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The impact of extracellular matrix viscoelasticity on cellular behavior

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Preface:

Significant research over the past two decades has established that extracellular matrix (ECM) elasticity, or stiffness, impacts fundamental cell processes including spreading, growth, proliferation, migration, differentiation, and organoid formation. Linearly elastic polyacrylamide hydrogels and polydimethylsiloxane (PDMS) elastomers coated with ECM proteins have become widely-used tools for assessing the role of stiffness, and results from these experiments are often assumed to reproduce the effect of the mechanical environment experienced by cells *in vivo*. However, tissues and ECMs are not linearly elastic materials – they in fact exhibit far more complex mechanical behaviors, including viscoelasticity, or a time-dependent response to loading or deformation, as well as mechanical plasticity and nonlinear elasticity. Recent work has revealed that matrix viscoelasticity regulates these same fundamental cell processes, and importantly can promote behaviors not observed with elastic hydrogels in both 2D and 3D culture microenvironments. These important findings have provided new insights into cell-matrix interactions and have given context as to how these interactions differentially modulate mechano-sensitive molecular pathways in cells. Moreover, these results indicate new design guidelines for the next generation of biomaterials that better match tissue and ECM mechanics for *in vitro* tissue models and applications in regenerative medicine.

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While indications of the impacts of the mechanical properties of culture substrates on cell behaviours have long been present, it is only in recent times that this concept has become widely accepted by the scientific community. Earlier studies demonstrating the impact of substrate mechanics on cell structure and proliferation were overshadowed by an emphasis on cell biology on genetics and biochemistry[1,2]. The situation began to change in the late 1990's when, using polyacrylamide hydrogels of varying elastic moduli coated with ECM proteins as cell culture substrates, Pelham and Wang showed that substrate stiffness affected cell-ECM adhesion, spreading, and migration[3]. Since this study, numerous groups have used polyacrylamide gels, and a variety of other material systems with tunable elastic moduli, to show that substrate stiffness impacts various other processes, including proliferation and apoptosis, stem cell differentiation, breast cancer progression and response to drugs[4–6]. Mechanistically, the current view is that cells exert traction forces using actomyosin-based contractility when coupled to substrates through integrin-based adhesions, or other cell-surface links, and they sense variations in substrate stiffness through differing magnitudes or extents of integrin and syndecan clustering and associated signaling, conformational changes in mechanosensitive proteins such as talin, vinculin, or lamin, activation of mechanosensitive ion channels (such as piezo1), and downstream activation of transcription factor activity[7–10]. While changes in ECM mechanics are sensed by cells over short timescales, these can impact long term cellular processes such as differentiation, fibrosis, and malignancy through continued sensing, mechanical memory, and changes in the epigenome[11–13]. Reported tissue elastic moduli vary from ~100s of Pascals in brain and fat tissue all the way up to 10s of GPa in bone[14,15]. Further, alterations in tissue mechanics are observed in development and in various diseases and have been linked to cell phenotype in these contexts[16,17]. Thus, the current consensus is that ECM stiffness plays a key role in regulating development, homeostasis, regenerative processes, and disease progression.

Living tissues and organisms appear as macroscopically solid objects, however they behave very differently to what one would expect of a perfectly elastic, or Hookean, solid when put under pressure or stretched. For example, whilst our skin and fat tissues eventually recover their shape after they are pinched or compressed, or after a wearable device is removed, they take time to do so. Tendons, when stretched slowly, are able to extend and then recoil back to their original size and shape, however, when rapidly extended, can further strain stiffen and eventually rupture[18]. Tissues are thus not purely elastic materials, like a rubber ball or a spring, because they exhibit a time-dependent mechanical response and dissipate a fraction of the energy it took to deform them, a property called viscoelasticity or poroelasticity, depending on the molecular mechanism. Macroscopically, loss of the ability to recover shape after applied mechanical stress or stretch is often a sign of injury, disease, or aging, as the affected tissues no longer recover shape after a bone break, a skin tear, or the drooping of the face after decades of gravitational stress[19]. However, even when tissues globally recover shape, local regions might not do so after forces are removed, experiencing irreversible or plastic deformations. Plastic deformation of the extracellular matrix is implicated in contributing to the conversion of an originally isotropic network of collagen fibers to a more aligned pattern that is often seen around tumors[20–22], and irreversible changes in cell-cell boundaries caused by cell-derived forces at junction sites have recently been shown to be essential features of pattern formation during development in Drosophila[23] and c-elegans[24].

Many soft tissues also exhibit nonlinear elasticity by strain-stiffening, or become increasingly difficult to extend as they are deformed, which may be advantageous in preventing large deformations that damage tissue[25]. For example, in blood vessel walls, distensibility at low strains accommodates pulsatile blood flow while increased stiffness at high strains provides elastic stability to prevent vessel rupture[26]. Biological tissues and ECMs thus exhibit complex, time and rate-dependent mechanical behaviors including a combination of viscoelasticity, poroelasticity, plasticity, and nonlinear elasticity (Box 1).

As cells interact with ECMs through dynamic processes that span a range of forces, from piconewtons up to hundreds of nanonewtons for individual cells, and span a range of timescales, from milliseconds to hours, it would be expected that time-dependent and strain-dependent mechanical responses in ECMs should impact cell-matrix interactions and mechanotransduction (Fig. 1). Indeed, an emerging body of evidence has demonstrated that these more complex mechanical characteristics of tissues and ECMs impact cells, sometimes in ways not anticipated from our previous understanding of mechanotransduction based on purely elastic substrates. Here, we review the complex mechanical behaviors of tissues and ECMs, discuss recent work elucidating the impact of ECM viscoelasticity on cells, and describe the potential for use of viscoelastic biomaterials in regenerative medicine.

Tissue and ECM mechanics are complex

Viscoelasticity has been found to be a near universal characteristic of living tissues and ECMs. In response to a mechanical perturbation, viscoelastic materials exhibit an instantaneous elastic response, characteristic of purely elastic solids, followed by a time-dependent mechanical response and energy dissipation or loss, both characteristics of viscous liquids. Viscoelastic materials will ‘creep’, or deform in a time-dependent manner, in response to the application of an external step stress or load, and undergo ‘stress relaxation’, or reduce stress levels in a time-dependent manner, in response to a step deformation. Further, under an imposed sinusoidal deformation, stress and strain are completely in-phase for a purely elastic material, due to all of the inputted deformation energy being able to be ‘stored’ and ‘recovered’ during each cycle without any loss, whereas for a purely viscous fluid they are completely out-of-phase, a result of all of the inputted deformation energy being dissipated or ‘lost’ by internal friction in the system as it flows. Viscoelastic materials exhibit a response between these two extremes, with the in-phase component of the response described as the storage, or elastic, modulus and the out-of-phase response described as the loss, or viscous, modulus. The magnitude of the ratio of the loss modulus to the storage modulus in viscoelastic materials typically depends on the frequency. Viscoelastic solids are differentiated from viscoelastic fluids by maintaining stress or elastic resistance at long times under a constant deformation, or by reaching an equilibrium deformation under loading at long times. Everyday examples of viscoelastic solids include jello (gelatin), a “stress ball”, and bread dough, while silly putty serves as an example of a viscoelastic fluid. One of the softest and most dissipative viscoelastic tissues in mammals is the brain, which has been extensively studied at time scales and deformation magnitudes that span the range relevant to blasts and concussions on the fast (ms) and high stress (MPa) limit to the deformation caused by tumor growth on the slow (weeks) and low stress (10 Pa) limit. Depending on the time scale and deformation, brain tissue can dissipate at least as much

