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REVIEW

SUBJECT COLLECTION: MECHANOTRANSDUCTION

Engineering the cellular mechanical microenvironment – from bulk mechanics to the nanoscale

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ABSTRACT

The field of mechanobiology studies how mechanical properties of the extracellular matrix (ECM), such as stiffness, and other mechanical stimuli regulate cell behaviour. Recent advancements in the field and the development of novel biomaterials and nanofabrication techniques have enabled researchers to recapitulate the mechanical properties of the microenvironment with an increasing degree of complexity on more biologically relevant dimensions and time scales. In this Review, we discuss different strategies to engineer substrates that mimic the mechanical properties of the ECM and outline how these substrates have been applied to gain further insight into the biomechanical interaction between the cell and its microenvironment.

KEY WORDS: Cellular biomechanics, Focal adhesions, Mechanobiology, Nanotopography, Viscoelasticity

Introduction

A variety of cellular processes, including cell morphology, polarisation, migration, stem cell differentiation and cancer malignancy, are regulated by mechanical stimuli. Therefore, engineering substrates that can mimic the mechanical properties of the cellular microenvironment of different tissues, including pathological conditions, is fundamental to the understanding of how cells respond to these mechanical cues.

Mechanical cues come in a variety of different forms, including nanotopography, distribution of adhesion ligands, absolute stiffness, spatio-temporal changes in stiffness, stress relaxation and forces transmitted through the tissue among others. Understanding the regulatory effect of the complex mechanical microenvironment on cells requires dissecting it into independent cues to study their individual contribution. To this end, a number of different approaches have been developed over the past two decades to develop cell culture substrates with defined mechanical properties that mimic those of native tissues. Early research focused on replicating matrix stiffness and cell morphology, which provided great insight into the effect of these mechanical signals on cell behaviour and spawned the field of mechanobiology.

Polyacrylamide (PAA) gels of different rigidities were initially developed by tuning the acrylamide:bis-acrylamide ratios in order to mimic the rigidity of different tissues (Engler et al., 2006; Pelham and Wang, 1997; Wang and Pelham, 1998). A number of materials have since been used to fabricate hydrogels of different rigidities, including alginate (Augst et al., 2006; Shi et al., 2017), hyaluronic acid (Burdick and Prestwich, 2011), fibrin (Man et al., 2011) and polydimethyl siloxane (PDMS) (Palchesko et al., 2012). Elastic micropillar array substrates can also be stiffness-tuned by changing the length of the pillars (Fu et al., 2010). These have the advantage of achieving different rigidity without altering the chemical composition or porosity of the substrate, but offer a discrete surface for attachment that is different to that of the continuous hydrogels. PAA gels of tuneable rigidity have been used to induce differentiation of mesenchymal stem cells into different lineages depending on substrate stiffness (Engler et al., 2006), and in cancer research to model the tumour stroma (Cortes et al., 2019a, 2018), to demonstrate that matrix rigidity can modulate chemoresistance (Rice et al., 2017) and to study the interaction between treatment and substrate stiffness (Cortes et al., 2019b, 2019c).

Seminal work in the field of mechanobiology also focused on controlling cell adhesion to the substrate and, ultimately, cell shape (McBeath et al., 2004). Cell shape, in turn, was found to regulate the formation of focal adhesions (FAs), the organisation of the actomyosin cytoskeleton and activity of the GTPase RhoA, which is involved in fibroblast activation and stem cell lineage commitment (McBeath et al., 2004).

While mimicking the stiffness of the matrix has provided a wealth of information about cellular behaviour, static hydrogels of uniform rigidity cannot recapitulate the complexity of the cellular microenvironment. For this reason, research efforts have focused on developing substrates with heterogeneous and dynamic properties, including spatial patterning (Tee et al., 2011; Tseng and Di Carlo, 2014) and dynamic stiffness (Rosales et al., 2017). Developing cell culture substrates that present physiologically relevant properties in both function and scale is therefore paramount to the study of mechanobiology at the cellular level.

Substrates engineered to recapitulate the mechanical complexity of the extracellular matrix (ECM) serve a number of functions. First, they can act as fundamental research platforms for the investigation of the cellular responses to the diversity of mechanical stimuli, as well as the mechanotransduction pathways and molecular mechanisms behind these responses. Second, these platforms can be used to study the role of these mechanical stimuli in physiological processes, and their contribution to the onset and progression of disease. Third, they help researchers discern the key mechanical cues the physiological microenvironment provides to direct cell behaviour and therefore inform the design of biomaterial scaffolds for tissue engineering.

In this Review, we discuss recent advances in the development of cell culture substrates that recapitulate mechanical stimuli from the cellular microenvironment, including spatiotemporal control of mechanical properties and its effect on cell migration and cellular mechanical memory, the distribution of adhesion ligands, and the viscoelastic stress relaxation of fibrous matrices. We then review novel platforms with defined nanoscale properties and liquid–liquid interfaces that have shed new light into the mechanical regulation of cell behaviour at the nanoscale.

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Stiffness gradients and durotaxis

As the field of mechanobiology develops, research has shifted from mimicking bulk mechanical properties like stiffness to substrates that offer more complex mechanical stimuli. One strategy to mimic the mechanical complexity of the native ECM is to generate hydrogels with heterogeneous stiffness. Stiffness differences can be readily accomplished using photolithographic techniques and photodegradable hydrogels, or photo initiator-mediated crosslinking. Here, the hydrogel is irradiated with UV light through a mask presenting a pattern of opacity, thereby achieving differential exposure of the hydrogel (Kidoaki and Matsuda, 2008; Nemir et al., 2010; Yanagawa et al., 2015). This approach can achieve stiffness patterns with micrometre precision.

This technique has been applied to generate ‘stiffness islands’ (i.e. regions of high rigidity in an otherwise soft gel) of different sizes. Fibroblasts cultured on large islands (200–1000 µm) expressed high levels of α smooth muscle actin (αSMA), consistent with stiffness-induced myofibroblastic activation, whereas in small islands (50–100 µm), where cell spreading is limited, fibroblasts remained inactive despite the high stiffness of the island, suggesting that cell spreading is necessary for mechanical activation (Guvendiren et al., 2014).

In the past decade, research on stiffness patterning of hydrogels has focused on the development of stiffness gradients. Hydrogels with a stiffness gradient offer an advantage over substrates with simple stiffness steps by presenting cells with a continuous transition in stiffness, which better replicates the heterogeneity in the ECM. These stiffness gradient hydrogels are widely used to study the rigidity-induced migration of cells, known as durotaxis (Lo et al., 2000; Roca-Cusachs et al., 2013), which has been observed for individual cells (Lachowski et al., 2017) and cell ensembles (Sunyer et al., 2016). These systems offer a platform to investigate the mechanotransduction pathways and molecular mechanisms underlying rigidity-guided migration, as well as to recapitulate the pathological stroma and to provide further insight into the biomechanical drivers of disease.

The majority of techniques to generate stiffness gradients aim at spatially modulating the cross-linking of a hydrogel. This can be achieved using a mask with an opacity gradient (Liu et al., 2010; Wong et al., 2003) or a moving opaque mask (Fig. 1A,B).

