Cell–extracellular matrix mechanotransduction in 3D

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Abstract |

Mechanical properties of extracellular matrices (ECMs) regulate essential cell behaviours, including differentiation, migration and proliferation, through mechanotransduction. Studies of cell–ECM mechanotransduction have largely focused on cells cultured in 2-dimensions (2D), on top of elastic substrates with a range of stiffness. However, cells often interact with ECMs in vivo in a 3-dimensional (3D) context, and cell–ECM interactions and mechanisms of mechanotransduction can differ in 3D. The ECM exhibits various structural features as well as complex mechanical properties. In 3D, mechanical confinement by the surrounding ECM restricts changes in cell volume and shape but allows cells to generate force on the matrix through extending protrusions and regulating cell volume as well as through actomyosin-based contractility. Furthermore, cell–matrix interactions are dynamic owing to matrix remodelling. Accordingly, ECM stiffness, viscoelasticity and degradability, often play a critical role in regulating cell behaviours in 3D. Mechanisms of 3D mechanotransduction include traditional integrin-mediated pathways that sense mechanical properties and more recently described mechanosensitive ion channel-mediated pathways that sense 3D confinement, with both converging on the nucleus for downstream control of transcription and phenotype. Mechanotransduction is involved in tissues from development to cancer and is being increasingly harnessed towards mechanotherapy. Here, we discuss recent progress in our understanding of cell–ECM mechanotransduction in 3D.

[H1] Introduction

Over the last several decades, it has been established that cell intrinsic mechanisms are not sufficient to explain cell behaviours, with the microenvironment playing a critical role in many, if not all, cellular functions. Tissues consist of cells and extracellular matrix (ECM). The ECM is a scaffolding that provides mechanical support and drives biological signalling in cells in tissues and has been recognized as a key aspect of the microenvironment that regulates cell behaviours and phenotype. As cells push and pull on ECM during various biological processes, the ECM initially resists these actions, as governed by ECM stiffness **[G]**. Stiffness is usually described by the elastic modulus, which ranges from 100s of Pascals (Pa) to several kilopascals (kPa) in soft tissues such as brain, adipose and breast tissue, and up to 10s of kPa in muscle[1,2] (Fig. 1a). As cells increase the magnitude of their pushing and pulling, the ECM resistance often increases nonlinearly or the ECM becomes stiffer, a behaviour described as nonlinear elasticity **[G]** [3]. As cells sustain their pushing and pulling over time, the resistance of the ECM to cell-induced deformation relaxes due to stress relaxation **[G],** and the ECM undergoes creep **[G]** under loading, as defined by the ECM viscoelasticity **[G]**. Finally, permanent deformations can set in following release of forces by the cell due to ECM mechanical plasticity **[G]**. These various mechanical properties of the cells impact intracellular signalling, transcription and phenotype through feedback from the ECM, whereby cells sense and respond to mechanical cues provided by the ECM — a process known as cell–ECM mechanotransduction.

Early studies of mechanotransduction focused on the impact of stiffness in 2D culture models on various processes such as cell migration, proliferation, malignancy and differentiation[4-8]. These established the concept of mechanotransduction and have proven to be relevant to various in vivo contexts. However, a 3D culture microenvironment is required for promoting biologically relevant behaviours in many contexts[9,10]. For example, a 3D microenvironment maintains a chondrogenic phenotype[11], distinguishes normal mammary epithelial cells from breast cancer cells with only the normal cells forming growth arrested organotypic acinar

structures[12], supports fibrillar adhesions in fibroblasts that are observed in vivo[9], boosts pluripotency of human embryonic stem cells[13], and regulates cancer cell angiogenic capability[14]. Importantly, an emerging body of evidence indicates that mechanotransduction in 3D can differ from mechanotransduction in 2D. In this Review, we discuss recent advances in our understanding of cell—ECM mechanotransduction in 3D. First, we start by reviewing the mechanics of various ECMs followed by the impact of ECM mechanics on various cell behaviours such as cell spreading, migration, differentiation and other fundamental biological processes. Then, we describe the nature of cell–ECM mechanical interactions in 3D, and the corresponding mechanotransduction mechanisms mediated through cell membrane receptor proteins and the nucleus. Lastly, we end the Review by describing the role of mechanotransduction in tissue development, disease and repair as well as the potential use of these findings towards mechanotherapy.

[H1] Mechanics of ECM Components

In this section, we discuss major ECM components (Fig. 1b-c) that are implicated in tissue mechanics and cell-ECM mechanotransduction.

[H2] Collagen-1

Type-1 collagen (col-1) is ubiquitously expressed and represents the most abundant protein in humans[15]. Col-1 forms fibrils and then fibres that range from 50 to a few hundred nanometers in thickness and can be many microns in length. These fibres crosslink together to form col-1 networks, and reconstituted col-1 gels are often used in 3D culture studies. Architecture and crosslinking of these networks vary substantially based on tissue type. As cells bind to col-1 fibres through integrin membrane receptors, heterodimers consisting of α and β subunits, with α2β1 and α1β1 integrins particularly implicated[16,17], these networks are central to many cell–matrix interactions.

Col-1 mechanical properties are complex and often exhibit varying levels of stiffness, nonlinear elasticity, viscoelasticity and plasticity. Individual col-1 fibrils exhibit a stiffness range of 300 MPa to 1.2 GPa, with the increase resulting from straightening and uncoiling of triple helical structures of col-1(ref. [18]). Reconstituted col-1 gels are microporous and exhibit elastic moduli on the order of 10s to 100s of Pa at the micro to macro scale. Col-1 networks initially resist external mechanical loading or deformation in shear or tension through resistance of individual fibres to bending, at low strain **[G]** values, and then stretching of rotated and aligned fibres, at higher strains[1,19] (Fig. 1d). This results in strain stiffening (nonlinear elasticity) with an almost order of magnitude increase in stiffness with strain (Fig. 1d), which depends sensitively on the length of fibres comprising the network[3,20]. Like most natural ECMs, col-1 networks are susceptible to degradation by proteases, particularly matrix metalloproteinases **[G]** (MMPs)[21].

Col-1 networks are formed from a combination of weak crosslinks within fibres and between fibres, physical entanglements, and covalent crosslinks[22,23]. Enzymes such as lysyl oxidase **[G]** and advanced glycation end products **[G]** facilitate covalent crosslinks between col-1 fibres. Increased crosslinking density restricts bending deformation of the fibres leading to increased stiffness. Under an applied mechanical stress or strain, unbinding of weak crosslinks within fibres or between fibres can lead to fibre lengthening or fibre reorientation and flow, respectively, giving rise to time-dependent viscoelastic responses such as creep or stress relaxation, and dissipating mechanical energy[22-24]. Unbinding of the crosslinks leads to fibre lengthening and matrix flow, corresponding to translational movement of the fibres, which are irreversible and stabilized by reformation of weak crosslinks and therefore associated with plastic or permanent deformation (Fig. 1d). Thus, in col-1, weak bonds whose breakage allows viscoelastic creep and stress relaxation also lead to plastic deformation, thereby linking viscoelasticity to plasticity.

[H2] Fibrin

Fibrin is the major constituent of blood clots. Fibrinogen is the precursor of fibrin, which is converted to fibrin fibres in the presence of thrombin and calcium during a blood clot. Fibrin forms a 3D branched fibrous network with the network exhibiting weak physical crosslinks and covalent crosslinks, facilitated by factor XIII[22,23]. Cells bind to fibrin through integrin receptors, α3β2 specifically. Fibrin gels typically exhibit elastic moduli on the range of 100s of Pa to the low kPas[25,26]. At the macroscale, col-1 and fibrin networks show similar mechanisms for stiffness, viscoelasticity, strain stiffening and plasticity owin to structural

similarities[3,22]. Fibrin gels are relevant for studying mechanotransduction in wound healing and also for various clinical applications.

[H2] Basement membrane

The basement membrane (BM) is a thin layer of ECM that separates epithelial and endothelial cells from the surrounding connective tissue, and also surrounds muscle and fat cells[27,28]. It is nanoporous and the major constituents are typically laminins, found in a layer facing cells, and type IV collagen (or col-IV), found in a layer facing the stroma, with the two layers linked by entactin. The BM has a thickness on the order of hundreds of nanometers to several microns. Curvature of the BM surrounding the epithelial cells may give rise to interesting 3D cell–ECM interactions (epithelial structures are typically 3D). Cells bind to laminins through various β1 containing integrins and α6β4 integrin. Stiffness measurements of different BMs vary from 100s of Pa to 10s of kPa, and BM exhibits nonlinear elasticity[29]. In tissues, cells may sense some combination of BM and col-1-rich stromal matrix mechanical properties, given the thickness of the BM[30]. Nonetheless, increased expression of laminin-crosslinking proteins such as Netrin-4 are associated with metastasis formation, thus indicating the role of stiffness of the BM in metastasis[31]. Reconstituted BM (rBM) matrices **[G]** comprise a homogenous nanoporous network formed from a mix of matrix proteins including laminin-111 and col-IV that is often used in 3D culture models of epithelia[32].