energy as it stores in elastically recoverable deformation[27], and at very long time scales it appears to flow like a glass or liquid[28]. Further, dissipation (and viscoelasticity) can resolve not only grey from white matter, but also different regions of the brain[29]. Other soft tissues are also viscoelastic, with rheological analysis showing that soft tissues generally exhibit loss, or viscous, moduli that are usually around 10 – 20% of their storage, or elastic, moduli at 1 Hz (Fig. 2a). Stress relaxation tests reveal that soft tissues, including liver, breast, muscle, skin, and adipose substantially relax their resistance to a deformation over timescales from tens to hundreds of seconds[30–36] (Fig. 2b). Even stiffer skeletal tissues including bone, tendon, ligaments, and cartilage are viscoelastic, with loss moduli at about ~10% of the storage moduli. Embryos at various stages of development[37], and regenerative structures such as fracture hematomas[30] or blood clots[38] also exhibit viscoelasticity.

Importantly, changes in viscoelasticity have been associated with disease progression. Determination of elastic moduli, the basis of palpation that can identify stiff tumors, is not efficient for identifying most types of brain tumors, but rather changes in their dissipative properties, as revealed by magnetic resonance elastography, can identify the margins of gliomas and other types of brain tumor in situ[39]. Further, changes in brain viscoelasticity have been linked to aging[40] and multiple sclerosis[41]. Similarly, breast cancer progression is associated with changes in both stiffness and energy dissipation[42]. Changes in viscoelasticity are likely to be associated with other types of cancers or other diseases, particularly those involving fibrosis or inflammation, as well as injuries, but data on these are largely missing, representing a critical gap in knowledge.

Materials that exhibit viscoplasticity represent a subset of viscoelastic materials, in that they exhibit permanent deformations when the applied stress exceeds a material ‘yield stress’ and remain at least partially deformed when the stress is removed. The response of these materials is viscoelastic to loads or deformations below their yield stress. For instance, molding clay and toothpaste are both viscoplastic. Reconstituted extracellular matrix materials used for cell culture, including common formulations of type-1 collagen gels, reconstituted basement membrane matrix, and fibrin gels, are typically viscoplastic[22,43] unless they are sufficiently crosslinked covalently by enzymes such as Factor XIIIa or lysyloxidase[44,45]. Tissue viscoplasticity has been characterized even less than tissue viscoelasticity, representing another critical gap in knowledge.

Numerous mechanisms underlie the dissipative properties of tissues and ECMs, with some of these mechanisms also leading to viscoplasticity. Tissues consist of cells, ECM, and extracellular fluid. The ECM, composed of fibrous protein polymer networks, typically type-1 collagen fiber networks, interspersed with highly hydrated, flexible polysaccharides and other large molecules, is thought to be a key regulator of tissue mechanics and viscoelasticity[46,47]. Dissipation in networks of collagen or fibrin fibers depends on the nature of the bonds that link one fiber to another[43,48]. Most network crosslinks are non-covalent and arise from numerous weak bonds with dissociation rates fast enough to allow stresses to relax, or allow material creep, on a relevant time scale. These weak bonds can also exhibit load-dependent dynamics[43], and the breaking of weak bonds under mechanical deformation or loading dissipates energy. Reformation of weak bonds following matrix deformation can stabilize the deformed state of the material, leading to plastic deformations.

Using a theoretical fiber network model of collagen, a phase diagram was derived that classified the dominant mechanisms of plasticity based on the rate and magnitude of deformation and the mechanical properties of individual fibers[21]. It was shown that the experimentally observed viscoplasticity of collagen networks is caused by the formation of new cross-links if moderate strains are applied at small rates or due to permanent fiber elongation if large strains are applied over short periods. Both slipping of bonds between collagen fibers, and sliding of collagen fibrils, have been observed *in vivo* for tissues under load, for example in skin[49] and tendon[50], respectively. Polymer entanglements may function similarly to weak crosslinks, as release of an entanglement dissipates energy and allows the matrix to flow. These weak crosslinks or entanglement interactions co-exist with more stable covalent crosslinks, which act to diminish liquid-like flow and mechanical plasticity of the matrix overall, but do not eliminate dissipation by unbinding of the weak bonds or by deformations that can change sample volume. Elastin fibers also act to promote elastic recovery at the tissue-scale[51,52]. Protein unfolding is another mechanism of energy dissipation and viscoelasticity[53,54], and has been reported in fibrin[55], spectrin[56,57] and intermediate filament[58] networks *in vitro*. The relative importance of these distinct mechanisms of dissipation will likely vary substantially in their relevance to the viscoelastic spectrum displayed by different tissues.

Since tissues are largely water, the flow of water within the ECM can cause significant viscous dissipation and what are termed poroelastic effects, depending on the mesh size or porosity of the tissue and the rate of loading. Dissipation due to poroelasticity occurs under tension or compression, and results from volume changes due to water flow into or out of the network[59]. Variations in cell number or density and ECM composition, density, and conformation in a tissue, enables fluid to be differentially held by or released from the matrix when under an externally imposed load or strain, resulting in variations in response. In contrast, shear deformations change shape but not volume of the sample, and dissipation due to water movement within the matrix is much lower. As a result, the time- or frequency-dependent viscoelastic modulus measured in uniaxial strain for the ECM is much greater than it is for shear strain[60]. Poroelastic effects superpose with other mechanical behaviours of tissues and ECM, including nonlinear elasticity, viscoelasticity, and viscoplasticity.

Similar mechanisms apply to viscoelasticity of the cytoskeleton of cells[61–63], with two important distinctions. The relatively impermeable cell membrane tends to prevent or retard poroelastic effects due to global cell deformation, but local contraction of the cytoskeleton can lead to intracellular poroelastic effects and transient pressure gradients that persist for biologically relevant times[64]. The second distinction is that covalent links between filaments of the cytoskeleton are very rare or non-existent. In addition, motor proteins apply random non-thermal forces to cytoskeletal filaments[65], moving them faster than they would under thermal agitation alone, with the result that the active cytoskeleton is more fluidized than one without motors[66]. Cellular viscoelasticity can also manifest at the tissue-scale. For example, rigor mortis, the stiffening and solidification of muscle that occurs after death, happens in part because the links between actin and myosin fibers become both more numerous and permanent rather than rapidly forming and dissociating, while the living sarcomere hydrolyses ATP so that the actin-myosin links rapidly form and dissociate.