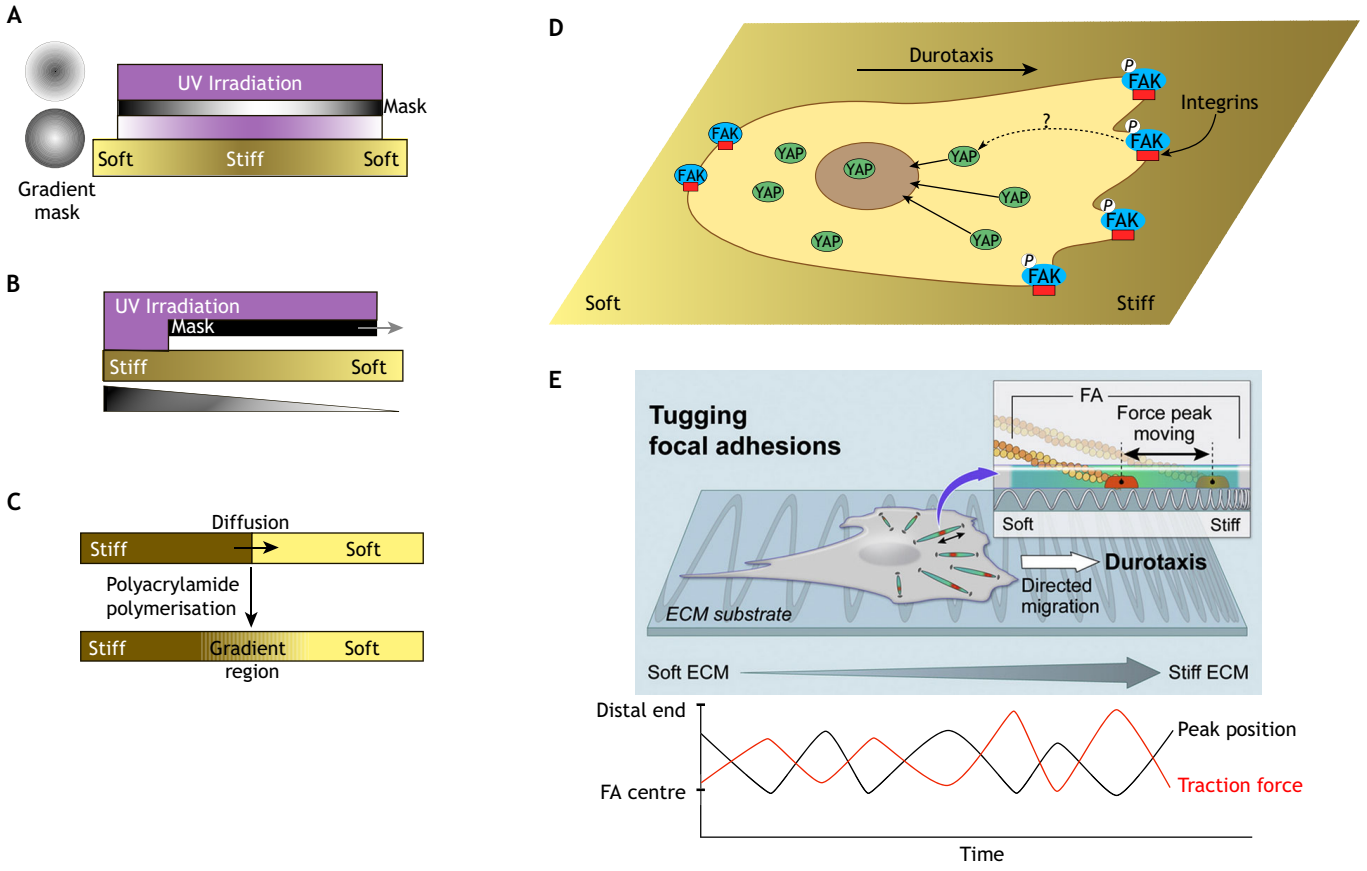

Fig. 1. Stiffness-gradient substrates used to study durotaxis. (A–C) Methods to generate substrates with a rigidity gradient. (A) Photo-crosslinking through an opacity gradient photomask. The gradient of the photomask results in differential exposure of the substrate to UV light and ultimately in differential stiffness. (B) Photo-crosslinking with a moving mask. A completely opaque mask is used to spatially control the exposure time of the gel to UV irradiation. (C) Diffusion at the gel interface. Two droplets of PAA with different acrylamide:bis-acrylamide ratios (resulting in different stiffness) are bought into contact during polymerisation. Diffusion between the two droplets generate a gradient of crosslinker that results in a smooth transition between the stiff and the soft gels. (D) Mechanism of single-cell durotaxis. Stiffness-dependent activation (phosphorylation) of FAK leads to an asymmetric distribution of mature FAs (represented by the phosphorylated FAK and integrin complex) and to cell polarisation. FAK activation and FA maturation on the stiff side of the substrate lead to mechanotransduction through YAP and result in cell migration towards higher stiffness. (E) Tugging FAs underlying durotaxis. A subpopulation of FAs with a dynamic profile of traction force distribution (tugging action, see inset) probe the substrate stiffness in the vicinity of the cell. In contrast to what is found for stable FAs, which have a static and centrally localised force peak, in these tugging FAs, the position of the peak force fluctuates from the distal tip of the FA to the centre in a cyclic manner, accompanied by an increase in the traction force (as represented in the graph underneath the diagram). These probing adhesions are fundamental for durotaxis, as they enable the cell to mechanosense matrix stiffness. Reproduced with permission from Plotnikov et al. (2012).

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The method of the moving mask offers higher flexibility in the substrate material, and the size and magnitude of the gradient, which can be controlled by the speed of the mask (García et al., 2015; Marklein and Burdick, 2010; Sunyer et al., 2016, 2012; Tong et al., 2016). Another technique, first reported in 2000 (Lo et al., 2000) and more recently by our group (Lachowski et al., 2017, 2018) and others (Lee et al., 2018a), consists of coalescing two drops of PAA prepolymer of different stiffness (i.e. different acrylamide:bis-acrylamide ratio) by bringing them into lateral contact during polymerisation. At the interface between the two droplets, diffusion of the bis-acrylamide crosslinker results in a smooth transition between the stiff and the soft regions (Fig. 1C). A similar method has been proposed based on sequential, rather than simultaneous polymerisation with a sloped morphology (Hadden et al., 2017). While this method does not allow for precise control of the gradient transition, it eliminates the need for a controlled photomask and UV irradiation, and is therefore more accessible. Another technique that enables the formation of stiffness gradients is density gradient multilayer polymerisation (DGMP), which uses inert density modifiers (e.g. sucrose or iodixanol) to achieve initial phase separation between polymer layers. Diffusion between segregated layers yields the desired gradient based on the settling time prior to photo-polymerisation (Joshi-Barr et al., 2013; Karpiak et al., 2012). Other methods to develop stiffness gradients have been explored, such as microfluidic gradient generators (Vincent et al., 2013) and electro-regulated ionic crosslinking (Yang and Liang, 2018), which has the advantage of allowing for dynamic control of the gradient.

The mechanisms by which cells sense stiffness gradients, polarise and migrate in response towards stiffer environments remain poorly understood. Durotaxis has been observed in a wide variety of cell types, including fibroblasts, mesenchymal stem cells (MSCs), vascular smooth muscle cells, pancreatic stellate cells and some cancer cells, and it may be a universal phenomenon that emerges as a result of stiffness-dependent migration persistence (Novikova et al., 2017). Collective durotaxis of cell monoloayers has also been reported (Sunyer et al., 2016). The strength of durotaxis (i.e. the tactic index and alignment of cell migration with the direction of the gradient) correlates positively with the magnitude of the gradient, but is independent from the absolute stiffness (Isenberg et al., 2009). The composition of the matrix also determines the capacity for cells to respond to the gradient. In particular, laminin inhibits durotaxis even when mixed with other proteins that promote it (e.g. fibronectin) (Hartman et al., 2017).

A minimum threshold gradient for cells to durotact has been observed, suggesting that there is a limit to the ability of a cell to mechanosense the stiffness gradient (Moriyama and Kidoaki, 2018). Interestingly, this threshold gradient increases with absolute local stiffness, indicating that the current mechano-active state of a cell can determine its durotactic response (Moriyama and Kidoaki, 2018) and that gradient mechanosensing is impaired at high rigidities. Consistent with this limit to cell polarisation, we recently demonstrated that durotaxis requires the asymmetric distribution of focal adhesion kinase (FAK, also known as PTK2) activation (Fig. 1D) (Lachowski et al., 2017, 2018), but is inhibited by constitutive activation of FAK.

At the level of FAs, durotaxis requires the formation of a subpopulation of FAs with a fluctuating force distribution described as tugging FAs (Fig. 1E) (Plotnikov et al., 2012; Plotnikov and Waterman, 2013). The formation of these tugging FA decreased with increasing substrate stiffness in a Rho-associated protein kinase (ROCK)-dependent manner, and was required for durotaxis but not haptotaxis, suggesting a role in mechanosensing of rigidity (Plotnikov and Waterman, 2013). Indeed, it has been proposed that these tugging FAs are a mechanism for mechanical exploration of the cell vicinity (Plotnikov and Waterman, 2013).

Currently, collective durotaxis and durotaxis in three-dimensional (3D) matrices are at the forefront of mechanobiology research, and a number of different fabrication methods are being explored to generate 3D stiffness gradients, including mechanical compression (Hadjipanayi et al., 2009), 3D bioprinting (Bracaglia et al., 2017) and microfluidics (Orsi et al., 2017; Sundararaghavan et al., 2009). This process of collective, 3D and rigidity-guided migration plays a fundamental role in embryonic development, wound healing and cancer progression, and these novel substrates with engineered stiffness gradients will be required to gain further insight into the process.