[H2] Hyaluronic acid

Hyaluronic acid (HA) is a linear polysaccharide that is present in almost every tissue. HA forms connected or interpenetrating networks with other ECM components such as col-1, as in skin or breast, or type-II collagen and aggrecan **[G]**, as in cartilage. Functionally, HA is a hydration molecule because of the large negative charge on its polymer chain that draws in water, and provides strong compression resistance. Cells bind to HA through CD44 **[G]** and RHAMM **[G]** receptors. While HA does not engage integrin receptors, it is implicated in mechanotransduction[33] and inducing specialized microtubule rich protrusive structures known as microtentacles **[G]** [34]. Ageing and disease conditions, such as osteoarthritis or cancer, have been shown to correlate with changes in molecular weight of HA, with higher molecular weight HA corresponding to pathology[35]. However, the links of molecular weight of HA to tissue mechanics and mechanotransduction are unclear. While naturally extracted HA does not form a gel, HA can be chemically modified with various crosslinking groups to form elastic or viscoelastic gels[36].

[H2] Fibronectin

Fibronectin is a glycoprotein that is found in connective tissues, and often implicated in mechanotransduction[37]. Each fibronectin protein has two Arginine-Glycine-Aspartate (RGD) sequences **[G]** which bind to several β1 and β3 containing integrins[17]. The RGD cell adhesion peptide motif is used in many synthetic-ECM-based cell culture studies[38-43]. In addition to the RGD domain which binds to cells, fibronectin also has col-1 binding domains which leads to formation of fibronectin–col-1 composite networks[44]. Unlike col-1 and fibrin, fibronectin does not form a fibrous network by itself or in the presence of any external chemical factors. Instead, it relies on cells to mediate fibronectin assembly of individual fibronectin molecules into insoluble elastic fibres. In this case, cellular forces open up the cryptic domains in fibronectin molecules that mediate crosslinking, leading to network formation[45]. Fibronectin coatings are used for 2D culture studies while fibronectin-rich matrices can be formed by fibroblasts[9]. The RGD cell adhesion peptide motif is used in many synthetic-ECM-based cell culture studies.

[H2] Other ECM proteins implicated in mechanotransduction

Beyond the components described above, ECM consists of various other proteins, often referred to as the matrisome, with some of these components implicated in mechanotransduction. For example, tenascin-C **[G]** is implicated in mechanotransduction in the context of glioblastoma brain cancer[46]. Proteomics of breast cancer tissues revealed the novel role of other types of collagens such as col-VI and col-XII in breast cancer

progression [47,48]. Specifically, col-VI has been shown to enhance cancer cell invasion, while col-XII is indicated in reorganization in col-1 which may be associated with col-1 remodelling during metastases.

[H2] ECM in tissues as composite networks

While the mechanics of individual ECM components are important, tissues typically consist of multiple interacting ECM proteins, networks and cells with properties emerging from the interactions between the different components. Indeed, reconstituted col-1, fibrin, and rBM have an elastic modulus ranging from ~10s to 100s of Pa, which are much softer than many soft tissues (~kPa). Recent studies have demonstrated that composites of HA and col-1 gels exhibited enhanced stiffness and delayed strain stiffening compared to col-1-only gels[49,50]. Further, the presence of elastin fibres, which are linearly elastic and highly resistant to degradation[51], is thought to drive elastic recovery of many tissues following bulk deformation[52]. Additionally, chemical interactions of proteoglycans **[G]** such as small leucine-rich proteoglycans and versican **[G]** affect col-1 organization[53]. However, clear mechanistic insights into the design rules by which various matrisomal proteins impact col-1 organization and mechanics remains largely unclear.

In addition to the contribution of ECM components to ECM mechanics, cell interactions with ECMs can also lead to emergent mechanical properties. For example, fibroblast contraction of nonlinear elastic col-1 gels leads to a stiffer matrix[54]. Alternatively, a composite of closely packed cells and col-1 exhibits compression stiffening similar to liver tissues, in contrast to compression softening of col-1 gels[55]. Taken together, these studies indicate the importance of understanding how ECM components interact together and with cells, as would occur in tissues, to govern the mechanical properties sensed by cells in mechanotransduction.

[H2] Tissue mimicking artificial ECMs for 3D culture

Reconstituted ECMs based on the natural ECM materials described above have several limitations in terms of their use for 3D culture studies of mechanotransduction. Many of these are much softer than soft tissues, with elastic moduli much less than 1 kPa (Fig. 1a; Supplementary Table 1). Further, stiffness, viscoelasticity, ligand density, and matrix pore size and architecture cannot be modulated independently, making it challenging to obtain definitive mechanistic insights into how these different properties influence cell biology. To address these shortcomings, engineered hydrogels with biologically relevant mechanics and signalling, and, importantly, independently tunable features, have emerged as powerful experimental platforms for 3D culture studies of mechanotransduction and led to key insights into mechanotransduction as described in the subsequent sections. Box 1 describes some of these platforms in more detail.

[H1] Impact of ECM mechanics on cells

In this section, we survey some key trends between variation in specific ECM properties and their impact on various biological processes, focusing on adherent cells (Table 1).

[H2] Cell spreading

Mesenchymal and epithelial cells spread in 2D and 3D by taking different morphologies such as spherical, spindle-like, or irregular shapes with different protrusions such as lamellopodia [G], filipodia [G], and invadopodia [G]. In 2D culture, increased stiffness generally promotes cell spreading[56]. In 3D, cell spreading proceeds in microporous ECM that are sufficiently stiff [57], but is restricted in nanoporous ECM. Covalently crosslinked elastic hydrogels typically restrict cell spreading in 3D[42,58-60]. Contrastingly, viscoelastic hydrogels with sufficient stress relaxation or plasticity[40,61,62], or hydrogels that undergo degradation due to hydrolytic or protease activity[42], allow cell spreading. Cell-adhesion ligands, such as RGD cell adhesion peptide motifs, are required for cell spreading in both 2D and 3D, though very high ligand density diminishes cell spreading[63]. Finally, ECM geometry, or structural features at the scale of tens to hundreds of microns such as curvature and patterning of cell–ECM adhesion ligands, also impact cell spreading in both 2D[64] and 3D contexts[65,66].

[H2] Cell migration

Cell migration occurs during development, wound healing, immune trafficking, and cancer metastasis[58,67]. On 2D substrates of increasing stiffnesses, migration often follows a biphasic pattern with higher levels of migration at intermediate stiffness values[68-70]. Further, different cells sense and migrate along positive and negative gradients of substrate stiffness — a process termed durotaxis[69,71,72]. In 3D ECMs, various properties have been implicated in regulating cell migration. If pore size of the ECM is above roughly 3 μm in diameter, cells can migrate robustly by squeezing through the pores[59,73,74], and migration can be guided by matrix architecture and alignment of fibres[75]. In ECMs with smaller pore sizes, cells can generate channels to migrate either using proteases to degrade the ECM[42,73,76], or forces to mechanically open channels to migrate through, if the ECM exhibits sufficient matrix mechanical plasticity, a property often linked to viscoelasticity[77-79]. Notably, interstitial fluid flow, which is dependent on matrix porosity, has also been shown enhance cell migration in 3D[80].

[H2] Matrix secretion

Cells not only degrade the ECM but also secrete nascent ECM[81-84] in 3D culture. Hydrogel viscoelasticity regulates ECM production by chondrocytes[84] and MSCs[85], with cells forming a more interconnected cartilage-like or bone-like ECM in fast relaxing hydrogels. Similarly, ECM degradability allows chondrocytes to form a cartilage-like matrix[86]. Overall, cells respond to ECM properties by secreting endogenous ECMs, resulting in a feedback mechanism that fine tunes cell–ECM interactions.

[H2] Stem cell differentiation

Stem cell differentiation occurs during development and homeostasis, and controlling stem cell differentiation is a critical goal in many applications in regenerative medicine[87]. Early 2D cultures studies showed that substrate stiffness regulates stem cell differentiation and illustrated the concept that matching native tissue stiffness promotes differentiation down that tissue-specific pathway in vitro in some stem cell types[56,88]. For example, culture of mesenchymal stem or stromal cells **[G]** (MSCs)[56] on stiff substrates, with a modulus approaching that of pre-mineralized bone, promotes osteogenic differentiation whereas culture on soft substrates, with a modulus approaching adipose tissue, promotes adipogenic differentiation[2,89,90]. Similar findings hold for differentiation of neural stem cells[91]. In other contexts, such as skeletal muscle stem cells, substrates with physiological stiffness can promote self-renewal[92]. Differentiation is also mediated by cell adhesion ligand density and type and viscoelasticity in 2D[90,93-97]. Finally, 2D ECM geometry has been shown to influence MSC differentiation[98] as well as embryonic stem cell differentiation into mesoderm [99] via regulation of cell shape and cytoskeletal tension.

In 3D, some similar trends emerge, with viscoelasticity and degradation playing a more prominent role. In viscoelastic ECMs, soft substrates promote adipogenic differentiation and stiff substrates promote osteogenic differentiation of MSCs, similar to the findings from 2D[39,40]. Osteogenesis is enhanced with faster stress relaxation[40], and stress stiffening of soft substrates can support osteogenic differentiation[100]. In covalently crosslinked hydrogels, degradability is required for osteogenesis[60]. Mechanical cues have been shown to impact fate in other stem cells populations in 3D, as well as monocyte differentiation[101-104]. Additionally, ECM architectural features such as fibre diameter and alignment have been shown to influence MSC differentiation[66].