Finally, many tissues exhibit nonlinear elasticity and do not display the simple linear relationship between stress and strain that characterizes most conventional Hookean solid materials used in engineering, such as concrete, aluminum, or steel. Analogous to a nonlinear elastic material, a coiled bungee cord or rope, an exercise band, or an accordion is easy to straighten out initially, but becomes increasing difficult to stretch as it becomes fully extended. In addition to their role in mediating tissue viscoelasticity, networks of cross-linked collagen fibers are thought to govern nonlinear elasticity. For both shear and tensile deformations, collagen networks behave like linear elastic materials up to a threshold level of strain, beyond which they strain-stiffen concomitant with the alignment of fibers in the direction of maximum tensile strain[25,67–71]. The alignment of fibers can enable force transmission over hundreds of micrometers, facilitating long-range communication between cells[70,72]. A theoretical fiber network model of collagen showed that strong coupling between modes of deformation can give rise to significantly higher strain-stiffening of the networks in triaxial and biaxial tensile loading compared to uniaxial loading[73]. Nonlinear elasticity is also observed in cytoskeletal filament networks, including actin, vimentin, and neurofilaments, but the origins of nonlinear elasticity in these networks may have a stronger contribution of entropic elasticity, due to the semiflexible nature of the filaments[25,74,75].

2D culture and the molecular clutch

The impact of substrate viscoelasticity on cells has been demonstrated powerfully through a set of 2D culture studies. In an early study, human mesenchymal stem cells (hMSCs) were cultured on collagen-coated polyacrylamide gels that had similar storage moduli, but varying loss moduli and creep responses[110]. Increased loss, or creep, in the substrates promoted cell spreading, focal adhesion formation, proliferation, and differentiation towards adipogenic, osteogenic, and smooth muscle cell lineages. Myosin and Rho-inhibition studies indicated the role of cytoskeletal tension in mediating the response to increased mechanical loss. In a follow-up study, increased activation of Rac1 and increases in motility and lamellipodial protrusions were found in hMSCs on substrates with higher loss and creep[122]. Another study compared fibroblasts and cancer cells cultured on covalently crosslinked, or elastic, versus ionically crosslinked, or viscoelastic and viscoplastic, alginate gels that presented RGD cell adhesion ligands. While cells were unable to spread on soft elastic gels, they were able to spread on soft viscoelastic gels through β1 integrin, myosin, and Rho, exhibiting robust focal adhesions and stress fibers and enhanced YAP activation, similar to their behavior on stiff and elastic substrates[123]. Increased spreading was associated with plastic deformation. To distinguish impacts of viscoelasticity versus viscoplasticity, viscoelastic but not viscoplastic substrates were formed using elastic polyacrylamide gels with linear acrylamide chains trapped inside[111]. An increased loss modulus, or faster stress relaxation, diminished fibroblast stiffness and cell spreading area, contrasting the results with viscoplastic alginate substrates. Similarly, hepatic stellate cells exhibited reduced spreading, stress fibers, and MRTF-A nuclear localization on viscoelastic compared to elastic substrates[124]. Interestingly, normal human hepatocytes also spread less and had lower motility on viscoelastic substrates, but hepatocellular carcinoma cells responded oppositely[125].

To explain these seemingly disparate results, computational modeling has been applied. The primary sensing apparatus of substrate stiffness for cells in 2D culture is thought to be the

myosin-actin-adhesion system, also known as the motor clutch module (Fig. 3), whose dynamics have successfully explained stiffness sensing of cells on elastic substrates[126–128]. To study the impact of ECM viscoelasticity on cell spreading, a generalized motor-clutch model that explicitly accounts for dissipative processes both in the ECM and in the cell has recently been developed[129]. In this model, myosin motors pull actomyosin networks at the leading edge of the cell towards the nucleus, generating actin retrograde flow. The retrograde flow is resisted by adhesion molecules that can randomly bind and unbind between actin bundles and ECM. At the cell leading edge, the polymerization of actin filaments, countered by retrograde flow, pushes the cell membrane forward, further resulting in the spreading of the cell. To account for processes that reinforce the adhesion (e.g., talin unfolding in the FA complex, which triggers recruitment of integrins[130]), the clutch binding rate is assumed to increase beyond a threshold level of force. Interestingly, the model shows that, for soft substrates, maximum cell spreading is achieved at an optimal level of viscosity in which the substrate relaxation time falls between the timescale for clutch binding and its characteristic binding lifetime. That is, viscosity serves to stiffen soft substrates on a timescale faster than the clutch off-rate, which enhances cell–ECM adhesion and cell spreading. On the other hand, for substrates that are stiff, the model predicts that viscosity will not influence cell spreading, since the bound clutches are saturated by the elevated stiffness. The model was tested and validated using experimental measurements on three different material systems and explained the different observed effects of viscosity on each substrate[129]. The clutch model has also been applied to describing myoblast interactions with purely viscous lipid bilayers[131].

3D culture and mechanical confinement

The role of matrix viscoelasticity has also been investigated in 3D culture. Culture dimensionality is known to impact cell structure, adhesions, signaling, and nutrient transport[132]. 3D culture supports various behaviors, including epithelial morphogenesis, maintenance of pluripotency in human embryonic stem cells, and the differentiated state in chondrocytes[133–135]. Culture dimensionality has also been specifically implicated in mediating mechanotransduction. For example, while 2D culture studies have implicated the YAP transcriptional regulator as a universal mechanotransducer, mediating the response of cells to stiffness in all 2D culture contexts[136], YAP-independent mechanotransduction is found in a 3D culture model of stiffness-induced breast cancer, which is consistent with analysis of human breast cancer patient samples[137]. Similarly, culture dimensionality impacts YAP/TAZ signaling in hMSCs[138]. YAP has been shown to play a role in mechanotransduction in some *in vivo* contexts, such as pancreatic cancer[139], highlighting that the importance of using 3D culture models depends upon the specific biological process.