Dynamic substrates and mechanical memory

Mechanical properties of the substrate change in response to disease, such as cancer progression, or treatment, such as anti-stromal therapies. Understanding how these changes in the local microenvironment affect cell behaviour, and how cells adapt to these changes, is critical to gain insight into the biomechanical aspects of disease and treatment. Dynamic hydrogels offer a platform to study the kinetics of mechanotransduction and the response of cells to changes in their mechanical microenvironment over time.

Dynamic hydrogels exhibit either a softening (Caliari et al., 2016b; Frey and Wang, 2009; Kloxin et al., 2010; Yanagawa et al., 2015) or stiffening (Caliari et al., 2016a; Guvendiren and Burdick, 2012; Liu et al., 2017) behavior, whereby the elasticity of the hydrogel either decreases or increases upon triggering. Photoresponsive hydrogels are of special interest due to the possibility of combining them with spatial patterning methods (e.g. photolithography). Photoresponsive dynamic hydrogels typically rely on UV-mediated crosslinking (stiffening) or UV photodegradation (softening) to change the mechanical properties of the substrate *in situ* (Tomatsu et al., 2011) (Fig. 2A,B). Some systems, such as a methacrylated hyaluronic acid (MeHA) hydrogel with a lithium acylphosphinate (LAP) initiator stiffen upon exposure to blue light, eliminating the need for cytotoxic UV exposure (Caliari et al., 2016a). In contrast to cells directly seeded on a stiff substrate, this system could replicate the dynamics and timescale of liver fibrosis, in which the ECM stiffness increases while cells remain attached to the substrate. Caliari et al. revealed that hepatic stellate cells respond more rapidly to *in situ* stiffening when initially cultured on a soft matrix compared to cells directly seeded on statically stiff matrices, including effects on cell spreading, nuclear translocation of YAP/TAZ family proteins and α-SMA expression (Caliari et al., 2016a).

Studies with dynamic substrates have also revealed that cellular behaviour is affected by past stiffness (Balestrini et al., 2012), a phenomenon called mechanical memory (Fig. 2C). Initial experiments demonstrated that lung primary fibroblasts and human (h)MSCs cultured on tissue culture plates and transferred to soft hydrogels exhibit a dose (time)-dependent mechanical memory (Balestrini et al., 2012; Yang et al., 2014); that is, the mechano-active phenotype promoted by the initial substrate stiffness (priming stiffness) persists even after cells are re-seeded onto substrates with different stiffness (secondary stiffness).

However, the need to employ enzymatic detachment to transfer cells between substrates can alter the cellular phenotype and therefore affect mechanical memory. Conversely, photodegradable softening hydrogels allow for *in situ* modulation of substrate

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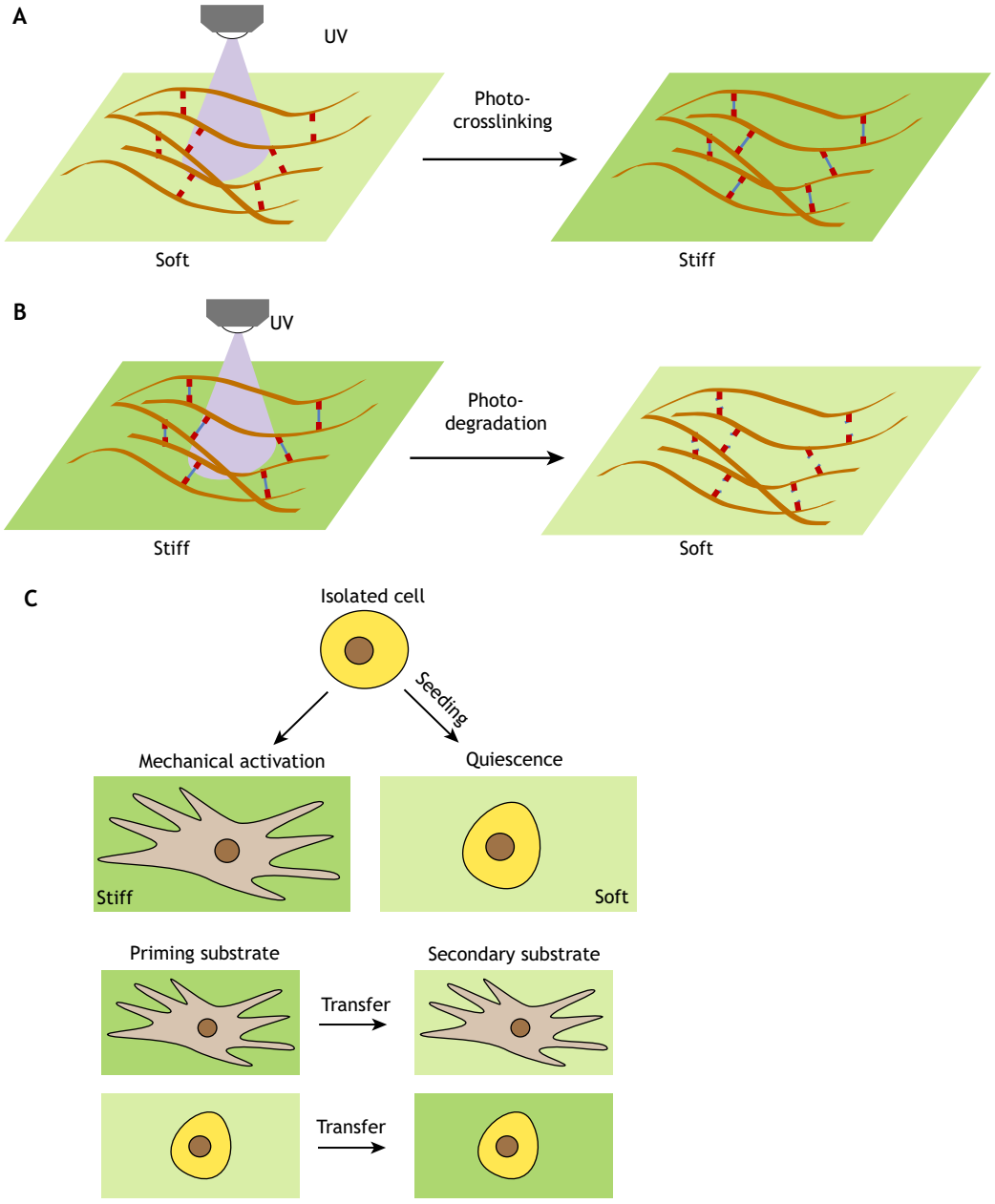

Fig. 2. Dynamic hydrogels reveal cellular mechanical memory. (A) Stiffening of a hydrogel through photo-crosslinking. The presence of a photoinitiator in the hydrogels enables the formation of chemical crosslinks between polymer chains (blue) upon exposure to UV light, leading to *in situ* stiffening of the substrate. (B) Softening of a photodegradable hydrogel. Here, the hydrogel is cross-linked by light-sensitive photocleavable crosslinks, which readily degrade upon exposure to UV light; this in turn decreases the stiffness of the hydrogel. The use of stiffening (A) and softening (B) substrates enables a dynamic control of the mechanical properties of the substrate. (C) Mechanical memory. Newly isolated cells seeded on either a stiff or soft substrate acquire a characteristic mechano-active or quiescent phenotype, respectively. Conversely, cells initially cultured on a primary or priming substrate maintain their mechano-active or quiescent phenotype, even when they are transferred to a secondary substrate with the opposite mechanical properties, that is, they exhibit mechanical memory.

stiffness, therefore eliminating the need to remove and reseed cells. By using a poly(ethylene glycol) di-photodegradable acrylate crosslinker (PEGdiPDA), Yang et al. identified the mechanoresponsive transcription factor YAP (also known as YAP1) as a key element of mechanical memory in hMSCs (Yang et al., 2014). In particular, nuclear accumulation of YAP persisted after softening of the hydrogel in stiff-primed cells. More recently, the microRNA miR-21 was identified as another effector of mechanical memory in MSCs (Li et al., 2016), which provides a previously unexplored link between fibrosis, oncogenesis, chemoresistance and mechanical memory (see Box 1) (Feng and Tsao, 2016; Kumarswamy et al., 2011).