[H2] Cell division

Every cell arises from a cell division event, and cell division underlies development and tumour growth. In 2D, increased stiffness promotes cell proliferation[38,105]. In 3D, covalently crosslinked elastic matrices that are nanoporous restrict cell division, while faster stress relaxation in viscoelastic matrices, or increased matrix degradability, allows cell division and proliferation in nanoporous matrices[40,103,106].

[H2] Apoptosis

Apoptosis, or programmed cell death, is necessary for proper tissue homeostasis and aberrant regulation of apoptosis is a hallmark of cancer. In 2D, soft substrates or confining geometries that limited spread area led to higher levels of apoptosis[38,64]. In 3D, apoptosis is triggered during cell migration through confining

pores[74] or through cell volume restriction in microwells in a stiffness dependent manner[107], and is also modulated by matrix viscoelasticity[41].

[H2] Morphogenesis

Morphogenesis is a complex multi-cellular process wherein cells self-organize to form 3D structures with specialized form and function[108]. While collective cell interactions are often emphasized in morphogenesis, there is increasing research emphasizing the role of 3D microenvironment, including matrix degradability and viscoelasticity as central factors regulating morphogenesis in organoid models. Organoids are multicellular structures that capture specific features of organs. rBM matrices, which are widely used for organoid culture are inherently viscoelastic and enzymatically degradable[109]. Degradable gels promote apicobasal polarization and lumen formation in MDCK cysts[110], support viability and formation of budded intestinal organoids containing differentiated cell types via symmetry breaking mechanisms,[111,112] but restrict progenitor cell phenotype in neural tubes cultures in vitro and in intestinal organoids[111-113]. Viscoelasticity modulates lumen formation in organoid cultures of human pluripotent stem cells (hPSCs)[41], in endothelial vasculogenesis[114] and during crypt budding in intestinal organoids[115]. Further, adhesion ligand density and type affect formation of intestinal organoids[111] and lumens in MDCK cysts[110] and hPSCs[41]. ECM geometry has recently been shown to guide intestinal organoid formation and improve reproducibility of organoids formed[65]. Finally, stiffness controls cell survival[111] and tissue budding[115] in intestinal organoids, and mimicking stiffness of native tissues enhances liver organoid formation[116].

[H2] Cancer progression

ECM mechanics regulate emergence of a tumour phenotype[117]. In 2D and 3D, increasing substrate stiffness over the range detected during breast cancer progression promotes induction of a malignant phenotype in models of normal mammary epithelium[118-122]. This same impact has been shown in other cancers as well, as we will describe later. ECM geometry also influences cancer progression and collective invasion by controlling cell shape and subsequently, cell phenotype in both 2D[123] and 3D microenvironments[84,124].

[H1]Cell–matrix interactions in 3D

Here we describe key features of cell–ECM interactions and force generation in 3D (Fig. 2), which ultimately underlie 3D mechanotransduction.

[H2] Cell–ECM adhesions

Cell adhesion signalling in 3D varies from that observed in 2D in several ways. Cells on 2D substrates form adhesions with ECM only on one surface, whereas cells can form adhesions in all directions in 3D, which impacts cell signalling. For example, mammary epithelial cells grow rapidly on 2D tissue culture plastic substrates, but form growth-arrested, organotypic acinar structures in 3D culture in rBM matrix[12]. Establishment of 3D matrix protein interactions, and not matrix per se, is sufficient for acinar morphogenesis as even on 2D rBM substrates cultured in media with the addition of soluble rBM proteins, thereby allowing cells to bind to these proteins in all directions, acini formation is observed[125]. How signalling activation across the entire cell surface leads to a different outcome than signalling across one bottom surface remains unclear.

Further, the structure of adhesions differ in 3D relative to 2D. Cells in most currently used 3D hydrogels (with some exceptions) typically do not form large focal adhesions **[G]** that are connected to robust actin stress fibres[126-128]. In nanoporous hydrogels, which unlike fibrillar hydrogels are more homogeneous and have nanometer-sized pores, distinct adhesive complexes are often not observed, whereas in fibrillar matrices, fibrillar adhesions **[G]** often form that co-localize with matrix fibres, and exhibit distinct phosphorylation of adhesion complex proteins such as paxillin and focal adhesion kinase **[G]** (FAK) relative to canonical focal adhesions[9,126,129]. By contrast, while adhesion complex formation also varies in 2D culture, substrates with sufficient stiffness and ligand density typically lead to formation of focal adhesions. Further, in 3D, cells typically lack the thick contractile actomyosin stress fibres spanning the cell length that are characteristic of 2D cell culture on stiff ligand-dense substrates[126]. Cells in 3D typically have an actin shell cortex at the cell

membrane, however, the molecular architecture of the actin meshwork and actomyosin machinery in the cell cortex remains unclear owing to limitations in the spatial resolution of microscopy for 3D culture.

[H2] Confinement

In 2D, cells can spread out on the substrate or change their volume unrestricted, whereas in 3D, changes in volume and shape are physically resisted, or confined, by the surrounding ECM (in addition, the ECM can restrict nutrient transport)[10]. Level of confinement is determined by ECM pore size[130] and properties such as viscoelasticity[40] and degradability[62]. A pore size of around 3 μm in elastic gels or structures with rigid pores serves as a barrier to cell migration because the relatively stiff nucleus cannot be deformed through smaller pores[73,74]. However, physiological matrices are not elastic with rigid pores, but are typically viscoelastic[52], exhibit mechanical plasticity[52,131] and are degradable[132], allowing expansion of pores by cellular forces and protease mediated degradation. For example, degradation of ECM can convert even an elastic matrix to a viscoelastic fluid-like matrix[133], opening up pores for cell migration[42,73,134] and promoting MSC spreading, force generation and differentiation[60]. Viscoelastic and viscoplastic matrices dissipate mechanical stresses, undergo matrix creep under loading, and exhibit irreversible deformations in response to force, allowing cells to generate space in the ECM, reducing confinement. As such, viscoelasticity and viscoplasticity enable cell spreading[135], cell volume expansion[61], cell growth for division[136], proliferation of multicellular structures[106], deposition of matrix extracellularly[84], and cell migration independent of proteases[77]. Thus, in 3D, cell confinement is governed by ECM pore size, viscoelasticity and plasticity, and degradability. Natural ECMs exhibit all these properties including viscoelasticity, plasticity and degradability, allowing cells in vivo to remodel the matrix and potentially alter confinement.

[H2] Cell–ECM forces

In 2D and 3D, contractile or pulling forces generated by actomyosin machinery represent a major modality of force generation, but several additional modalities of force generation become accessible to cells in 3D. 3D traction force microscopy **[G]** techniques have enabled quantification of cell generated forces by measuring matrix deformations and estimating stresses using theoretical models of matrix properties[137-139]. These studies, and other studies using these techniques, show that cells exert contractile forces on ECMs through integrin-based adhesions in 3D. Indeed, it has long been known that fibroblasts cultured in a collagen gel will continuously contract the gel[140]. Owing to the differing structure of both the adhesions and the contractile actomyosin machinery, the capabilities of contractile force generation in 3D likely differ from 2D, though the precise differences remain unclear.

In 3D, cells also generate protrusive forces. Polymerization of branched actin networks generates protrusive forces[141]. In 3D, using adhesions or steric support of the matrix, cells apply these protrusive forces onto the ECM, in the form of structures such as invadopodia **[G]**, filopodia **[G]**, or lamellipodia **[G]**[58,77,142,143]. Alternatively, extension of the microtubule-based spindle generates outward protrusive forces during mitosis[136,144]. Any process resulting in shape change in confining 3D ECMs must necessarily generate some combination of protrusive and contractile forces.

Similarly, any process involving increased cell volume in confining 3D matrices necessarily requires force generation. Cell volume is a tightly controlled parameter that is crucial for cell survival and function[145,146]. Cell volume is governed by the combination of osmotic pressure and hydrostatic pressure differentials between the intracellular and extracellular space. To increase volume, cells generate osmotic pressure by increasing the concentration of ions inside the cells via activity of ion pumps and channels[145,147]. This draws water into cells both by diffusion across the cell membrane and transport via water channels such as aquaporins, thereby causing volume expansion[145]. Cell volume expansion can occur both globally and locally. For example, in 3D confining matrices, nuclear entry into thin protrusions acts as a piston[148] to pressurize protrusions by opening ion channels which subsequently generates osmotic and hydrostatic pressure to expand the protrusion and expand the ECM pore to create a track allowing cell migration[78]. Water flux however does not necessarily indicate volume changes as directed water flow across the cell can drive cell migration[149]. Overall, cells generate localized contractile and protrusive forces on ECM, as well as global outward forces during cell volume expansion, in 3D.