Various studies have explored the impact of matrix viscoelasticity on cells in 3D culture. Increased stress relaxation, enhanced creep, or a higher loss modulus in RGD-coupled PEG gels[31], RGD-coupled alginate gels[30,119], and interpenetrating networks of hyaluronic acid and collagen[116] promotes spreading of adherent cells such as myoblasts, fibroblasts, and MSCs. Faster stress relaxation and increased loss also promote cell cycle progression and completion of mitosis in single cancer cells and fibroblasts, as well as osteogenic differentiation of MSCs[30,107,140]. Transcriptional responses are cell type specific, with

human cortical progenitors and MSCs being sensitive to different ranges of stress relaxation and initial elastic moduli[141]. Maintenance of neural progenitor stemness is also facilitated by hydrogels with fast stress relaxation, while being inhibited in covalently crosslinked hydrogels[142]. In addition, chondrocytes and osteogenically differentiated MSCs can form wide volumes of interconnected cartilage-like or bone-like matrix, respectively, in viscoelastic hydrogels that exhibit fast stress relaxation[30,143]. Notably, viscoelastic hydrogels used in theses 3D culture studies are all viscoplastic.

Matrix viscoplasticity has been implicated in enabling mechanical remodeling of the matrix structure for cells cultured in 3D in collagen gels both locally[20,22,144,145] and in microtissues[146]. The impact of viscoplasticity on cancer cell migration was explicitly tested in interpenetrating networks of reconstituted basement membrane matrix and alginate[147]. Cancer cells were found to be able to migrate through the nanoporous matrices in a protease-independent manner when the matrices exhibited sufficient mechanical plasticity. Cells mechanically opened up channels in the matrix using invadopodial protrusions, independent of proteases, and then migrated through the channels.

The impact of hydrogel viscoelasticity and viscoplasticity on cell spreading, proliferation, matrix deposition, and migration in 3D culture indicates a link to the concept of mechanical confinement. Many cellular processes involve changes in cell volume, shape, or movement (Fig. 4a). When these processes are physically restricted in 3D by the surrounding ECM or cells, the cells are considered to be mechanically confined[148,149]. The established view has been that pore size and matrix degradability are key regulators of mechanical confinement[148]. For example, in the context of cancer cell migration, it had been shown that rigid pore sizes below ~3 μm block migration, with cells unable to squeeze their stiff nucleus through smaller pores[150–152]. Note that PEG, alginate, and hyaluronic acid based hydrogels typically have nanometer scale pores. With rigid or elastic pores, matrix degradation was required for the cells to overcome confinement and migrate. However, given sufficient viscoelasticity or viscoplasticity, cells can overcome confinement to grow in size, deposit matrix, change their morphology as they spread or undergo mitosis, and migrate. This provides the new perspective that in addition to pore size and degradability, matrix mechanical viscoplasticity governs confinement (Fig. 4b). During cell-matrix remodeling, these properties are coupled: cell remodeling of viscoplastic matrices alters pore size[147], degradation of the matrix changes its viscoelastic properties[153], and changes in the matrix architecture likely impacts both viscoplasticity and degradability.

In viscoelastic and viscoplastic 3D matrices, various mechanisms of mechanotransduction have been reported. As with 2D culture, actomyosin based contractility coupled to the matrix through integrin mediated adhesions, and integrin-ligand clustering, are implicated[30,154]. While in principle, some of these impacts could likely be explained by molecular-clutch based models, these models have not yet been extended to 3D contexts involving mechanical confinement. Another mechanism involves cell volume expansion. Chondrocytes, MSCs, and cancer cells expand their volume, or grow as part of the cell cycle, in matrices with fast stress relaxation, but the volume expansion is restricted in matrices that exhibit slow stress relaxation, or are more elastic[107,143,155]. In MSCs, volume expansion activates TRPV4 stretch-activated ion channels, and the signaling cascade

induced by the resulting calcium influx drives nuclear localization of RUNX2, but not YAP, to promote osteogenic differentiation in MSCs[155]. Similarly, growth during the G1 phase of the cell-cycle activates a TRPV4-PI3K/Akt-$p27^{kip1}$ signaling axis to promote cell cycle progression in cancer cells[107]. Restriction of cell volume expansion promotes Il-1β signaling in chondrocytes, resulting in an osteoarthritic phenotype[143]. Finally, as matrix remodeling and deposition are often enhanced in matrices with increased viscoplasticity, the mechanical microenvironment to which cells respond is time-dependent, and cell-matrix interaction becomes a dynamic and potentially iterative process.

Viscoelastic biomaterials in medicine

One potentially impactful application for these findings lies in the design of biomaterials for regenerative medicine. This field originated with the goal of regenerating tissues and organs, or engineering replacements, for those damaged or lost to disease or trauma[156]. Biomaterials are typically utilized for cell and drug delivery, to spatially organize transplanted and resident cells, for regulation of gene expression, and to guide tissue structure and function in various regenerative, tissue and immune-engineering applications[157]. The demonstrated impact of matrix viscoelasticity on cell proliferation, gene expression, fate, and migration highlights it as potentially a key design parameter for biomaterials-based applications. Indeed, FDA-approved, tissue engineering products (e.g., Apligraft™ engineered skin, Infuse™ bone regeneration devices) are often based on viscoelastic matrices. Advances in materials processing techniques such as 3D printing, which often utilizes viscoelastic materials[158,159], have allowed tissue and organ structure and properties to be more faithfully recapitulated. The utility of engineered tissues as improved models for basic studies of development and pathology, test beds for toxicology analysis, and improved drug screening have also led to significant interest in the development of microphysiological systems (e.g., tissue-on-chip) and cultured organoids[160,161]. These can more faithfully recapitulate tissue and organ biology than standard, 2D cell culture models, while also enabling the study of human biology as versus the animal biology of classic preclinical studies.

There is both direct evidence, and significant correlative data, that viscoelasticity is an important design parameter for biomaterials used in regenerative medicine. The first demonstration that matrix stiffness regulates regeneration utilized the transplantation of stem cells within viscoelastic hydrogels[162]. Strikingly, the impact of stiffness on stem cell fate in those gels related to the ability of cellular traction forces to remodel the polymers comprising the hydrogels[154], suggesting that in fact it was the viscoelasticity of the gels that was key to their impact on cell fate *in vivo*. A subsequent study directly examined the impact of viscoelasticity by transplanting cells in hydrogels of matched initial elastic moduli, but varying rates of stress relaxation. Hydrogels with more rapid stress relaxation led to greater bone regeneration[163]; the optimal relaxation rate corresponded to that of human fracture hematomas isolated from patients[163], which provide the environment in which bone regeneration naturally occurs. Similar viscoelastic hydrogels delivering inductive proteins were also found to promote extensive bone regeneration, likely due to the ability of host cells to readily invade the gels[164,165]. The beneficial impact of hydrogels in various applications including cartilage regeneration, vocal cord regeneration, and amelioration of

pathologic remodeling of the myocardium following myocardial infarction may also relate to their viscoelastic properties[166–169].