Changes in substrate stiffness can also be accomplished by using cells migrating on a substrate that presents a stiffness step: as cells migrate, they are exposed to a different substrate stiffness, resulting in spatiotemporal changes in substrate stiffness (Nasrollahi et al., 2017) (Fig. 2D). This method enables the dynamic analysis of cell migration velocity and mechanics, rather than analysing static cells. However, as cells have to migrate on a substrate with heterogeneous stiffness, they are exposed to combination of both spatial and temporal effects that could complicate the analysis of underlying mechanical memory. Nevertheless, by using such a platform, cancer cells were found to display persistent mechanical activation after

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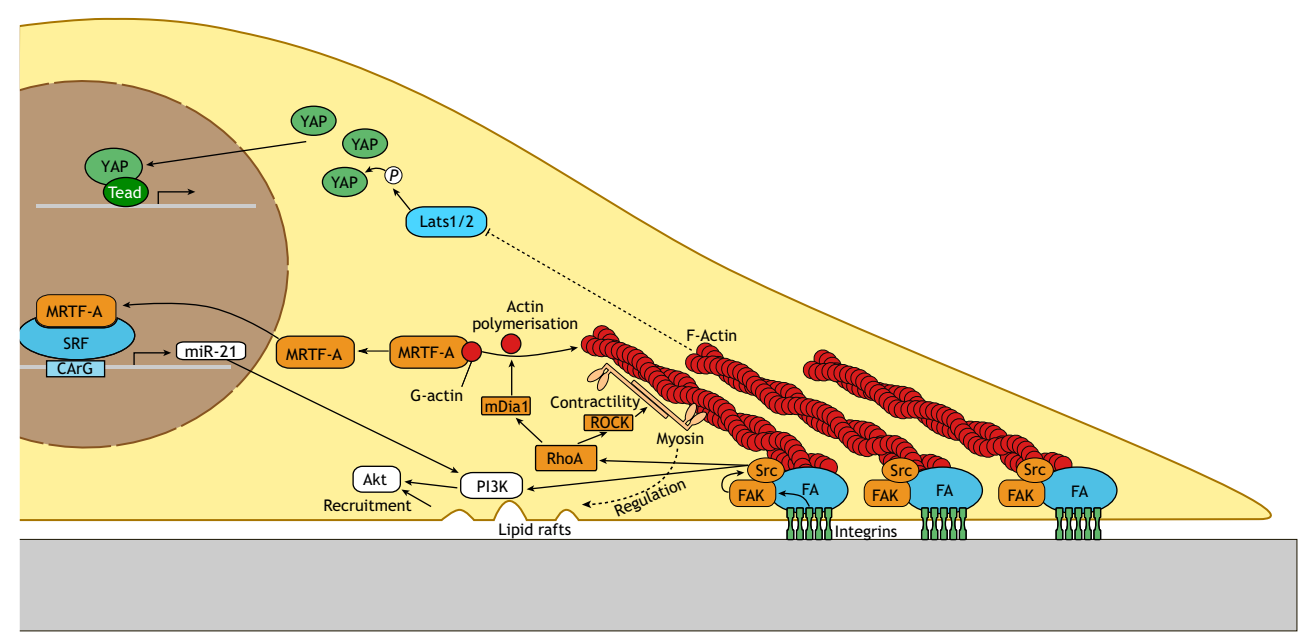
Box 1. Mechanical memory pathways

YAP and miR-21 have been identified as effectors of mechanical memory, that is the process whereby the mechanical activation persists after cells are transferred to a soft substrate (Caviglia et al., 2018; Yang et al., 2014). Engagement of integrins on a stiff substrate leads to the activation of mechanotransducers in FAs, such as FAK and Src. Phosphorylation of FAK and Src lead to the activation of the RhoA pathway, which increases actomyosin contractility through ROCK1 and ROCK2 proteins and drives actin polymerisation (F-actin formation) through diaphanous-related formin-1 (mDia1). Actin polymerisation in turn modulates the nuclear translocation of myocardin-related transcription factor A (MRTF-A) directly through the dissociation of the G-actin–MRTF-A complex. F-actin formation also regulates YAP nuclear translocation indirectly by inhibiting the activity of large tumor suppressor kinase 1 and 2 (Lats1/2), which, when active, inhibits YAP nuclear translocation (see figure). RhoA can also activate YAP nuclear translocation independently of Lats1/2 through the non-canonical Hippo pathways.

YAP plays well-known and fundamental roles in mechanosensing and mechanotransduction (Dupont et al., 2011), and its accumulation in the nucleus is characteristic of a mechano-activated phenotype. It was recently reported that the nuclear accumulation of YAP can result from direct force application to the nucleus (Elosegui-Artola et al., 2017). Moreover, YAP nuclear accumulation during mechanical priming has been found to correlate with increased expression and nuclear localisation of runt-related transcription factor 2 (RUNX2) and biased hMSC differentiation towards the osteogenic lineage in a bipotential (osteogenic/adipogenic) medium, which could have important implications in tissue engineering (Yang et al., 2014).

MRTF-A has been shown to act through the SRF to bind to the CArG box on the miR-21 promoter, increasing miR-21 transcription. MiR-21 is a fibrosis-associated miRNA (fibro-miR) that has been found to be upregulated in a variety of solid tumours. MiR-21 can modulate the phosphoinositide 3-kinase (PI3K)/Akt pathway (Caviglia et al., 2018; Jiang et al., 2010; von Erlach et al., 2018), which has a well-known oncogenic role (Aoki and Fujishita, 2017). Mechanical memory can also indirectly regulate PI3K/Akt activity through the cell morphology-dependent formation of lipid rafts. Through these mechanisms, parallel YAP and MRTF/SRF pathways provide a link between mechanotransduction, mechanical memory and cancer malignancy.



cells migrated to a soft substrate (Nasrollahi et al., 2017). YAP accumulation was accompanied by higher expression of actin and phospho-myosin light chain (pMLC), as well as a larger FA area and faster migration of the leading edge, consistent with a mechano-active state (Nasrollahi et al., 2017).

Mechanical memory is critical both to the understanding of cell biology and to its application in tissue engineering. For instance, the persistent mechanical activation of myofibroblasts could contribute to the self-sustenance of fibrosis and could potentially compromise the success of antistromal therapies that target the fibrotic ECM. Similarly, novel anti-stromal therapies should aim at disrupting the mechanical memory pathways to prevent relapse. Mechanical memory is also crucial in tissue engineering, where mechanical pre-conditioning could be critical to control cell function in the biomaterial scaffold.

Switchable hydrogels are a subset of dynamic hydrogels that are amenable to both photo-crosslinking and photodegradation; they can be either softened or stiffened, depending on the trigger and can also revert from one state to the other (Lee et al., 2018b; Rosales et al., 2015, 2017). Switchable hydrogels are an ideal platform to study mechanical memory, since the cellular response to both softening and stiffening can be analysed using the same substrate without changing the chemical composition of the hydrogel. Azobenzene-based hydrogels are some of the most common photo-switchable hydrogels, but they still require cytotoxic levels of UV irradiation (Lee et al., 2018b) To overcome this limitation, systems that are responsive to two different bands of visible light (Zhao et al., 2018), or that incorporate near-infrared (NIR)-responsive nanoparticles (Mandl et al., 2018), have been developed. As an alternative to photoresponsive hydrogels, switchable substrates have also been generated using other ‘smart hydrogels’, including pH-responsive (Yoshikawa et al., 2011), enzyme-activated (Liu et al., 2017), temperature-responsive (Uto et al., 2014) or redox-switchable (Fadeev et al., 2018) hydrogels.

Switchable substrates have been used to study the dynamic response of cells to changes in their microenvironment. For instance, hMSCs on stiff hydrogels exhibit a decrease in nuclear YAP upon softening of the substrate, but recover initial nuclear YAP levels upon re-stiffening. Switchable hydrogels also offer a unique opportunity to study the response of cells to repeated

stiffening–softening cycles that recapitulate the microenvironment of diseases characterised by repeated injuries, such as liver fibrosis (Caliari et al., 2016b; Kisseleva et al., 2012), atherosclerosis (Friedman et al., 1975) or cancer (Argyris, 1985).