[H2] ECM stresses in 3D

Cellular forces on ECMs in 3D result in complex mechanical stress fields, which in turn act upon other cells as well as trigger feedback mechanisms in cells applying these forces. These force fields in the matrix can be interpreted in the form of a combination of hydrostatic and deviatoric components. Hydrostatic stresses **[G]** are dilational in nature or act to change ECM volume, or from the cellular perspective, oppose or promote cell volume changes. For example, as tumour cells proliferate in spheroids in 3D, hydrostatic stresses are generated which mechanically oppose tumour growth[150,151]. Deviatoric stresses **[G]** , on the other hand, are distortional in nature and act to change morphology while preserving volume. Such distortional stresses are necessary for matrix remodelling and fibre alignment, which in turn promote cell spreading and migration[19,24]. The extent of cell deformation and morphology change due to these stresses are dependent on cell mechanical properties. Cells actively regulate their cytoskeletal and nucleoskeletal proteins as well as fixed charges (the net electric charge of all intercellular components that cannot freely diffuse out of the cell) and concentration of macromolecules, determining the viscoelasticity, poroelasticity, **[G]** and non-linearly elasticity of the cell[152,153]. Such emergent mechanical properties of a cell result in a net bulk modulus which determines to what extent the cell is able to resist a change in volume and a shear modulus which determines the cell's resistance to distortion. The cell bulk modulus is on the order of MPa to GPa, whereas the shear moduli are on the order of kPa[154]. Thus, volume changes in cells must be regulated actively, since the bulk modulus is so much higher than typical physiological stresses in soft tissues resulting from cell generated or externally applied forces. These moduli determine a cell's ability to change volume and navigate confining environments. For example, nuclear stiffness governs cell migration through confining spaces[74,155-157]. Overall, cell mechanical properties in conjunction with matrix stresses dictate cell morphology and behaviours such as migration and differentiation in 3D.

[H2] Cell–ECM mechanical feedback

As cells interact with physiological ECMs in 3D, the interactions are dynamic as matrix properties change over time due to matrix viscoelasticity (relaxation, creep), plasticity, degradation and matrix deposition. Natural ECMs dissipate forces and flow over time due to viscoelasticity and degradability, and undergo permanent deformation due to plasticity. Further, these interactions depend on force or deformation scales (or magnitudes) due to nonlinear elasticity of the ECM, whereby matrix resistance increases with increased magnitude of force or deformation, thereby supporting the generation of higher cellular forces and forming a positive feedback loop. Thus, timescales and magnitudes of cellular forces exerted on their 3D microenvironment are critical. For example, actomyosin contractile forces applied on ECM fibres generate distortional stress and locally align fibres[158]. In non-linear, elastic ECMs such as collagen, this fibre alignment increases local stiffness of the matrix which in turn promotes higher force generation and increases cell stiffness revealing a positive mechanical feedback loop between cells and matrix[159]. Interestingly, fibroblasts exploit non-linear elasticity of the ECM and mechanical feedback loops to migrate by generating fibre alignment and higher forces at the front of a migrating cell as compared to the rear[160]. By contrast, in linear elastic ECMs such feedback loops are absent which prevent higher force generation and lamellipodia formation[57,161]. Similar feedback loops are observed between cellular pushing forces and matrix viscoelasticity or plasticity[62,77,162,163]. In confining nanoporous ECMs, cancer cells apply protrusive forces to deform the ECM and in sufficiently plastic ECMs, such forces lead to permanent matrix deformations, which in turn promote growth of protrusions and result in larger pores for cell migration. In addition, deposition of matrix by cells, for example MSCs secreting matrix proteins such as fibronectin, laminin and collagen, leads to formation of an endogenous ECM that the cell interacts with, and can mediate mechanotransduction[82,164]. Thus, dynamic cell–ECM interactions and positive cell–ECM feedback loops orchestrate cell behaviours in 3D.

[H1]Mechanisms of 3D mechanotransduction

In this section, we discuss mechanisms of 3D mechanotransduction mediated through two major classes of membrane proteins, integrins and mechanosensitive ion channels **[G]**, and how these two pathways converge on the nucleus (Fig. 3). Many of these 3D studies were performed in hydrogels with independently tunable stiffness, stress relaxation and degradability (see Box 1).

[H2] Integrin-mediated mechanotransduction

Similar to their role in 2D (Box 2), integrins have been identified as central regulators of mechanotransduction in 3D, often through integrin clustering. For example, increasing stiffness of col-1 rich gels used to culture mammary epithelial cells in 3D from 100s of Pa to kPa (as is observed during breast cancer progression) — through either increased col-1 density or crosslinking, — promotes a malignant phenotype characterized by elevated proliferation and invasion into the surrounding matrix through α5 and β1 integrins and integrin clustering[119,120]. Integrin binding to stiff ECM in dense col-1 gels leads to activation of FAK, activated Rho signalling **[G]** , and increased cell contractility, causing integrin clustering[119,120,165]. These events lead to downstream activation of the Ras-mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways, both activated during breast cancer progression, which promote the growth of a tumour and invasion[165]. Interestingly, an opposite trend was observed when mammary epithelial cells were cultured in interpenetrating networks of rBM and alginate, used to mimic pre-invasive breast cancer where cells are surrounded by BM; in this scenario, increased stiffness inhibited formation of clustered α6β4 integrin-containing adhesions, thereby promoting an invasive phenotype through activating Rac1 and PI3K [122]. Together these data indicate that stiffness- and ligand-mediated clustering of multiple integrin types plays a critical role in 3D mechanotransduction in cancer cells and cancer progression.

Integrin-mediated mechanotransduction also plays a central role in how stiffness, degradability, stress relaxation and stress stiffening regulate differentiation of stem cells. MSCs encapsulated in ionically crosslinked RGD-coupled viscoelastic alginate hydrogels with an elastic modulus in the range of 11-30 kPa differentiated into osteoblasts, in an integrin-mediated manner, whereas those cultured in lower stiffness gels underwent adipogenesis. The differentiation state was associated with integrin clustering, with maximum levels of integrin clustering associated with optimal osteogenic differentiation. Integrin clustering was driven by contractility mediated forces. Interestingly, in covalently crosslinked non-degradable HA-hydrogels, hydrogel stiffness did not impact differentiation, with adipogenesis observed from 1 kPa to 100 kPa. Instead, degradability of the HA hydrogels was required for osteogenesis, with MSCs able to exert larger integrin-mediated tractions with increasing degradability[60]. Further, in another study, increased rate of stress relaxation in RGD-coupled alginate hydrogels with a 20 kPa modulus promoted enhanced osteogenic differentiation of MSCs, and differentiation was associated with integrin clustering[40]. Importantly, cell morphology was decoupled from osteogenic differentiation in all of these studies, in contrast to the result in 2D where 2D morphologies are distinct for adipogenic, and osteogenic differentiation [56]. Finally, stress stiffening in 0.2-0.4 kPa polyisocyanate hydrogels also led to integrin clustering in MSCs and shift of differentiation from adipogenesis to osteogenesis[100]. These studies are consistent with the concept that the extent to which the matrix can be remodelled, facilitated by either viscoelasticity or degradability, and some minimum level of tension across the integrins, mediated by the stiffness and nonlinear elasticity, are required for osteogenesis. In vasculogenesis of endothelial progenitor cells (EPCs), ECM viscoelasticity along with hypoxic environment promote the aggregation of EPCs through contractility-mediated integrin clustering[114,166].

While there are similarities between 2D and 3D integrin-mediated mechanotransduction pathways, key differences have been implicated. This is suggested by the differences in adhesion structures and the actin cytoskeleton, with cells not exhibiting focal adhesions or stress fibres in most 3D conditions, as described in the previous section. Indeed, cell morphology was decoupled from osteogenic differentiation of MSCs in 3D [39,40,60], in contrast to the result in 2D[56], and some studies have even demonstrated integrin-mediated osteogenic differentiation in 3D is independent of contractility[100,167]. However, in contrast to the well-known pathways and events following integrin–ECM binding in 2D culture, there is limited available information on downstream molecular events following integrin engagement in 3D. Further, the application of the molecular clutch mechanism, broadly implicated in 2D mechanotransduction (Box 2), towards 3D contexts remains to be tested. These highlight the gaps in our understanding of integrin-mediated mechanotransduction in 3D.

[H2] Confinement sensing by cell volume regulation

Recent work has pointed towards the role of active regulation of cell volume and activation of mechanosensitive ion channels in 3D mechanotransduction, particularly in the context of sensing mechanical confinement. For example, chondrocytes increase their overall volume with increased stress relaxation of alginate hydrogels without integrin-binding ligands, and increased cell volume promoted enhanced cartilage

matrix deposition and the chondrogenic phenotype[84]. Similarly, cell volume is shown to regulate various intracellular events such as actin organization, nuclear accumulation of histones, YAP/TAZ **[G]** localization, nuclear shape, and focal adhesions for MSCs cultured in 3D PDMS microwells[168]. Cell volume expansion is possible in degradable hydrogels or hydrogels with fast stress relaxation, but necessarily restricted in more confining elastic hydrogels. Hence, cell volume regulation has been recognized as an important mediator of 3D mechanotransduction.