A key question is whether viscoelasticity has been a hidden variable that explains much past work in the biomaterials field more broadly. Some of the most widely used and successful biomaterials in regenerative medicine, including collagen gels, hyaluronic acid, and supramolecular assemblies[170] are physically-crosslinked hydrogels (e.g., collagen and hyaluronic acid). The most widely used biomaterial for intestinal organoid formation in vitro, reconstituted basement membrane matrix, is also a physically-crosslinked viscoelastic hydrogel, as are others used to promote formation of skeletal muscle, liver, and neural organoids[171–174]. While there have been a number of studies aiming to delineate the impact of matrix degradation on tissue regeneration, a provocative possibility is that the impacts might, at least in part, relate to the viscoelastic behavior of these biomaterials. Several early studies concluded that more rapidly degrading hydrogels led to greater tissue regeneration than more slowly degrading gels[175,176]. However, those studies utilized alterations in polymer molecular weight to regulate gel dissolution, and these changes will also alter viscoelasticity. A number of studies examining 3D mechanotransduction have utilized covalently-crosslinked hydrogels and concluded that degradation of the gels was key to how cells interpreted gel cues[177,178]. However, the cellular activity leading to degradation of these materials will likely transition the local matrix to a more *viscoelastic* state. In addition, cells may be interacting with the matrix molecules they themselves deposit[117], which might provide a viscoelastic substrate. Similarly, recent efforts to develop a synthetic analog to the naturally-derived, physical hydrogels for organoid formation demonstrate that gel degradability is critical to designing synthetic replacements[179,180]. While little is known regarding the role of viscoelasticity in the fate and functional state of cells of the innate and adaptive immune system, a recent study has implicated purely elastic covalently-crosslinked synthetic matrices, as contrasted to those fabricated with naturally derived physically-crosslinked viscoelastic extracellular matrix, as leading to inflammatory as versus regeneration-promoting immune cell responses[181]. Clearly, significant research will be required to delineate the specific roles of viscoelasticity, other physical properties and chemical composition in the cellular and tissue response to various biomaterials mediating tissue repair and formation.

Future outlook

Viscoelasticity is a near universal feature of living tissues and ECMs, and a rapidly expanding body of evidence is establishing that cells sense and respond to the viscoelastic properties of ECMs, challenging the current stiffness-centric view of cell-matrix mechanotransduction. There is a fundamental need for additional measurements of the viscoelasticity and viscoplasticity of tissues during development, and adult and pathologic tissues, as such measurements are currently quite limited. The change in viscoelasticity and viscoplasticity associated with diseases will be of particular interest, especially at the microscale relevant to cells. As both 2D and 3D culture studies have shown that changes in matrix viscoelasticity drive broad changes in proliferation, gene expression, migration, and differentiation, it is likely that changes in tissue viscoelasticity will play a role in disease progression and this relation could serve as a potent target for therapeutic approaches. More

work is needed in the future to explore the relationships between viscoelasticity and viscoplasticity and higher order behaviours in development, tissue genesis and repair and disease aetiology.

While the impact of substrate viscoelasticity on cell spreading in 2D culture is increasingly understood, the impact of viscoelasticity must also be considered in the context of other physical cues of the matrix. Architectural features, including geometry, porosity, and topology (e.g., nanoscale roughness) have all been demonstrated to impact various aspects of cell behavior[182–186]. However, these have typically been studied in the context of high moduli, purely elastic matrices. It is unclear how cells will interpret these cues in the context of viscoelastic matrices. Cells generate forces and deformations on substrates in a highly dynamic manner, leading to a complex time-dependent mechanical response of the substrates, which may significantly alter the original architectural and the feature sizes to which cells respond. While externally applied stresses (e.g., compressive and shear forces) conveyed to cells from their matrices also regulate cellular gene expression and tissue structure and function[187,188], their impacts have often been studied in the context of purely elastic substrates. Dissipation of externally applied forces by viscoelastic matrices is likely to diminish the magnitude and distance of action of these cues and may alter the mechanotransduction pathways they trigger.

Mechanistic understanding of mechanotransduction in viscoelastic and viscoplastic matrices in 3D is still limited. New tools and approaches that enable one to decipher cell-matrix interactions with greater spatiotemporal resolution are needed. This is particularly important in viscoplastic matrices as cell interactions with the matrix would be expected to dynamically alter local matrix architecture, ligand density, and viscoelasticity. Super-resolution imaging in 3D, molecular force sensors, and materials with dynamically tunable mechanical properties are emerging technologies that may address this need and provide a detailed readout of the dynamic molecular scale interactions and forces that occur between cells and viscoplastic matrices[189–192], helping to develop a more holistic view of cell-matrix signaling. In addition, most synthetic hydrogel systems used in this field are nanoporous and do not capture the fibrillarity and ligand presentation of native ECMs.. Incorporation of collagen fibers into synthetic hydrogels[116,121], or use of synthetic approaches to generating collagen-like fibers[193], may help address this important limitation. Further, integrating advances in chemical synthesis routes that permit explicit control over composition, architecture and precise positioning of functional groups[194,195] (e.g. RAFT, DNA origami) and real time, non-invasive tuning of properties[191], with adaptive manufacturing processes that can program material composition and architecture across varying length scales[159] likely will provide new material systems to explore the impacts of viscoelasticity and viscoplasticity both *in vitro* and *in vivo.* New synthetic semiflexible filament networks made from self-assembling helix-forming monomers or by electrospinning represent a novel class of materials that can more closely mimic the elastic properties of native ECM[196,197] as well as incorporate energy dissipation[198] and plastic deformation[199]. In addition, there are major gaps in our understanding of how matrix viscoelasticity impacts signaling pathways and regulation of transcription in 3D. Mechanical cues generally regulate genome architecture[200], and a recent study found that matrix stiffness impacted genome accessibility in a 3D culture model of breast cancer, which mediated induction of malignancy by

enhanced stiffness[13]. The connection between matrix viscoelasticity and cell signaling, transcription factor activation, and the epigenome is an area ripe for study.

While biomaterials design has historically operated in the dark, relative to the importance of viscoelasticity, viscoelasticity is likely to be a key technical specification in many applications moving forward (Fig. 5). Success will likely involve mimicking the mechanical characteristics of developing tissues, as this is often used as the model for regenerative strategies. The role of viscoelasticity in regulating the biology of the various cell types regulating regeneration, possibly including pluripotent stem cells, tissue resident stem and differentiated cells, and immune cells will also need to be delineated to rationally design materials to enhance tissue regeneration. Biomaterial design may also require decoupling of the local viscoelastic properties that cells sense, from the larger, tissue-scale properties required to achieve mechanical stability of the regenerating or engineered tissue. Thus, the advent of biomaterials with controlled viscoelasticity may be transformative in improving the success of biomaterials applications in regenerative medicine.