Surface patterning and nanotopography

Cells not only respond to the stiffness of their environment, the distribution of mechanical and biochemical cues within the 3D space of the ECM is also a fundamental regulator of cell polarisation, migration, and shape, which in turn modulates the biomechanical interaction between the cell and its microenvironment and, ultimately, directs cell function. Such patterning can broadly take two forms: (1) spatial patterning of biochemical cues, for example adhesion ligands, and (2) development of quasi-3D topographical features (3D topographical features in a 2D culture substrate). The former aims at spatially controlling cell attachment, morphology and migration, while the latter focuses on mimicking the complex nanotopography of the native ECM.

One method to precisely control cell morphology is to confine cell attachment by patterning adhesion molecules (e.g. fibronectin) into a specific arrangement. Patterning adhesion ligands into islands of different geometries and dimensions on a non-adhesive background has shown that cells acquire the morphology of the island (McBeath et al., 2004; Parker et al., 2002). Different methods have been developed to deposit these adhesion molecules into a precise pattern. These include stamping or transfer techniques, such as microcontact printing (Major and Choi, 2018), *in situ* polymerisation (Rape et al., 2011; Vignaud et al., 2014), photolithography and pattern lift-off (Moeller et al., 2018; Sorribas et al., 2002), as well as photochemical patterning such as deep UV activation (Tseng et al., 2011). Moreover, these techniques can be readily applied to hydrogels with different stiffness to combine both surface patterning and substrate stiffness. However, it has been reported that different stamping methods can result in differences in ligand–substrate adhesion, which in turn can affect cell behaviour, so special care must be taken to select the chemistry of the surface ligand to avoid masking the effect of the topography on cell behaviour (Hu et al., 2018).

The distribution of adhesion ligands also regulates cell polarisation and migration through haptotaxis, the guided migration of cells following a gradient of surface-bound cues and a fundamental process in 3D migration (McCarthy et al., 1983; Moreno-Arotzena et al., 2015). For this reason, techniques that enable precise control over the pattern and concentration of adhesion ligands are a useful platform to research haptotaxis. In recent years, a novel technique, called block copolymer micelle nanolithography (BCMN), has gained interest for its ability to pattern ligands with nanometre precision (Glass et al., 2003). In this method, polystyrene-b-poly(2-vinylpyridine) diblock copolymer micelles containing $HAuCl_4$ self-assemble into a highly ordered monomicellar layer on a solid support. Subsequent plasma treatment leads to removal of the polymer and deposition of the $HAuCl_4$ precursor into gold nanodots, which can be readily functionalised with adhesive ligands through gold–thiol interactions. The spacing and dimensions of the gold nanodots can be controlled by tuning the size of the micelles, which in turn depend on the copolymer molecular mass (Fig. 3A).

The first use of this technique aimed to control the distance between RGD-motif-containing integrin ligands and monitor its effects on cell adhesion (Arnold et al., 2004, 2008), and found that stable cell adhesion and spreading through the formation of FA was severely impaired above a critical inter-ligand spacing of 73 nm. More recently, a modified method has been developed to dynamically control inter-ligand spacing by transferring the gold nanodot array to a stretchable poly(N-acryloyl glycinamide) (PNAGA) hydrogel (Fig. 3B) (Deng et al., 2017). Here, stretching of the hydrogel allowed the inter-ligand spacing to be varied in the direction of stretching (anisotropically) from 35 nm to up to 112 nm. Consistent with previous results (Arnold et al., 2004, 2008), cells on stretched substrates displayed less stable adhesion. However, cells on stretched anisotropic hydrogels exhibited higher polarisation and migration speed in the direction orthogonal to the stretching (where the Poisson effect reduces inter-ligand spacing to ∼25 nm), suggesting that the formation of unstable FAs favours motility in the direction of the shorter inter-ligand spacing (Fig. 3C) (Deng et al., 2017).

Previously, this critical inter-ligand spacing has been attributed to the dimensions of adaptor proteins involved in the lateral clustering of integrins, which impede effective clustering at high inter-ligand spacing (Cavalcanti-Adam et al., 2006, 2007). However, it has recently been shown that the critical inter-ligand spacing threshold is not universal, but depends on the substrate stiffness (Oria et al., 2017). By transferring the BCMN pattern of gold nanodots to polyacrylamide gels of different stiffness, the authors demonstrated that the optimal spacing decreased with increasing substrate rigidity owing to higher force generation. As the force in a FA increases, integrins are recruited in a vinculin-dependent manner (Dumbauld et al., 2013), thereby increasing the FA area and redistributing the load to maintain a constant stress (Balaban et al., 2001), which prevents the failure of the integrin–ECM bond. High ligand spacing limits integrin recruitment, leading to FA collapse (Fig. 3D) (Oria et al., 2017). Similarly, the minimum ECM area required for effective assembly of a FA is dynamic and depends on cell contractility and the activity of mechanoresponsive cytoskeletal components (Coyer et al., 2012), such as talin-1 (Haining et al., 2018, 2016) and vinculin (Dumbauld et al., 2013).

Inter-ligand spacing also affects cell protrusion dynamics, with lamellipodia displaying faster and more frequent protrusion–retraction cycles in response to higher inter-ligand spacing (Cavalcanti-Adam et al., 2007), pointing towards a role for lamilipodia in haptotatic mechanosensing. Interestingly, a reduction in myosin II contractility and depletion of mature FAs through ROCK protein inhibition does not affect haptotaxis (King et al., 2016), suggesting that the mechanism of haptotaxis relies on the protrusion of probing lamellipodia with nascent focal complexes, which enable the cell to mechanosense its vicinity. Recent work has demonstrated that lamillipodia are responsible for stiffness sensing through stiffness-dependent stabilisation of nascent FAs, independently of myosin contractility (Oakes et al., 2018).

The role of lamellipodia in haptotaxis has also been investigated using microfluidic-generated fibronectin gradients, which revealed the critical function of these structures for the ability of the cell to migrate following surface-bound cues (King et al., 2016). Cells on fibronectin gradients displayed differential (polarised) protrusion dynamics, with lamellipodia extending further and presenting a longer lifetime in the direction of the gradient. This polarisation depends on the differential activation of Arp2/3 through the WAVE2 complex, which is responsible for actin filament branching and protrusion of lamellipodia. Interestingly, a similar procedure to generate 3D fibronectin gradients demonstrated that the same pathway regulates haptotaxis in 3D (Fig. 3E) (King et al., 2016).

A second category of substrate-patterning strategies aims at recapitulating the physical nanotopography of the ECM. These approaches rely on micro- and nano-fabrication techniques to

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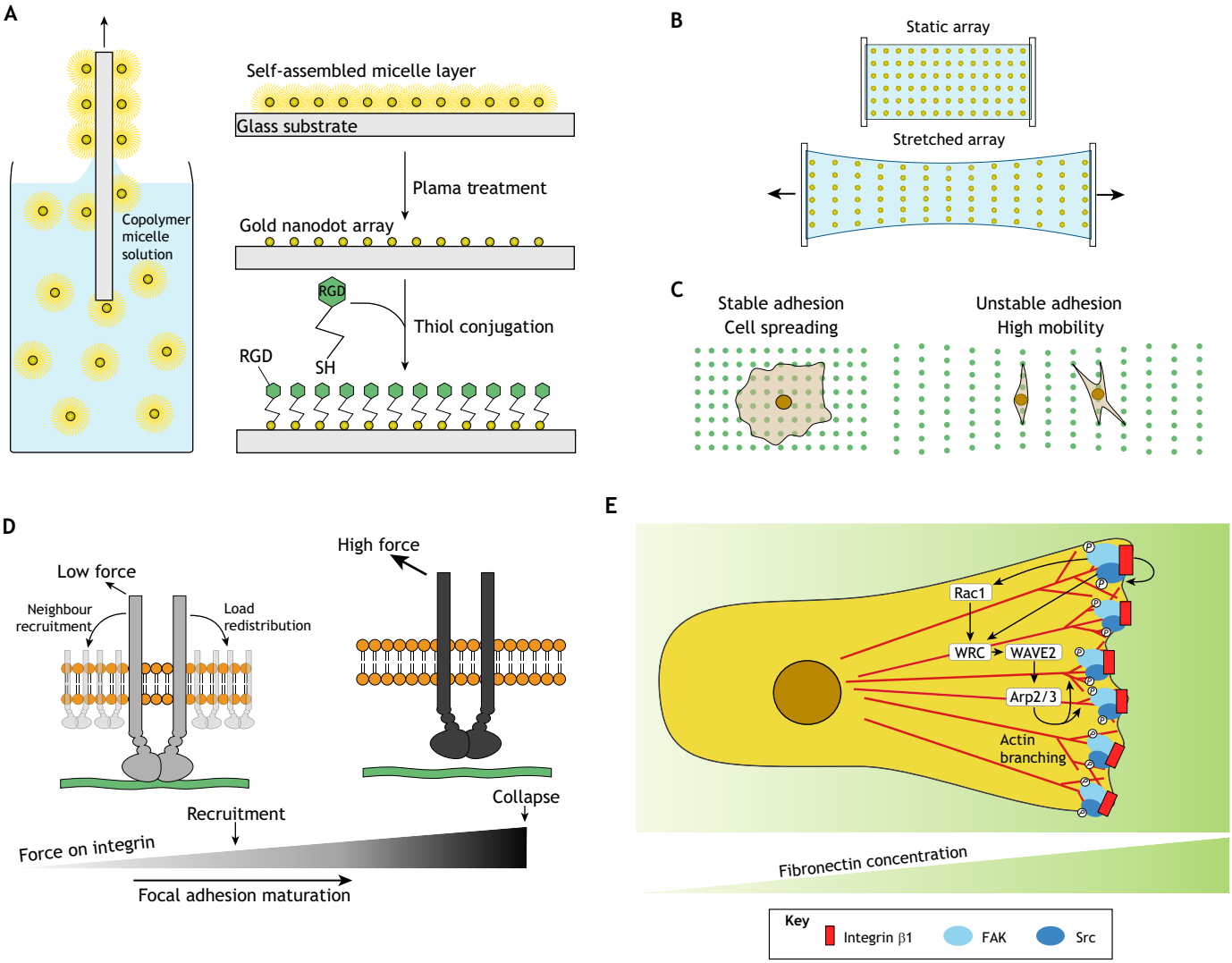