Mechanosensitive ion channels, particularly TRPV4 channels, have emerged as key sensors of changes in cell volume. In MSCs cultured in RGD-coupled alginate hydrogels of different time scales of stress relaxation with same initial stiffness, faster stress relaxation leads to increased cell volumes associated with activation ofTRPV4 ion channels[61]. Increased calcium influx through TRPV4 activation drives nuclear localization of RUNX2, a transcription factor involved in osteogenic differentiation. Similarly, in the context of cancer cell proliferation in confining alginate hydrogels, cell growth during the G1 phase of the cell cycle activated a TRPV4–PI3K–AKT–p27 signalling axis that drove S-phase progression and proliferation[106]. However, increased confinement in more elastic gels blocked cell growth and activation of this pathway. Further, TRPV4 was linked to cell volume expansion and mechanical confinement-sensing in chondrocytes, and controlled phosphorylation of GSK3β (glycogen synthase kinase 3β), an enzyme associated with osteoarthritis[169]. In myofibroblast activation, increased stiffness induces TRPV4 activation and YAP nuclear localization suggesting a regulatory role of TRPV4 in mediating YAP nuclear shuttling and signalling[170]. Connecting cell volume to activation of mechanosensitive ion channels is membrane tension[171]. Piezo1, a mechanosensitive ion channel central to force-sensing in many contexts[172], has also been implicated in some of these studies[106,171], but its role in responses to ECM-mediated confinement remains less clear. Together these studies indicate that confinement regulates cell volume expansion, and as cell volume expands, membrane tension increases, activating mechanosensitive ion channels, allowing the passage of ions across the membrane, which in turn activate various signalling pathways to drive mechanotransduction.

Several key questions remain regarding this mode of mechanotransduction. For example, it is unclear what initiates cell volume expansion in many of the aforementioned studies. Moreover, it has been observed that integrins are also implicated in mechanotransduction in parallel with mechanosensitive ion channel mediated mechanotransduction. For example during MSC differentiation[40,60], cells utilize integrin-binding and clustering in addition to TRPV4-mediated volume expansion to regulate their differentiation pathways, suggesting the possibility of interactions or crosstalk between integrin-mediated and ion channel-mediated pathways. In another connection, the findings on the role of mechanosensitive ion channels in cell–ECM mechanotransduction complement findings on the role of these channels in force-mediated mechanotransduction, including application of stretch and shear, indicating the potential for crosstalk between these distinct modes of mechanotransduction[173,174].

[H2] Nuclear mechanotransduction

As the center of transcription, the nucleus is a key element of mechanotransduction pathways downstream of both integrin and mechanosensitive ion channel mediated routes. The membrane of the nucleus is mechanically linked to the actin cytoskeleton through linker of nucleoskeleton and cytoskeleton (LINC) complexes comprising of nesprins and SUN (also known as Sa1p in yeast and UNC-84 in *C. elegans*). Forces from actomyosin based contractility, which acts extracellularly on ECM in both 2D and 3D environments, also can be transmitted to the nucleus through LINC complexes leading to mechanical deformations of the nucleus[175]. However, a clear mechanism associating cellular forces to nuclear deformation in 3D is still unclear, in part due to lack of clarity over the actin cytoskeletal structure for cells in 3D. Below we discuss key nuclear changes observed in cells cultured in 3D and linked to mechanotransduction.

[H3] Nuclear morphologies. 2D studies have demonstrated change of nuclear morphology as a clear indicator of mechanotransduction associated with various outcomes, such as epigenetic remodelling, shuttling of transcription factors such as YAP/TAZ and cell fate changes[175]. However, nuclear morphologies in 3D differ significantly from those observed in 2D cell culture models. For example, projected nuclear areas and circularity are similar for mammary epithelial cells and pluripotent stem cells in vivo and in 3D culture models[41,126], but significantly higher in 2D[154]. Further, both increased stiffness and increased degradability leads to increased nuclear wrinkling in 3D[127,176], whereas increased stiffness has opposite effect on nuclear wrinkling

in 2D[176]. Mechanosensitive ion channels in the nucleus, recently implicated in cell migration in confining microenvironments[155,156], could potentially play a role in responding to morphological changes in the nucleus in 3D. Together these indicate that nuclear mechanotransduction mechanisms in 3D are likely different from 2D.

[H3] Regulation of transcription factors. RNA-seq studies show that changes in hydrogel stiffness, stress relaxation and ligand type and density each are associated with large changes in gene expression in different cell types[126,177]. Whereas YAP/TAZ coregulators have been described as universal mechanotransducers in 2D culture studies, their role in 3D mechanotransduction is context dependent. Increased stiffness, faster stress relaxation, and increased degradability promote enhanced YAP/TAZ nuclear localization in MSCs in 3D[40,176]. However, YAP localization was decoupled from osteogenesis induced by stiffness [40,61]. Further, in a 3D culture model of pre-invasive breast cancer, YAP was not required for mechanotransduction, consistent with in vivo observations[126]. Instead, transcription factors such as STAT3, p300, and Sp1 were implicated[126,127]. Contrastingly, YAP is found to play a role in 3D mechanotransduction in gastric cancer. Moreover, in 2D, YAP/TAZ localization was observed in the absence of nuclear wrinkling[176], while in 3D[178], nuclear wrinkling is correlated with YAP/TAZ localization. In neural stem cell differentiation, stiff ECM is associated with nuclear localization of transcription factor EGR1 in 3D but not in 2D, and this occurs in the absence of integrin-based adhesions[179]. In MSCs, soft ECM increases the clustering of inflammatory receptor, tumor necrosis factor-α (TNF-α), which further activates nuclear factor kB (NF-kB) and expression of chemokines and cytokines that promote monocyte recruitment and differentiation[180]. These set of studies highlight the role of transcription factors in mediating mechanotransduction in 3D. However, how these different transcription factors are regulated by mechanical properties of the ECM, remains a key open question.

[H3] Epigenetic regulation of chromatin accessibility. Several recent studies have pointed to a key role of chromatin accessibility in mediating mechanotransduction. In the 3D culture model of pre-invasive breast cancer, increased ECM stiffness induced broad changes in chromatin accessibility mediated by histone deacetylases 3 and 8, with chromatin becoming much more open[127]. Increased chromatin accessibility then allowed binding of the Sp1 transcription factor, which led to downstream changes in gene expression that functionally mediated the malignant phenotype. In a gastric cancer study, increased ECM stiffness led to YAP translocation into the nucleus in coordination with DNA demethylation and increased chromatin accessibility of YAP promoter region, together inducing the tumorigenic phenotype[178]. Interestingly, this is in contrast to recent 2D studies where chromatin accessibility in fibroblasts decreased with increased stiffness, which led to compaction of chromatin promoting persistent activation of fibroblasts[181]. In addition to stiffness, matrix degradability affected chromatin organization in neural progenitor cells, where chromatin accessibility was increased with enhanced degradability[182]. Thus, chromatin accessibility has emerged as a key regulator of 3D mechanotransduction, though how this is regulated and functions in different contexts , and why effects may be different from 2D studies, remain to be determined.

[H1] Mechanotransduction in tissues

In this section, we describe some selected examples of how cell–ECM mechanotransduction is thought to play a role in tissue physiology (Fig. 4, 5), highlighting the ubiquity and importance of 3Dmechanotransduction in vivo.

[H2] Development

Cell–ECM interactions and mechanotransduction are implicated in multiple stages of development[183,184]. During peri-implantation development, BM secretion enables apicobasal polarization and formation of epiblast cavity[185]. During gastrulation, localized degradation of the BM mediates migration of primitive streak, establishing the body axes in mice[186] (Fig. 4), whereas differences in cell stiffness drive flow of cells and internalization of the gastrulating furrow[187]. Fluidization and rigidity transition of tissues — whereby tissues switch from being solid-like to fluid-like and vice versa — are recurring themes for processes that shape tissues during development and have recently been linked to changes in ECM-dependent confinement [188-190]. Further, migrating neural crest cells generate a stiffness gradient via N-cadherin mediated cell-cell interactions that they then follow using durotaxis[191]. At later stages of development, compaction of

col-1–rich ECM by cell intercalation and contractility enables budding and branching of epithelia in salivary glands[192] and in mesenchymal condensates (densified mesenchymal structures that deform during epithelial morphogenesis)[193].

[H2] Homeostasis and regeneration

In adults, mechanotransduction-regulated behaviours maintain homeostasis and normal activity. Fibroblasts in stromal tissues maintain or modulate a state of tensional force by contracting against their surrounding col-1-rich stromal ECM to maintain mechanical homeostasis, a state of mechanical equilibrium[194,195]. Differentiation or maintenance of stemness in stem cells, or maintenance of normal phenotypes in differentiated cells, are often supported by stiffness, and in some cases viscoelasticity, of the niche[56,84,88,92]. When mechanisms maintaining normal tissue function become disrupted, diseases such as fibrosis and cancer, both discussed in the following sub-sections, can occur[196], as can other diseases such as osteoarthritis[169], polycystic kidney disease[197] and aneurysms[198].

Mechanotransduction is also implicated in regenerative processes. For example, the hematoma that follows a bone fracture is highly viscoelastic, and such viscoelasticity has been found to be necessary for infiltration of MSCs and promoting osteogenesis of the MSCs in vivo[40,78,199] (Fig. 5b).