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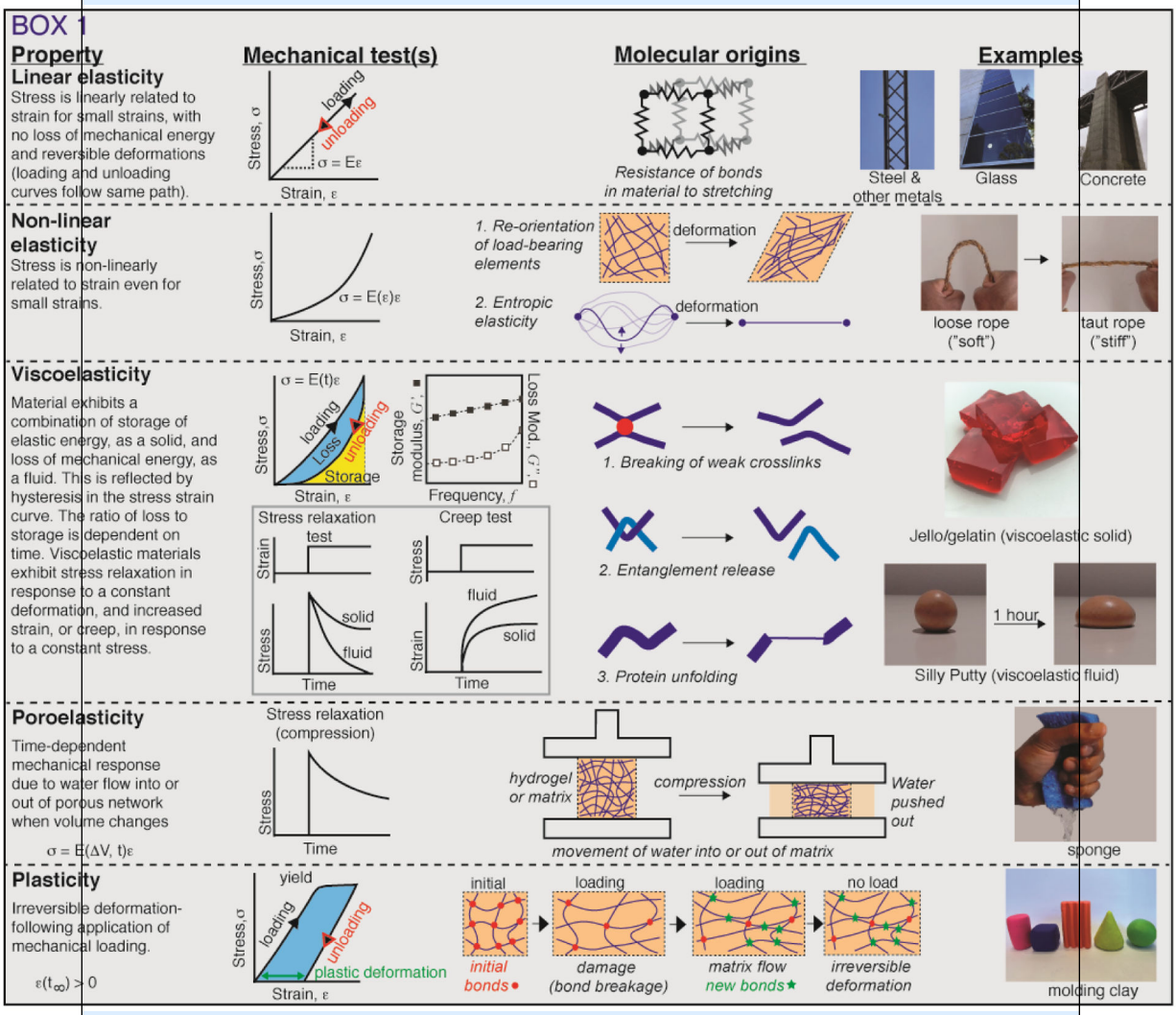
Box 1| Materials and mechanical concepts: linking material structure to functional responses under load.

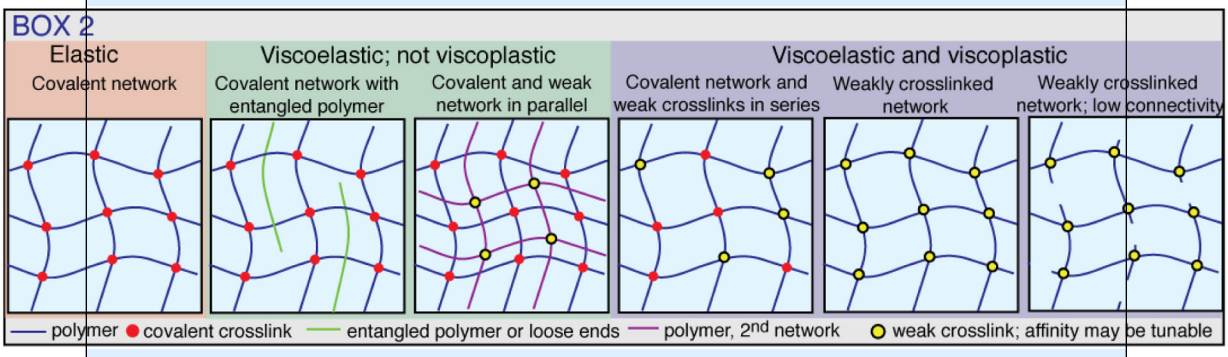
Materials can be categorized by how they deform (or change shape) in response to mechanical loading, typically in a stress strain test. Mechanical stress is defined as the force per unit area, with units of Pascals (N/m^2) and can be in shear or normal. Strain is a normalized measure of deformation. Constitutive equations describe the relationship between stress and strain for a given material. Biological tissues and ECMs can exhibit a combination of nonlinear elasticity, viscoelasticity, poroelasticity, and plasticity. Materials that are both viscoelastic and plastic are considered to be viscoplastic.



BOX 2: Biomaterials with tunable viscoelasticity

To reproduce both the elastic and dissipative properties of tissues in simplified bioengineered materials used for cell culture, several novel approaches based on the principles of polymer physics have recently been reported. Polymers that are inert to cell binding and not susceptible to degradation by mammalian proteases are typically used, with cell-adhesion peptide motifs or protein coupling to the polymer serving as tunable design parameters. A purely elastic hydrogel involves formation of an ideal covalent polymer network, as uncrosslinked polymers and loose ends lead to energy dissipation[109]. In contrast, non-ideally crosslinked polymer networks, such as polyacrylamide crosslinked to just beyond the gel point, form materials with incomplete crosslinking that allow for loss and creep[110]. Varying the concentrations of acrylamide (monomer) and bisacrylamide (crosslinker), or inclusion of non-crosslinked linear acrylamide polymers into crosslinked polyacrylamide gel[111], allows formation of a set of gels with the same storage modulus, but varying loss moduli.