Fig. 3. Surface patterning techniques used to study haptotaxis. (A) Schematic illustration of substrate surface patterning using block copolymer micelle nanolithography (BCMN). Micelles formed of a block copolymer [polystyrene-b-poly(2-vinylpyridine)] containing a precursor self-assemble onto a rigid substrate (e.g. glass). Plasma treatment of the self-assembled micelle monolayer removes the micelles, producing a highly precise gold nanodot array that can be readily functionalised with adhesion ligands through thiol–gold conjugation. Spacing between nanodots can be tuned by controlling the molecular mass of the polymer. (B) A stretchable nano-dot array can be used to control ligand spacing. A gold nanodot array produced by BCMN is transferred to a stretchable poly(N-acryloyl glycinamide) hydrogel. (C) Short inter-ligand spacing (<70 nm) enables cell spreading through the formation of stable mature FAs. Stretching of the ligand array increases the inter-ligand spacing anisotropically. A high inter-ligand spacing (>70 nm) prevents the formation of stable FAs, but increases cell mobility and polarisation in the direction orthogonal to the stretching. (D) Model of a stiffness-dependent inter-ligand spacing threshold. As FAs mature and the force on integrins increase, neighbouring integrins are recruited, which redistributes the load. If the inter-ligand spacing is too high, recruitment and redistribution of the load is impaired, resulting in FA collapse. Because the generation of traction force depends on substrate rigidity, cells on stiff substrates require a higher density of ligands to effectively distribute the load. (E) The distribution (density gradient) of cell adhesion ligands on a substrate can direct cell migration, a process known as haptotaxis. In fibroblasts, haptotaxis is initiated through the engagement of β1 integrin with fibronectin; this leads to the activation (phosphorylation) of FAK and Src family kinases at nascent focal complexes and activation of Rac1, which in turn modulates the WAVE regulatory complex (WRC)–Arp2/3 axis. Arp2/3 governs the dynamics of lamellipodia protrusions, with increasing rate of protrusion formation and stability towards the direction of increased fibronectin concentration.

generate grooves, pits or other nanoscale features on the surface of the culture substrate. It has been shown that such nanotopography features can regulate cell morphology, alignment, contact guidance of migration (Ray et al., 2017) and stem cell differentiation (McNamara et al., 2010). For an in-depth analysis of nanofabrication methods, nanotopography and its effect on cell function and behaviour, we direct the reader to excellent reviews on the topic (Bettinger et al., 2009; Ermis et al., 2018; Park et al., 2018; Zhang et al., 2015).

Indeed, based on findings using these approaches, a new mechanism for directed migration, termed topotaxis, has been proposed (Park et al., 2016, 2018). Topotaxis refers to the migration of cells based on the density of topographical cues. Interestingly, the direction of cell migration is not universal but depends on their cortical stiffness, suggesting a role for cell deformation during migration (Park et al., 2016., 2018) (see Box 2) Although topotaxis remains poorly understood, substrates with defined nanotopography will be key to elucidating the mechanism underlying this novel method of directed migration.

Viscoelasticity and stress relaxation

Although the behaviour of cells cultured on purely elastic matrices of different stiffness is well characterised, most tissues and natural

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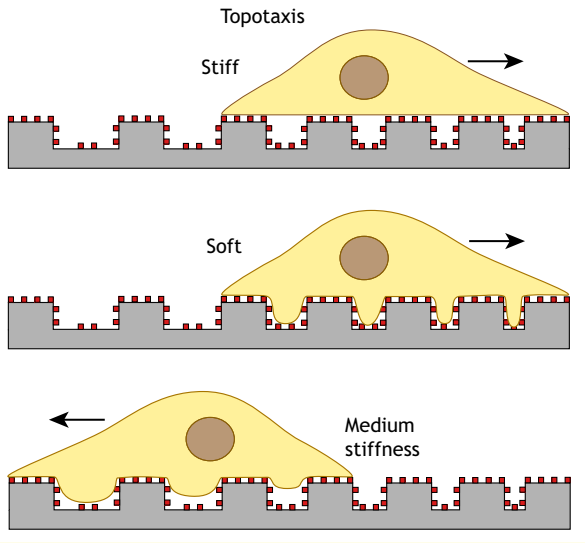
Box 2. The mechanisms of topotaxis

Topotaxis was recently described as the migration of cells either up or down a gradient of density of topographical features (e.g. posts or ECM fibres). Interestingly, the direction of migration depends on the cell elasticity (Park et al., 2016; Park et al., 2018), which suggests that this mechanism of migration is the result of the interaction between cell compliance and available contact area. Stiff cells are unable to deform around the topographical features and can only access adhesion ligands exposed on the surface. As a result, stiff cells migrate towards higher densities of adhesion features (top panel in the figure). More-compliant (soft) cells can adapt to the nanotopography, thereby accessing cryptic adhesion ligands that are inaccessible to stiff cells. As a result, soft cells also migrate towards higher densities of topographical features, as this maximises contact between the cell and the adhesion ligands displayed on the topography (middle panel). In contrast, cells of intermediate stiffness favour regions of sparse fibres because these enable the cell to deform and maximise contact with the topography, whereas a higher density of fibres prevent the deformation of these cells (bottom panel). This mechanism suggests an optimal density of topographical features that would result in maximum contact area for a given cell compliance, therefore leading to topography-guided cell clustering.

It is possible that topotaxis is a specific case of haptotaxis. Indeed, cells undergoing topotaxis migrate towards areas of higher effective density of adhesion ligands (defined as the density of ligands that the cell can access), which depends on the local topography and the ability of the cell to access the adhesion ligands by deforming around the topography. Recent discoveries regarding the role of fibre architecture, ligand recruitment and remodelling of the ECM in regulating cell behaviour in 3D matrices indicate that topotaxis may be a fundamental mechanism for 3D cell migration.



ECMs are fibrous in nature and exhibit viscoelastic behaviour, including frequency-dependent elastic modulus and stress relaxation (Chaudhuri et al., 2016). Therefore, expanding our knowledge of the mechanical microenvironment by incorporating the effect of stress relaxation is fundamental to gain a better understanding of the interaction between cells and ECM *in vivo*.