[H2] Ageing and fibrosis

Ageing disrupts tissue properties and alters homeostatic mechanosensation further reinforcing disease phenotypes[200]. Skin BM exhibits characteristic thinning with increasing age[200], whereas BMs in the retina and blood-brain barrier thicken [201]. In stromal matrices that fill up soft tissues and are rich in type-1 collagen, buildup of advanced glycation end products and increased non-enzymatic crosslinking of col-1 results in pockets of stiff matrix while matrix metalloproteinase-driven matrix degradation softens other regions of the stroma, resulting in highly heterogeneous collagen networks with low solubility[200] resulting in fibroblast senescence[202] and reduced motility[203]. These changes alter epithelial and mesenchymal cell phenotypes, and result in positive feedback between changes in cell phenotype and ECM structure which increase the risk of diseases such as fibrosis and cancer[200,204,205].

Tissue fibrosis involving excessive deposition of ECM and aberrant tissue mechanics and is implicated in many deaths[206]. Persistent activation of fibroblasts to a myofibroblast phenotype is responsible for poor prognosis of fibrotic diseases, and is driven by altered mechanosensing[181]. As opposed to regenerative wound healing, fibrosis leads to scar formation wherein excess secretion[57] and alteration of collagen architecture[207] leads to differentiation of fibroblasts to myofibroblasts, which in turn reinforce the fibrotic niche[208] (Fig. 5c). Fibrosis of the bone marrow, or myelofibrosis, increases marrow stiffness and reduces stress relaxation, promoting monocyte differentiation towards dendritic cells, thus promoting a pro-inflammatory microenvironment and disease progression[104].

[H2] Cancer

Although at the root of cancer are mutations that activate oncogenes and inactivate tumour suppressor genes, as well as changes in gene copy number resulting from genomic instability, there has been increased recognition that the tumour microenvironment, including ECM mechanics, plays a key role in restraining or promoting tumour progression[101,209]. In breast cancer, enhanced mammographic density, associated with increased ECM stiffness, has been a well-known risk factor for disease progression[210,211]. Increased ECM stiffness is associated with increased col-1 density and elevated crosslinking of the col-1, and likely results from the activity of cancer associated fibroblasts and tumor associated macrophages[212,213]. Increased ECM stiffness promotes a more proliferative and invasive phenotype in breast cancer[118-122,126] (Fig. 5d). Matrix mechanical plasticity and degradation of the stromal matrix mediate formation of collagen tracks that carcinoma cells utilize to migrate away from the tumour[73,77,214]. Similarly, increased stiffness and more fibrillar col-1 has been linked to other cancers including pancreatic ductal adenocarcinoma[215,216], glioma brain cancer[46], colorectal cancer[217], lung cancer[218], hepatocellular carcinoma[219] and cutaneous squamous cell carcinoma[220]. Changes in viscoelasticity are also associated with breast cancer progression[221], brain cancer[222], and liver cancer[223], and likely other cancers, and also regulate tumour spheroid growth in vitro[106]; however the functional significance of these changes in viscoelasticity to disease progression in vivo are unclear.

[H1] Conclusions and perspectives

Mechanotransduction of cells in 3D impacts various cellular behaviors, which play a key role in many aspects of tissue physiology from development to disease. 2D culture models are sufficient for capturing critical aspects of mechanotransduction in vivo in some contexts; in other contexts, 3D culture is required. In 3D culture, it has become clear that stiffness, viscoelasticity, plasticity, and degradability are key parameters regulating cell behaviours (Table 1). Confinement has emerged as a key aspect of the mechanical microenvironment in 3D. Mechanosensitive ion channel-mediated sensing of confinement can, at least in some cases, complement integrin-mediated mechanotransduction pathways to regulate nuclear morphologies, chromatin accessibility and transcription factor activity, which in turn regulate gene expression and cell phenotype.

Despite these major insights, key gaps in our knowledge of cell–matrix mechanotransduction in 3D remain, as we have highlighted throughout the Review. Beyond integrins and mechanosensitive ion channels, it remains relatively unclear how mechanical cues are transduced in the 3D context. Specifically, subcellular cytoskeletal structures, downstream mechanotransduction molecules, the specific pathways, chromatin remodelling enzymes and events and set of transcription factors that mediate mechanotransduction remain to be uncovered. Promisingly, there are a large set of tools emerging that can potentially be applied to 3D culture. Spatiotemporal control of local hydrogel properties[224] and single cell microencapsulations[225] provide more tailored control of local cell microenvironments in 3D. Super resolution imaging techniques[226] suitable for 3D can reveal the structure of integrin-based adhesions, the actin cytoskeleton, and the actomyosin machinery of cells in 3D, as well as actin adaptor proteins such as talin and vinculin, and the spatiotemporal dynamics of Rho GTPase activation as indicated by FRET **[G]** based sensors[227]. Further, genome-wide assays such as RNA-seq and single-cell RNA-seq for gene expression or ATAC-seq **[G]** for chromatin accessibility, and genome-wide CRISPR screens tailored for 3D assays[228] can identify mechanotransduction regulators unique to 3D.

In addition to the need for deeper mechanistic insights, there is also a critical requirement for studies on in vivo relevance. This has been done in a number of contexts often by associating measured tissue mechanics with biological signatures (i.e. gene expression or transcription factor activation) predicted from 3D culture studies, or by perturbing key mechanotransduction regulators in vivo, such as FAK or YAP, and examining the downstream effect. Clever approaches to directly modulate stiffness and viscoelasticity in vivo, or perturb novel downstream regulators, could further increase confidence in the relevance of the causal, mechanistic insights reported in in vitro studies. Furthermore, although the role of mechanotransduction pathways in development and cancer have been studied heavily and are becoming increasingly clear, the role of mechanotransduction behaviours in other contexts including homeostasis, regenerative processes, ageing, various other diseases, and in immune cell activity require significant additional effort.

These future efforts in mechanotransduction are likely to be very impactful. Beyond providing a fundamental understanding of cell and tissue physiology, these studies can advance the rapidly emerging area of mechanotherapy, which holds great promise for medicine. In a recent mice study[229], fibrosis induced by stiff (~MPa) silicone implants, which are used in breast mastectomy, was reduced by encapsuling the stiff implants in soft silicone implants (~kPa). Relatedly, another study[230] had the objective to reduce fibrosis associated with skin grafting . Targeting mechanotransduction using a small molecule FAK inhibitor, the fibrotic nature of the skin was substantially reduced with increased healing of wounds. In the context of ovarian fibrosis, targeting fibrotic col-1 using antifibrosis drugs restored ovulation in mice[231].

ECM stiffness and degradability have been the clinical targets in the context of cancer[232]. However, results have been mixed. In the context of stiffness, inhibition of lysyl oxidases (NCT00195091), β_1 integrin mediated mechanotransduction (NCT02683824), and FAK (NCT03727880) are undergoing clinical trials. In the context of ECM degradation, MMP inhibition was not effective in preventing cancer progression, potentially owing to off target effects as well as alternative mechanisms of invasion of cancer cells, not relying on ECM proteolysis. MMP inhibition also has severe side effects[232]. Future efforts could potentially target other downstream effectors of mechanotransduction as well as viscoelasticity and viscoplasticity in combination with degradation.

Injecting cells within biomaterial carriers into injured or diseased tissues is a common approach in regenerative medicine[233]. Varying stiffness, degradability and viscoelasticity have shown to optimize the in

vivo healing response. For example, promoting viscoelasticity of ECM in vivo was shown to increase bone formation by MSCs[199]. Interestingly, most biomaterials used in translational applications tend to be viscoelastic or degradable[52]. These highlight the importance of mechanotransduction in guiding the selection and use of biomaterials in these applications.

Table: Impact of ECM mechanics on cells.

ECM property / Cellular process	Stiffness	Viscoelasticity	Degradability	Non-linear elasticity	Pore size	Ligand type & density	Geometry
Spreading	2D[63,69,234]: + 3D[39,60,235,236]: #	2D[95,96,237,238]: + 3D[40,61,62,135]: +	2D: **?** 3D[42,60,103]: +	2D: **?** 3D[57]: +	2D: **n/a** 3D[239,240]: +	2D[63,97,241]: # 3D[40,241]: #	2D[64,89,242]: # 3D[65,243]: #
Migration	2D[68-71,191,244-246]: # 3D[245,247]: #	2D[248]: + 3D[77,78]: +	2D: **?** 3D[42,73,249]: +	2D: **?** 3D[158,159]: +	2D: **n/a** 3D[73,74]: +	2D[241,250]: # 3D[241]: #	2D[251]: # 3D[252]: #
Differentiation	2D[56,88,90-92]: # 3D[39,40]: #	2D[95,96]: # 3D[39,40,61]: #	2D: **?** 3D[60,103,253]: #	2D: **?** 3D[100]: #	2D: **n/a** 3D[239]: #	2D[90]: # 3D[111]: #	2D[99,242]: # 3D[65,254]: #
Division	2D[105,255]: + 3D[105]: +	2D[238]: + 3D[106]: +	2D: **?** 3D[103,256]: +	2D: **?** 3D: **?**	2D: **n/a** 3D: **?**	2D: **?** 3D[40,41]: #	2D[64,257]: # 3D[258]: #
Apoptosis	2D[38,259]: - 3D[107]: -	2D: **?** 3D[41]: -	2D: **?** 3D: **?**	2D: **?** 3D: **?**	2D: **n/a** 3D[74]: -	2D: **?** 3D[260]: #	2D[64]: # 3D: **?**
Tumour phenotype	2D[261,262]: + 3D[118-122]: +	2D: **?** 3D: **?**	2D: **?** 3D[263,264]: +	2D[265]: + 3D: **?**	2D: **n/a** 3D[73,74]: #	2D: **?** 3D[122]: #	2D[123,266]: # 3D[124,214,266]: #
Morphogenesis	2D: **?** 3D[111,115,116]: #	2D: **?** 3D[41,114,115]: +	2D: **?** 3D[110-112,267]: +	2D: **?** 3D: **?**	2D: **n/a** 3D[268]: #	2D: **?** 3D[41,110,111]: #	2D: **?** 3D[65]: #
Matrix secretion	2D: **?** 3D[181]: +	2D: **?** 3D[40,84,85]: +	2D: **?** 3D[82,83,86]: +	2D: **?** 3D: **?**	2D: **n/a** 3D[83]: #	2D: **?** 3D: **?**	2D: **?** 3D: **?**