Other approaches are based on hydrogel materials that are formed, at least in part, with weak (dynamic or physical) crosslinks between the polymers. Viscoelastic polyethylene glycol (PEG) hydrogels have been formed using dynamic covalent hydrazone bonds, boronate bonds, or thioester exchange[31,112–114]. In alginate gels, weak ionic crosslinking leads to viscoelastic gels[115]. Viscoelastic hyaluronic acid-based hydrogels can be formed by using hydrazone bonds or guest-host crosslinking[116,117]. Weak crosslinks can also be programmed into peptide-based hydrogels[118]. In these networks with weak bonds, viscoelasticity can be modulated independent of the initial elastic modulus by some combination of varying the following parameters: molecular weight of the constituent polymer; coupling of inert molecules to the constituent polymer as spacers; affinity of the weak bonds; ratio between weak and covalent bonds; and the total number of bonds[30,116–121]. Networks formed exclusively from weak crosslinks are expected to be viscoplastic, whereas single or double networks formed with a combination of covalent and weak crosslinks may or may not exhibit viscoplasticity at the bulk scale, depending on the molecular architecture.



Box 2 Figure|.
Strategies for forming hydrogels that are elastic, viscoelastic but not viscoplastic, or viscoelastic and viscoplastic.

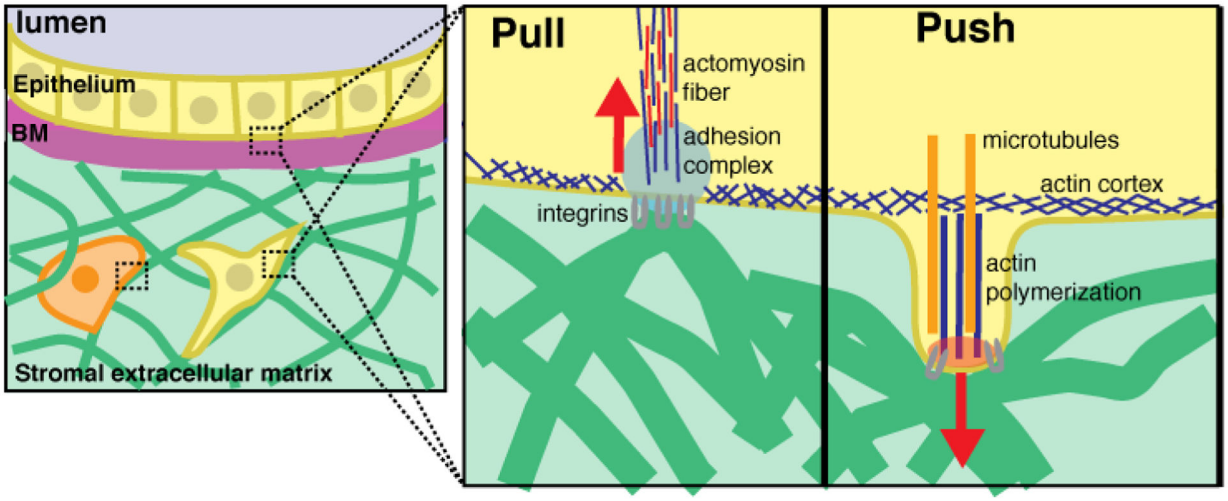


Figure 1|. Mechanical interactions between cells and extracellular matrices.

Cells interact with ECMs mechanically, including by pulling, often through actomyosin-based contractility coupled to the ECM through integrin-based adhesions, and pushing, often through actin polymerization and microtubules. The mechanical properties of ECMs mediate these interactions resulting in cell mechanotransduction and impacting cell behaviors.

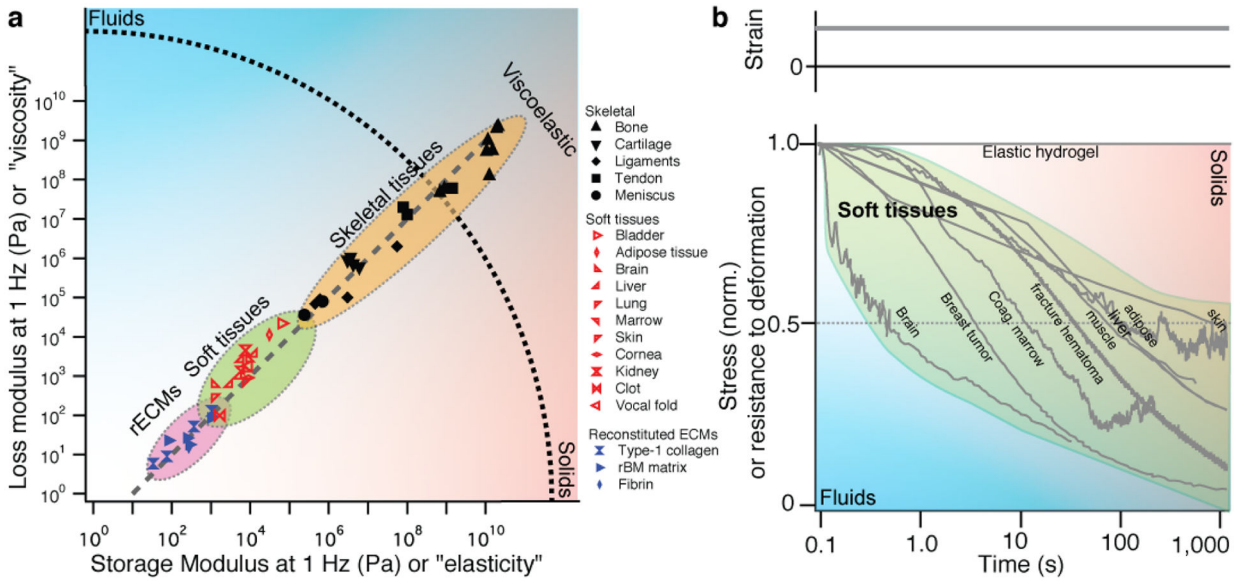


Figure 2|. Biological tissues and extracellular matrices are viscoelastic and exhibit stress relaxation in response to a deformation.

a, Plot of loss modulus at ~1 Hz, a measure of viscosity (or dissipation), versus storage modulus at ~1 Hz, a measure of elasticity, for skeletal tissues, soft tissues, and reconstituted ECMs (rECMs). Grey dotted line indicates a loss modulus that is 10% of storage modulus. Data was taken from a set of randomly selected publications[28,33,35,43,76–106]. Shear storage and loss moduli were converted to storage and loss moduli by assuming a Poisson ratio of 0.5, and thus multiplying by a factor of 3. **b,** Stress relaxation tests on the indicated tissues. Data from refs.[14,30,31,107,108]. Data for **a** and **b** result from various modalities of measurement (shear, compression, tension), various measurement tools (mechanical testers, nanoindentation, AFM, shear rheometry), and tissue of different animal origins (human, rat, mouse, bovine, sheep, porcine, canine).