The viscoelastic character of fibrous matrices originates from their ability to dissipate the elastic energy induced by cellular traction forces through the remodelling of the fibre architecture. The ability for the ECM to be remodelled fundamentally affects how cells interact with their microenvironment, and sense its mechanical properties and exert forces on it. For these reasons, cell behaviour on viscoelastic matrices fundamentally differs from the behaviour of cells in elastic matrices with no stress relaxation or remodelling (Fig. 4A). Hydrogels with tuneable viscoelastic properties have been synthesized with a variety of materials, including alginate (Chaudhuri et al., 2015, 2016), poly(ethylene glycol) (PEG) (McKinnon et al., 2014) and PAA (Cameron et al., 2011, 2014), as well as artificial polypeptide networks (Dooling et al., 2016; Mujeeb et al., 2013).

Cells cultured on soft gels with high relaxation rates exhibit increased spread, adhesion, nuclear YAP localisation and osteogenic differentiation in MSCs; phenotypic features that are characteristic of mechano-activated cells on stiff substrates (Cameron et al., 2011; Chaudhuri et al., 2015, 2016). According to the current paradigm of mechanosensing, the mechanical resistance of a substrate sensed by cells on stress-relaxing hydrogels would be lower than on elastic substrates owing to energy dissipation over time. However, the aforementioned evidence suggests that cells on viscoelastic substrates sense higher resistance, resulting in higher mechano-activation.

One interpretation of these findings is that, on gels with a higher stress relaxation rate, cellular traction forces remodel the matrix within a timescale of less than an hour (Kim et al., 2017), leading to changes in the availability of cell adhesion ligands (e.g. fibronectin) or fibres (e.g. collagen), which have been shown to affect cell spreading, regardless of stiffness (Engler et al., 2004). Indeed, computational models predict that clustering of adhesive ligands due to matrix remodelling in stress-relaxing substrates can account for the apparent increase in stiffness sensing, particularly on soft substrates (Fig. 4A) (Chaudhuri et al., 2015; Kim et al., 2017).

Recently, hydrogels have been developed with independently tuneable stiffness and viscous dissipation through a combination of cross-linked (elastic) and linear (viscous) PAA (Charrier et al., 2018). By presenting ligands only on the cross-linked elastic component, this system provides viscoelasticity, but does not enable ligand clustering. In contrast to previous viscoelastic platforms that are amenable to ligand clustering, cells exhibited a lower spreading area and FA size with increasing viscous dissipation, which is consisted with time-integrated stiffness sensing (Charrier et al., 2018). This evidence further supports the ligand-clustering model of mechanosensing.

The seemingly contradictory behaviour of cells on viscoelastic hydrogels is also observed for cells in fibre networks, particularly within 3D matrices (i.e. cells on softer matrices present higher mechano-activation). For instance, on electrospun methacrylated Dextran (DexMA) matrices with photoirradiantion-mediated crosslinking that allows for tuning of fibre properties, more cell-mediated remodelling of the fibre architecture was observed in soft gels compared to stiff ones (Baker et al., 2015). This fibre recruitment was accompanied by an increase in the number and size of FAs, higher FAK phosphorylation and increased cell proliferation, consistent with the mechano-active behaviour.

In 3D matrices, fibrillarity and viscoelasticity are both required for cell to form FAs (Chaudhuri et al., 2016). Collagen–hyaluronic acid interpenetrating polymer networks (IPNs) offer both characteristics, owing to their dynamic covalent crosslinking, as well as tuneable mechanical properties (Lou et al., 2018). These IPN matrices have been used to study FA formation, matrix remodelling and mechanosensing in 3D (Lou et al., 2018).

Mathematical models of fibrous dissipative matrices that allow for cells to break crosslinking between fibres predict that FA size depends on traction force-mediated fibre recruitment to the cell periphery (Cao et al., 2017). In these fibrous matrices, fibre recruitment is analogous to adhesion ligand clustering. Consistent with experimental results, in

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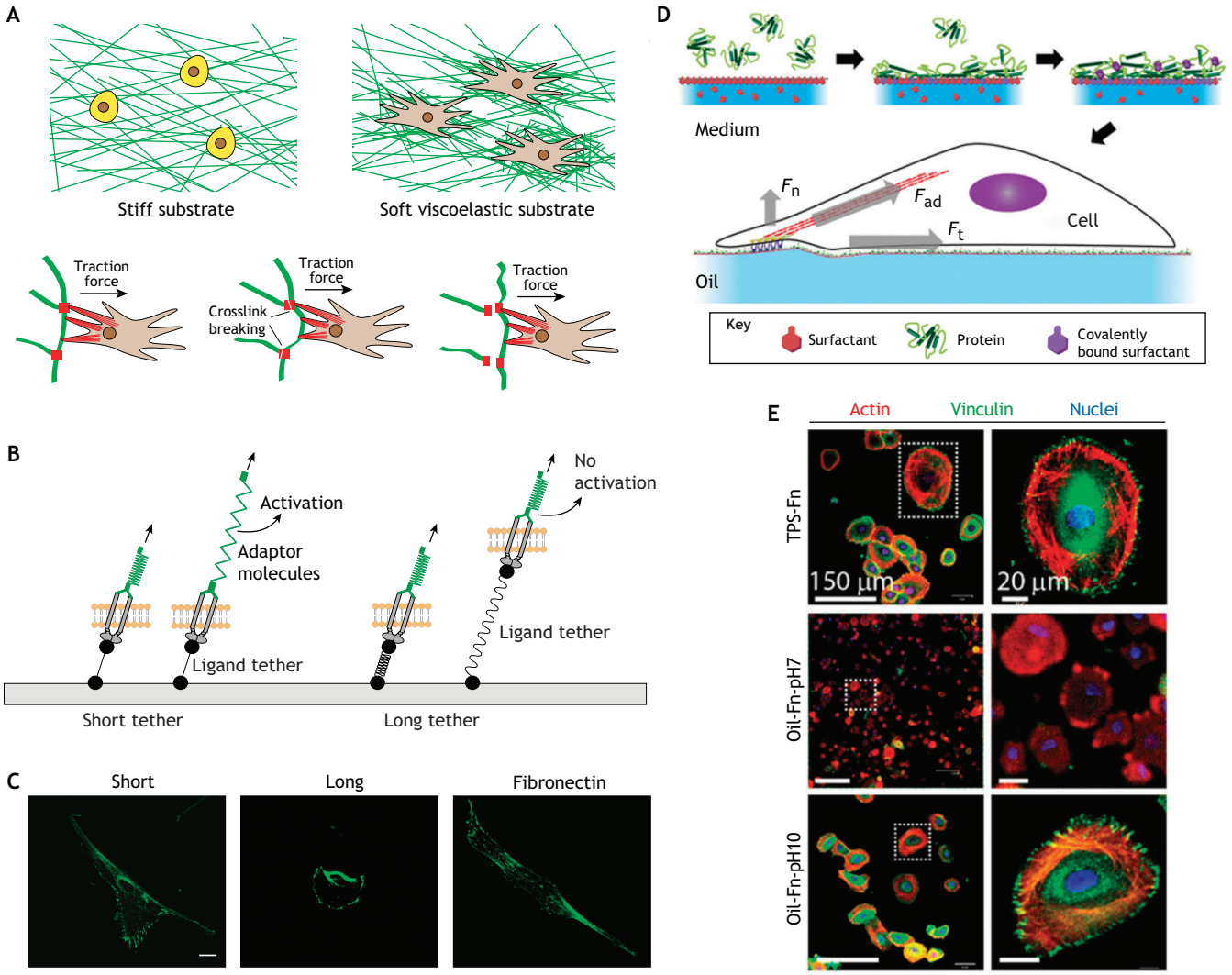

