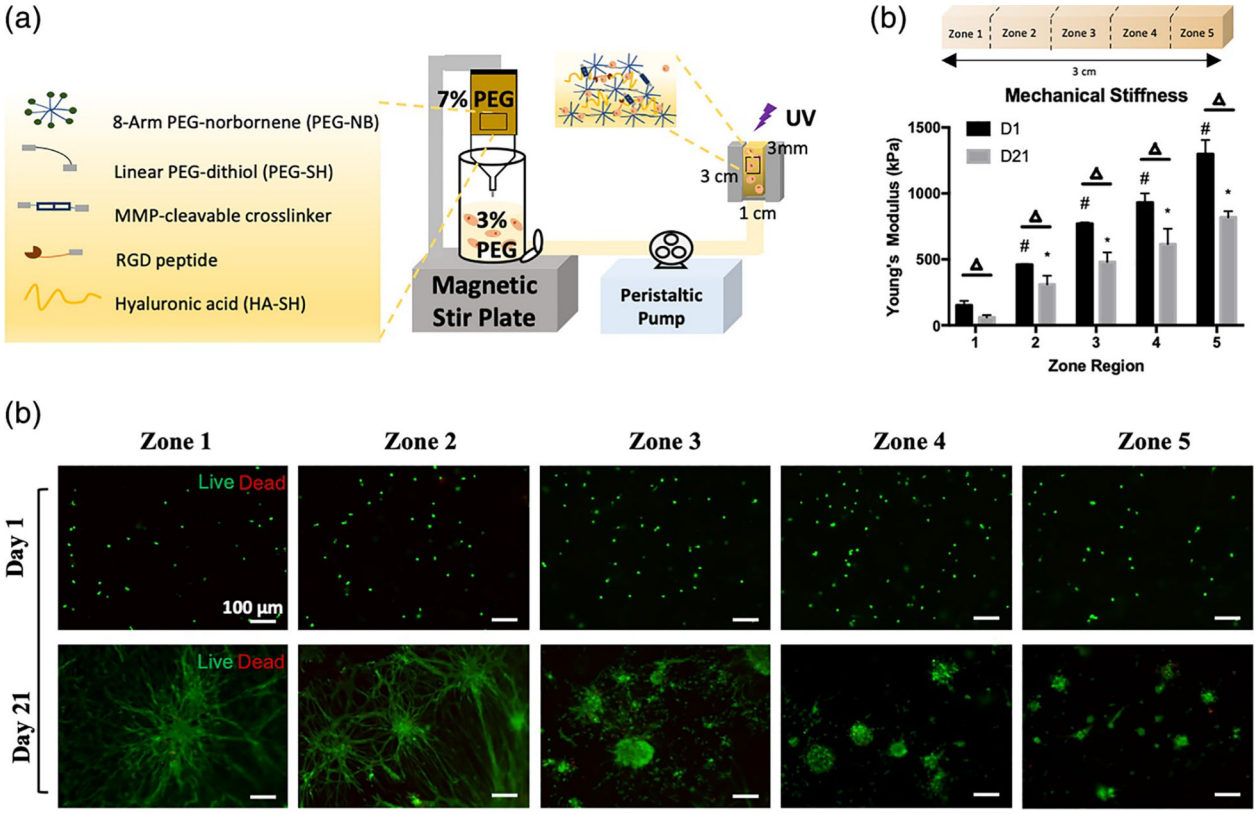

Figure 9:

(a) Schematic representation of the hydrogel composition and syringe-pump/gradient maker system used to create the 3D gradient hydrogel. The vertical cylindrical chamber holds 3% (w/v) hydrogel precursor solution. The syringe pump holds the 7% (w/v) hydrogel precursor solution. The peristaltic pump pushes the mixed solutions through to form a gradient in the customizable mold. (b) Unconfined compression test of PDTX GBM-laden cellular gradient hydrogels stiffness (Pa) on day 1 and day 21 (n = 3). # one-way ANOVA followed by Tukey's post hoc t-tests, $p < .05$ against D1 zone 1 stiffness; * one-way ANOVA followed by Tukey's post hoc t-tests, $p < .05$ against D21 zone 1 stiffness; Δ multiple students' t-tests between D1 and D21 in each zone, $p < .05$. (c) Live/dead assay of PDTX GBM cells 1 day and 21 days after encapsulation in gradient hydrogels. Live: green; dead: red. Scale bar = 100 μm. Figure adapted with permission from Zhu et *al.* [195].