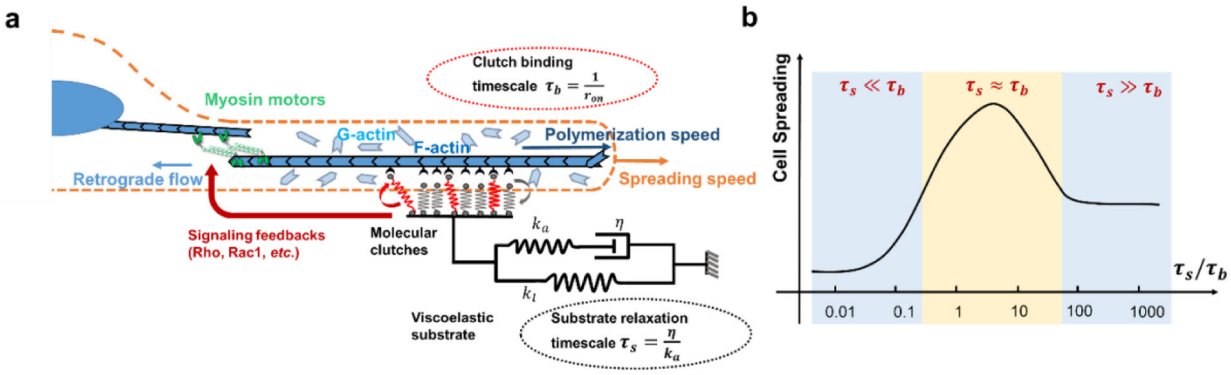


Figure 3|. The molecular clutch model of mechanotransduction explains the impact of matrix viscoelasticity on cell spreading in 2D.

a, Schematic of molecular clutch model of mechanotransduction as applied to viscoelastic substrates. Adapted from Ref.[129]. **b,** Molecular clutch model simulations predict optimal cell spreading when the timescale for stress relaxation is similar to the clutch binding timescale.

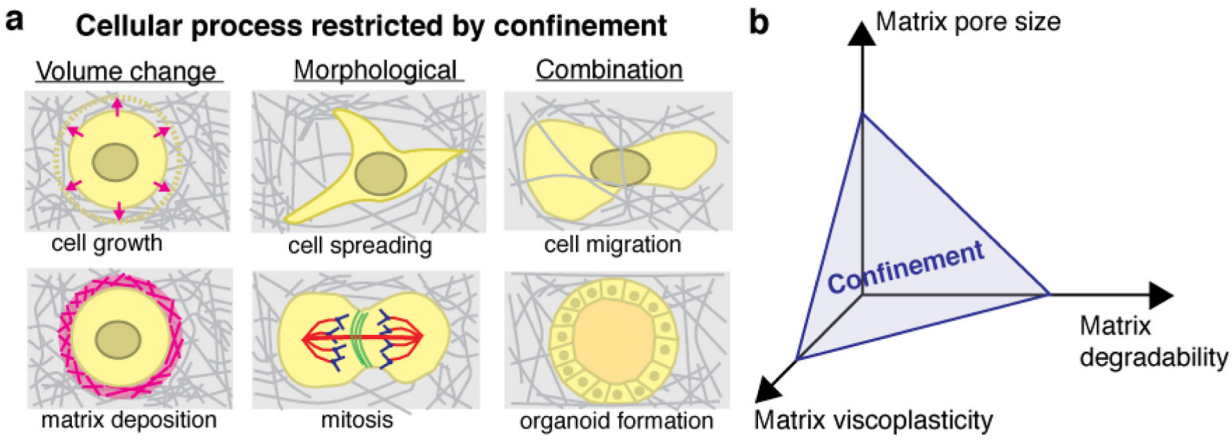


Figure 4|. Matrix viscoplasticity mediates mechanical confinement in 3D culture.

a, In confining 3D matrices, processes that involve volume change, morphological changes, or a combination of both are restricted. **b,** Confinement is governed by a combination of matrix pore size, matrix degradability, and matrix viscoplasticity. A sufficiently large value for any one of these properties releases confinement.

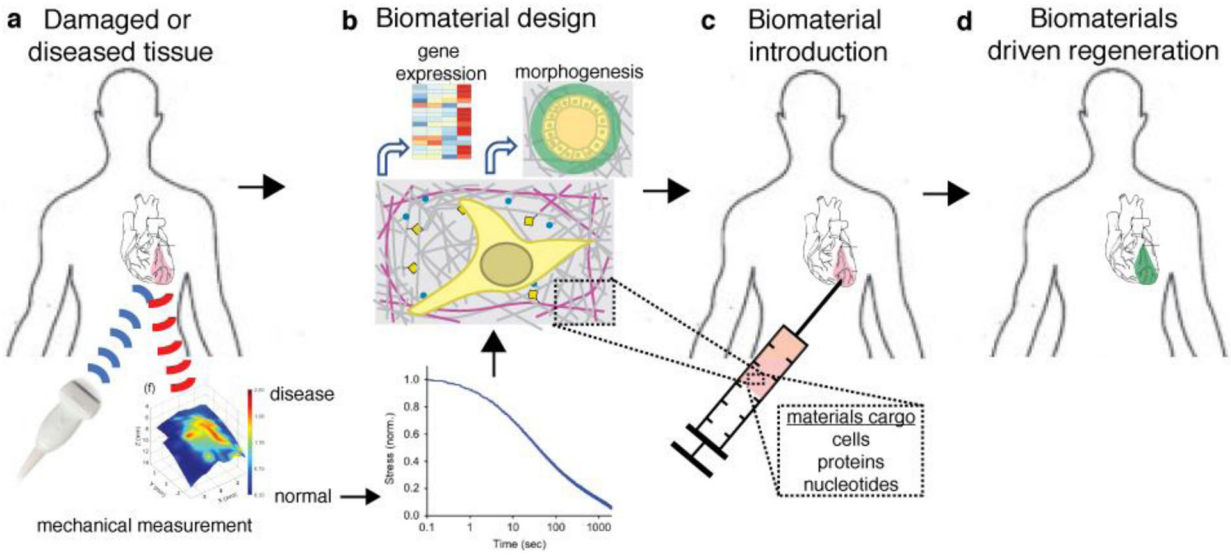


Figure 5|. Designing viscoelastic biomaterials for regenerative medicine.

a-b, Advanced imaging is utilized to detect the mechanical properties of the tissue, damaged and normal, in order to design materials with appropriate viscoelastic properties to guide the desired pattern of gene expression from interacting cells and morphogenesis. **c-d,** Introduction of the material, either alone or carrying various regeneration-promoting cargoes (e.g., cells) will then lead to (right panel) regeneration of the damaged tissue and reconstitution of function.