Table 1: Impact of ECM mechanics on cells. ECM properties such as stiffness, viscoelasticity, degradability, non-linear elasticity, pore size, ligand type, density and geometry regulate various cell behaviors. **n/a**: not applicable (ECM pore size is an important consideration in 3D but is not meaningful in 2D). "+" indicates positive correlation, "-" indicates negative correlation, "#" indicates a complex relationship and "**?**" indicates an unknown relationship, that is yet to be thoroughly investigated. Selected references for known impacts are included.

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Figure 1. Tissue mechanics, ECM components, and cell-ECM mechanical interactions. **(a)** Stiffness and half time of stress relaxation for various soft tissues, and the range of those properties accessible with reconstituted ECMs or synthetic ECMs (i.e. hydrogels). Data were taken from refs. [1,40,52,84,122,135,199,269]. Stress relaxation half times, which provide a measure of viscoelasticity, are defined as the time for the stress to relax to half its original value in response to a constant deformation. Synthetic ECMs, including alginate hydrogels and PEG hydrogels, can be modified to mimic the physiological mechanics of soft tissues (check Box 1). **(b)** Schematics of structural components of major polymeric (left) and non-polymeric (right) ECMs in tissues. **(c)** Schematic of an epithelial monolayer with basement membrane underneath, and a stromal cell surrounded by fibrous ECMs such as col-1 and elastin in the underlying connective tissue. **(d)** As cells interact with ECM, these interactions are mediated by mechanical properties of the ECM including stiffness, nonlinear elasticity, viscoelasticity, and plasticity. As the cell push/pull on the ECM, the ECM may resist the cellular force through bending and stretching of the ECM fibers (left top). With increased forces from the cell, the ECM may stiffen (i.e. exhibit greater resistance) due to local alignment in fibers (right top). Over the time of force application, the ECM may undergo creep and stresses may relax due to detachment of weak crosslinks and fiber rearrangements (bottom right). Once the cell detaches from the ECM, the ECM may retain permanent deformations resulting from reformation of weak crosslinks that lock in changes in fibre position and alignment (bottom left).

Figure 2. Cell–matrix interactions in 3D. Cells in 3D are confined in all directions due to restrictions imposed by the matrix and can form cell–matrix adhesions on all surfaces contacting ECM. Cells exert contractile, protrusive, and volumetric forces on the matrix, generating dilational and distortional stresses in the ECM, which in turn regulate various cell behaviours (Table 1). Hydrostatic stress is volumetric or dilational stress that acts to increase or decrease the volume of an object on which it acts, without changing its shape. Deviatoric stress is distortional stress that acts to change the shape of an object on which it acts, without changing its volume. Hydrostatic and deviatoric stresses combine to produce net 3D stress fields which cells perceive. These stress fields directly deform cells and influence their behaviour including proliferation, migration and differentiation. In addition to this, such stresses change over time depending on ECM properties such as viscoelasticity, degradability and plasticity, and form a positive feedback loop with the cell-generated forces.

Figure 3. 2D and 3D Mechanotransduction. A) Cells sense substrate stiffness by exerting contractile forces on 2D substrates with stress fibres through focal adhesions, which activates various proteins such as FAK, talin, Rho and ROCK at the adhesion site. Activation of these proteins leads to adhesion maturation and stress fibre formation and contractility, which in turn transmits forces to the nucleus via the linker of nucleoskeleton and cytoskeleton (LINC) complex, resulting in changes in nuclear envelope tension and nuclear pore opening. This allows the nuclear entry of proteins such as YAP transcriptional regulator leading to downstream impact on cell phenotype . Moreover, in 2D, a cell can spread laterally without encountering any mechanical confinement. B) Cells embedded in the ECM sense stiffness and viscoelastic properties of the matrix through integrin binding, activation, and clustering, while sensing confinement, viscoelasticity and plasticity through cell volume changes and ion channel activation which leads to Ca^{2+} ion influx. Additionally, ECM stiffness/viscoelasticity and confinement regulate activation of various proteins, such as FAK, ROCK, MLCK, pathways, such as those involving PI3K, ERK, and Rho, and transcriptional regulators such as YAP, p27, Sp1, RUNX2, and EGR1. However, clear mechanistic links between the ECM properties and activation of these proteins, pathways, and transcription regulators remain unclear. Unknown connections in the pathways are indicated by question marks. Both mechanisms of mechanotransduction converge on the nucleus and regulate the activation of transcription factors (TFs), which are facilitated by chromatin remodelling and control cell behaviour .

Figure 4. Mechanotransduction in development. During development, basement membrane secretion and degradation triggers mouse epiblast lumenogenesis and gastrulation respectively. Post-implantation, trophectoderm cells in the mouse embryo, secrete a basement membrane around the epiblast which triggers polarization and lumen formation in the epiblast. Later during gastrulation, gastrulating cells, also called primitive streak cells, secrete proteases to locally degrade the basement membrane layer and enable migration.

Figure 5. Mechanotransduction in tissues. (a) In homeostasis, healthy ECM and fibroblasts help maintain normally functioning epithelium by maintaining optimal ECM mechanical properties such as stiffness and viscoelasticity. (b) Following a bone fracture, viscoelasticity of fracture hematoma promotes infiltration pf mesenchymal stem cells (MSCs) and stiff bone surface promotes differentiation of MSCs into bone-producing osteoblasts. (c) Myofibroblast differentiation and heterogeneous ECM occurring during ageing results in loss of epithelial integrity and function. As tissue fibrosis proceeds with ageing, normal fibroblasts differentiate into a myofibroblast phenotype and heterogeneously secrete and deform the ECM. Such a heterogeneous matrix promotes further myofibroblast differentiation and results in altered ECM mechanical properties. Epithelial cells sense these altered ECM properties and undergo transcriptional changes causing loss of epithelial integrity and function. (d) During cancer progression, cancer-associated fibroblasts remodel the ECM into a dense, stiff matrix. This increase in ECM stiffness, in combination with other cues and genetic changes in the cancer cells, leads to activation of a malignant phenotype in epithelial cells. These cells then undergo sustained proliferation, breach the basement membrane during invasion, migrate into the stromal matrix and eventually can metastasize.

Box 1: In vitro materials for 3D culture: choosing the right system

Here, we provide a brief guide for choosing a 3D culture system for mechanotransduction studies, focusing on commercially available materials (see figure). Natural matrices such as collagen, reconstituted basement membrane (rBM) matrix (i.e., Matrigel or Geltrex), and fibrin are easy-to-use and broadly mimic in vivo stromal matrix, BM, and blood clot environments respectively, and are often used for 3D culture. These matrices tend to be soft, with an elastic modulus ranging from tens to hundreds of Pa, are inherently viscoelastic and degradable (Supplementary Table 1), and provide biologically relevant signalling[52]. Fibrin and collagen are micro-porous and have a fibrillar architecture while rBM matrix is nano-porous with a non-fibrillar architecture. Key limitations are that these materials have limited independent tunability of mechanical properties such as stiffness, viscoelasticity, fibre length and porosity, limiting their usefulness for studies of mechanotransduction. Synthetic and chemically modified natural hydrogel systems based on polyethylene glycol (PEG), alginate, agarose and hyaluronic acid provide much more tunability. Cell adhesion ligand (i.e., RGD peptide) density, stiffness, viscoelasticity and degradability can often be modulated independent of other parameters. These materials are usually nano-porous and nonfibrillar. However, these materials do not present full physiological signalling ligands and tuning some properties requires more complex chemistries than are commercially available[270-273]. Due to their high tunability, however, these materials are especially useful for translational applications or for mechanotransduction studies . Finally, in applications where tunability is desired but the engineered material is not sufficient to elicit the desired cell behaviour, interpenetrating networks of the engineered biomaterial with the relevant ECM matrix of interest can be considered. For example, interpenetrating networks of collagen and agarose[247], rBM-matrix and alginate[77,122], and collagen and alginate[235] (in various proportions) offer tunability of stiffness and in some cases viscoelasticity while promoting biologically relevant behaviours. The reader is directed to various recent references for more information on choosing hydrogel platforms suitable for their needs[108,274,275].