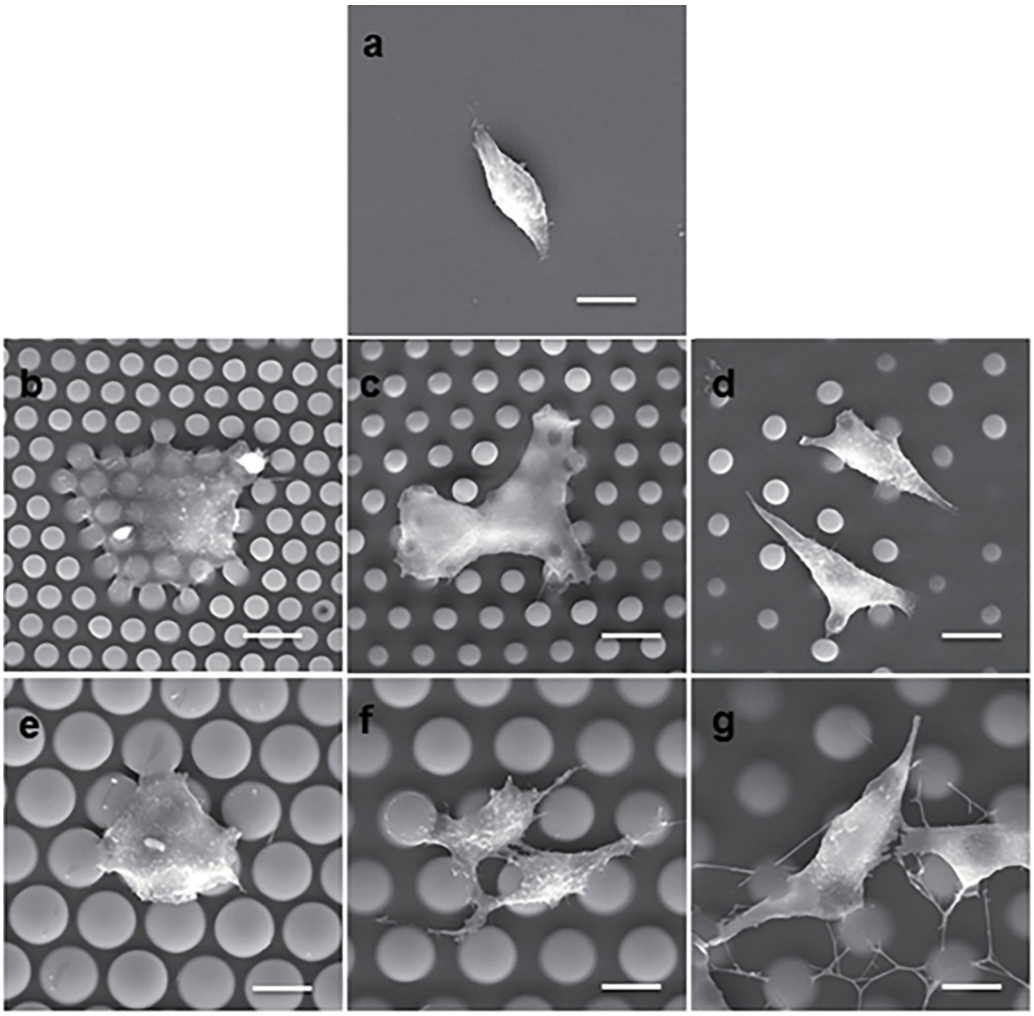

Figure 10:

Cellular morphology on micropillar arrays and planar. A: Micropillar-induced cellular morphogenesis. (a) An SEM micrograph shows A549 cells on the flat PDMS substrate. (b–g) SEM micrographs show A549 cells on the 4–2 μm (b), 4–4 μm (c), 4–7 μm (d), 10–2 μm (e), 10–4 μm (f), and 10–7 μm (g) micropillar arrayed substrates. Scale bar =10 μm. Figure reproduced with permission from Xu et *al.* [220].