Box 2. Mechanotransduction: Lessons from 2D

Many studies of mechanotransduction in 2D have converged upon a central mechanism by which cells sense stiffness (Fig. 3a). The predominant 2D culture mechanotransduction platform has been cells cultured on collagen1- or fibronectin-coated elastic polyacrylamide substrates with independently tunable stiffness[69,90]. Cells first bind to the substrate through β1 containing integrins, initiating formation of an adhesion complex, and activating actomyosin contractility, focal adhesion kinase (FAK), and the Rho pathway[119]. As a cell first spreads on the substrate through actin polymerization, or during processes involving actin polymerization such as filopodial extension or lamellipodial protrusion, a molecular clutch mechanism responds to stiffness[276]. In this mechanism, changes in stiffness mediate engagement of the molecular clutch, involving talin[277], that connects polymerizing actin filaments to integrin-based adhesions, governing whether there is productive

extension of the cell membrane versus retrograde flow of actin. Over time, nascent adhesions can mature into focal adhesions[278], containing talin, vinculin, and FAK, which connect to clustered integrin binding ligands[38] extracellularly and highly contractile actin stress fibres intracellularly[279]. Stress fibres connect to the lamin-containing nucleus through the linker of nucleoskeleton and cytoskeleton (LINC) complex, with higher contractile forces typically observed on stiffer substrates[119]. Tension across mechanosensitive proteins such as talin[280], vinculin[281] and lamin[2] impacts their activation and binding interactions, thereby converting changes in stiffness to biological signalling. Further, tension from stress fibres causes deformation and stretching of the nucleus, which opens up pores in the nucleus, allowing translocation of the YAP transcriptional regulator into the nucleus, and subsequent activation of transcriptional pathways, regulating processes such as proliferation and differentiation[282-284].

Various other mechanisms and relations have been described in 2D. There is a strong correlation between cell spreading area and ECM stiffness, and limiting cell spreading area on substrates with high stiffness can lead to mechanotransduction behaviours observed on soft substrates[234,242,282]. Cell adhesion ligand density, spacing of the ligands and architecture of ECM ligands can play a key role in mediating mechanotransduction[63,240,285]. Viscoelastic and viscoplastic substrates are amenable to ligand clustering and longer timescale of molecular clutch binding, leading to spreading, migration, and differentiation behaviors similar to that observed on stiffer elastic substrates[96,238,248]. By contrast, viscoelastic substrates that are not viscoplastic can lead to the opposite trend[95,286]. In one study, increased stiffness reduced cell volume, and modulating cell volume directly controlled mechanotransduction outcomes[154]. In addition to YAP, MRTF-A **[G]**, which responds to actin polymerization, has been implicated in mediating stiffness sensing at the transcriptional level[287,288]. Further, stress fibres also affect chromatin accessibility through their contractility via redistribution of histone deacetylase 3 to the nucleus [289]. Finally, recent studies have found distinct mechanotransduction behaviours in epithelial monolayers, finding for example that monolayers can sense shallow stiffness gradients more robustly than single cells during durotaxis[71], suggesting the role of collective cell behaviour in mediating 2D cell–ECM mechanotransduction. Many excellent reviews cover 2D mechanotransduction in more detail (e.g. refs. [4,5,8]).

Glossary

Advanced glycation end products (AGEs)
AGEs are proteins or lipids that are glycated when exposed to sugars, and are a biomarker implicated in many diseases such as diabetes and atherosclerosis.

Aggrecan
Aggrecan is the major proteoglycan in the articular cartilage which provides hydration to the cartilage.

Arginine-Glycine-Aspartate (RGD) sequences
A three peptide cell-matrix adhesion motif derived from ECM proteins such as fibronectin and vitronection that serves as a binding site for integrins such as αvβ3, α5β1 and αIIbβ3

ATAC-seq (Assay for Tranposases-Accessible Chromating using Sequencing)
A genome wide assay that identifies accesible DNA regions/genome wide chromatin accessibility.

Biglycan
Biglycan consists of a protein core and two glycosaminoglycan (GAG) chains, and is found in connective tissues.

Creep
Creep, a behavior of viscoelastic materials, is the time-dependent deformation or strain of a material under constant force/stress.

Deviatoric stresses
Distortional mechanical stresses that act to change the shape of an object on which they act, without changing its volume.

FRET
Fluorescent Resonance Energy Transfer (FRET) involves energy transfer between two light-sensitive molecules, and the efficiency of this energy transfer is inversely proportional to the sixth power of the distance between the molecules. This can be used to study small changes in distances between molecules.

Fibrillar adhesions
Fibrillar adhesions are cell-ECM adhesions whose shapes are elliptical in nature, often forming along fibrous ECM.

Filipodia
Actin-rich protrusions that are long and thin, and can be highly dynamic.

Focal adhesion kinase (FAK)
A receptor tyrosine kinase protein that localizes to focal complexes at cell-ECM adhesion sites and plays a crucial role in the several intergin-dependent mechanotransductive pathways.

Focal adhesions
Large cell–matrix adhesions typically formed by cells cultured on stiff 2D substrates, characterized by clustered integrin receptors, localization of proteins such as paxilin, talin, vinculin, phosphorylated FAK and thick actomyosin stress fibres, mediating strong cell-substrate adhesion.

Hydrostatic stresses

Volumetric or dilational stresses that act to increase or decrease the volume of an object on which they act, without changing its shape.

Invadapodia
Actin-rich structures that are present at the basal surface of cells, and thought to degrade and apply forces to ECM.

Lamellipodia
Thin sheet-like protrusions composedof a branched network of actin filaments. Extension of lamellipodia at the leading edge is often implicated in driving cell migration.

Lysyl oxidase **(LOX)**
An enzyme that converts lysine molecules into highly reactive aldehydes that form crosslinks in ECMs such as col-1 and elastin.

Nonlinear elasticity
Elasticity is the ability of a material to retain its initial shape/configuration following the application and release of external deformation or loading. In nonlinear elasticity, stress is nonlinearly related to strain even at small strains. By contrast, in linear elastic materials, stress of a material is linearly related to the strain until the material starts to yield.

Matrix metalloproteinases (MMPs)
Cell-secreted enzymes that are capable of proteolytically degrading various ECM components.

Mechanical Plasticity
A material property that defines the extent to which it undergoes permanent or irreversible deformation following the application and release of external deformation or loading.

Mechanosensitive ion channels
Ion channels that open or close in response to cell membrane stretch or tension. TRPV4 and Piezo1 are examples of mechanosensitive ion channels implicated in mechanotransduction.

Mesenchymal stem or stromal cells (MSCs)
Multipotent stem or stromal cells that are found in the bone marrow, which have been reported to differentiate into osteoblasts, adipocytes, chondrocytes, myocytes and neurons.

Microtentacles
Microtubule-based membrane protrusions which are often obseved in detached circulating tumor cells.

MRTF-A
Myocardin related transcription factor A. A transcription factor that plays a key role in mediating smooth muscle cell differentiation.

Poroelasticity
A material property that describes the interaction between fluid flow and solid deformations within the porous material. Cells and ECMs are usually poroelastic in nature.

Proteoglycans
Supramolecules that posses protein as a core and a side chain of sugars.

Reconstituted BM (rBM) matrices
Commercially available matrices such as Matrigel or Geltrex, that are derived from Engelbreth–Holm–Swarm (EHS) tumor, a mouse sarcoma, and are commonly used for in vitro cell culture and contain laminin, collagen IV, entactin, perlecan and other components.

RHAMM
Receptor for hyaluronin-mediated motility (RHAMM) is a protein which bounds to hyaluronan.

Rho signalling
A cell signalling pathway involved in regulation of wide variety of cell processes such as cell morphology, survival, proliferation, and adhesion.

Stiffness
Stiffness describes the resistance to deformation of a specific structure, which is dependent on the Young's modulus and geometry of the structure. Hence, stiffness is regarded as property of a specific structure while Young's modulus is an inherent property of the material.

Strain
Strain is a measure of localized deformation in a material, with uniaxial strain typically defined as the ratio of deformation to the original length of the material.

Stress
A measure of force per unit area, which has units of Pascals (Pa).

Stress relaxation
A behaviour of viscoelastic materials referring to the time-dependent change in stress in a material under constant deformation.

Tenascin-C
A multimodular ECM glycoprotein which is expressed in various tissues during development, disease, or injury.

Traction force microscopy
A technique that takes experimentally measured the force-induced displacement field of a substrate with known mechanical properties and uses these to computationally determine the cell–ECM forces applied to the substrate.

Versican
A large ECM proteoglycan that is found in various tissues such as blood vessels, and skin.

Viscoelasticity
Describes materials that exhibit some behaviours characteristic of elastic solids and some of viscous liquids, and is characterized by a time-dependent mechanical response (i.e. creep or stress relaxation).

YAP/TAZ
Transcriptional coactivators that shuttle between the nucleus and cytoplasm and play an important role in mediating mechanotransduction, particularly in 2D. When translocated to the nucleus, YAP/TAZ do not directly bind to DNA but regulate gene expression through their binding to transcription factors of the TEAD family.

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Competing interests
The authors declare no competing interests.

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eTOC
Mechanical cues from the extracellular matrix (ECM) regulate cell fate and behaviour through cell–ECM mechanotransduction. Studies of cell–ECM mechanotransduction have largely focused on cells cultured in 2D, and only recently have we begun to unravel how these processes occur in 3D — a context native to most cells in vivo.