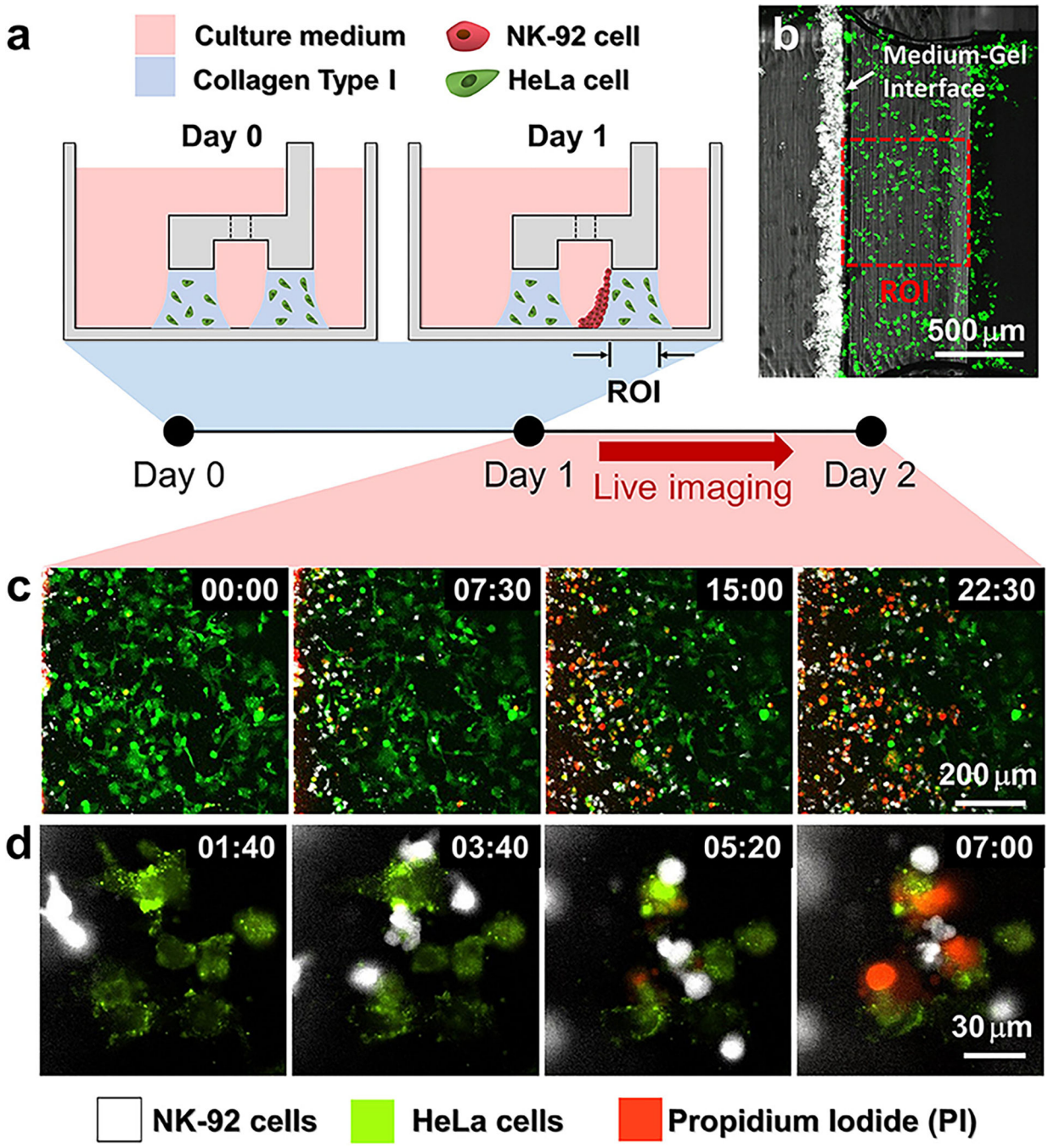

Figure 11:

Procedure of 3D cytotoxicity assay and its outputs. (a) Schematic process of the assay. HeLa cells embedded in collagen were patterned under low rails (Day 0). After 24 h of cultivation, NK-92 cells were loaded into a microchannel formed by the hydrogel. By tilting the device at an angle of 90°, NK-92 cells were deposited on a collagen block (Day 1) and cultured for additional 24 h to observe migration and cytotoxic activity of NK cells. (b) Initial state of the assay (Day 1). (c,d) Live monitoring of migration and cytotoxic activity of NK-92 cells.

Time is indicated in HH:MM in the top right corner of each image. Figure reproduced with permission from Park et *al.* [109].