Fig. 4. Viscoelasticity and other nanoscale mechanical properties of substrates. (A) Viscoelastic fibrous matrices can dissipate the elastic energy induced by cellular traction forces through reorganising their fibre architecture, which enables cells to recruit matrix fibres to their vicinity. This remodelling is achieved when cellular traction forces break the crosslinks between fibres. Matrices that are softer and with shorter relaxation times present weaker crosslinks and are therefore more amenable to matrix remodelling and fibre recruitment, whereas stiffer or purely elastic matrices cannot be remodelled. The changes in the matrix nanoarchitecture caused by fibre recruitment in turn promote the mechanical activation of cells. (B) One type of nanoscale mechanical property of a substrate is ligand tether length, which determines the mechanical response of cells. Cellular mechanosensing is responsive to the molecular compliance of adhesion ligands, independently of the bulk substrate stiffness. Short ligand tethers present higher resistance to deformation, leading to the activation of adaptor molecules, and thus induce a mechanoresponse. Long tethers are compliant due to their ability to unfold, thereby decreasing mechanical feedback to the cell and preventing mechanical activation of the cell, even on stiff substrates. (C) Effect of tether length on actin cytoskeleton and FAs (as visualised by paxillin puncta) on cells cultured on glass functionalised with either short, medium and long tethers. Cells on short and medium tethers exhibit higher spreading and FA size; this response is similar to cells plated on fibronectin. In contrast, cells on long tethers have a round morphology and decreased FA size, which is characteristic of quiescent cells on soft substrates. This suggest that nanoscale mechanics dominates the response of cells to their substrate. Scale bars: 20 µm. Reproduced with permission from Attwood et al. (2016) where it was published under a Creative Commons Attribution license (CC BY 4.0). (D) Formation of a protein nanosheet on a liquid–liquid (oil–cell culture medium) interface. The addition of pro-surfactant to the oil drives the self-assembly of a nanometre-thick (15–20 nm) protein layer. Such a protein nanosheet is stable and mechanically strong, enabling the culture and spreading of cells at the liquid–liquid interface. The mechanical properties of the nanosheet can be tuned by changing the pH during self-assembly, with pH 10 and pH 7 resulting in stiff and soft nanosheets respectively. F_{ad}, adhesion force; F_n and F_t, forces applied by the cell in the normal and tangential directions, respectively. Reproduced with permission from Kong et al. (2018a) where it was published under a Creative Commons Attribution license (CC BY 4.0). (E) HPKs cultured on fibronectin (Fn)-coated tissue culture plates (TPS-Fn, top) and protein nanoshets, assembled at either pH 7 (soft; middle) or pH 10 (stiff; bottom). HPKs on stiff nanosheets show a higher degree cell spreading; they form FAs and assemble an actin cytoskeleton, similar to cells on tissue culture plates, as seen in the higher magnification view on the right. In contrast, HPKs on soft nanosheets present a lower degree of cell spreading, decreased FAs and disorganised actin fibres. Reproduced with permission from Kong et al. (2018b) where it was published under a Creative Commons Attribution license (CC BY 4.0).

matrices that allow for higher fibre remodelling (e.g. weaker crosslinking), lower stiffness effectively leads to fibre recruitment and an increase in the size of FAs, whereas in matrices with fixed crosslinking that do not allow for sufficient fibre recruitment, the FA size positively correlates with stiffness, as observed in elastic hydrogel substrates (Baker et al., 2015; Cao et al., 2017).

The growing body of evidence thus points to a model in which the ability of a cell to recruit and remodel matrix fibres, as well as ligand clustering, but not stiffness, play a critical role in regulating cell biomechanical behaviour in 3D matrices. Interestingly, fibre recruitment and ligand clustering depend on cell contractility, thus reinforcing the mechano-activation state of the cell, as highly contractile cells can efficiently recruit fibres and are thus exposed to a mechano-activating microenvironment.

At the moment, it is unclear whether cells can sense the dissipation or stress relaxation independently of substrate stiffness and through distinct mechanosensing mechanisms, or whether they share a common mechanotranduction pathway. Despite being poorly understood, matrix viscoelasticity has been found to be a fundamental biomechanical determinant of chondrogenesis (Lee et al., 2017), MSC differentiation (Chaudhuri et al., 2016) and increased nuclear localization of YAP (Chaudhuri et al., 2015). It is therefore paramount to develop viscoelastic substrates to decouple the effects of stiffness, stress relaxation, fibre remodelling and ligand density in order to elucidate the mechanisms underlying the effect of viscoelastic fibre remodelling on cell adhesion dynamics, spreading and cell differentiation.

Nanoscale mechanical properties

Much of the research in mechanobiology has focused on the response of cells to bulk mechanical properties (e.g. stiffness) derived from continuum mechanics. However, there are some studies that have highlighted the importance of nanoscale mechanics in regulating cell behaviour (Attwood et al., 2016; Kong et al., 2017). These recent findings support the idea that the biomechanical interaction between cells and their microenvironment is governed by the nanoscale mechanical properties of their immediate vicinity, without much interaction from the bulk mechanical properties.

For instance, in functionalised stiff substrates, the mechano-active state of the cell depends on the properties of the tether between the RGD ligand and the glass surface. Tether length regulates FA size, cell spreading, cell surface density and adhesion kinetics, with long and short tethers producing a response that is consistent with soft and stiff substrates, respectively (Fig. 4B,C) (Attwood et al., 2016). Magnetic modulation of the RGD ligand mobility through conjugation with magnetic nanoparticles achieves similar results, with cells acquiring a mechano-active phenotype when presented with low-mobility ligands, regardless of substrate properties (Wong et al., 2017). Furthermore, remote magnetic oscillation of RGD-decorated superparamagnetic iron oxide nanoparticles has been shown to regulate cell adhesion in a frequency-dependent manner, with low frequency oscillations resulting in the osteogenic differentiation of hMSCs (Kang et al., 2017b), as well as adhesion and M2 polarisation of macrophages, both *in vitro* and *in vivo* (Kang et al., 2017a). More recently, magnetic nanocages have been used to remotely control the availability of adhesion ligands (Kang et al., 2018a,b).

Cells are even able to form stable adhesions and spread on substrates that lack bulk mechanical stability, such as the surface of liquids. The culture of cells on liquid–liquid interfaces has been demonstrated through the formation of a mechanically stable protein nanosheet (15–20 nm in thickness) at the interface (Kong et al., 2018a,b, 2017). These nanosheets are formed via pro-surfactant-assisted self-assembly of proteins, and they enable cells to form mature FAs and to spread and to proliferate on the surface of liquid substrates (e.g. oil). These nanosheets can be assembled from bovine serum albumin (BSA) (Kong et al., 2018a) or poly-L-lysine (PLL) (Kong et al., 2018b), and their interfacial shear stress moduli can be tuned by modifying the concentration of pro-surfactant and the pH during self-assembly, respectively. Moreover, PLL polymer nanosheets can be coated with fibronectin, which enables the adhesion and culture of adherent stem cells, such as MSCs and human primary keratinocytes (HPKs) (Fig. 4D) (Kong et al., 2018b).

Both MSCs and HPKs cultured on strong (i.e. with a high interfacial shear stress modulus) PLL nanosheets interfaces (pH 10.5) attach to and spread on the polymer nanosheet in an integrin β1-dependent manner. The cells also form mature FAs, including recruitment of vinculin, talin and paxillin, exhibit a fully developed actomyosin cytoskeleton and form lamellipodia and filopodia, as observed for cells cultured on conventional tissue culture plates (Kong et al., 2018b). Conversely, such a mechano-active phenotype was not observed in cells cultured on soft nanosheets, or in those with a compromised actomyosin cytoskeleton, which confirms the role of the latter in the adhesion of cells to the nanosheets. The nanoscale mechanics of the nanosheet also regulates cell proliferation and stem cell fate, despite the lack of any bulk mechanical properties. Indeed, HPKs cultured in differentiation medium (FAD; Ham's F12 medium/Dulbecco's modified Eagle medium at 1:3) on strong nanosheets presented high levels of involucrin, a marker of cornified differentiation, whereas those grown on soft interfaces remained non-cornified (Fig. 4E) (Kong et al., 2018b). These results suggest that cells can indeed sense and respond to nanoscale substrates such as nanosheets on liquid interfaces.

Interestingly, none of the studies discussed above have investigated the simultaneous effect of nanoscale and bulk mechanical properties. It is possible, therefore, that cells respond to a combination of multi-scale mechanical cues, and that the forces that arise at different scales have distinct roles in regulating cell behaviour (Chaudhuri et al., 2015). Although cells cultured on liquid–liquid interfaces appear to exhibit adhesion mechanics similar to those on stiff substrates, the mechanotransduction pathways underlying this response have not been characterised. The development of novel biomaterials with tuneable and distinct bulk and nanoscale mechanical properties, as well as new methods to characterise nanoscale mechanics (Megone et al., 2018) will be fundamental to better understand multi-scale mechanosensing.

Conclusions – towards a new framework for mechanobiology

As outlined above, parallel developments in the field of mechanobiology and biomaterials have enabled researchers to analyse the response of cells to substrates that better mimic the mechanical properties of the ECM. A spatiotemporal control of mechanical properties and adhesive ligand presentation can direct cell attachment and migration, or modulate the mechano-active state of the cell through mechanical priming. Another step towards understanding the interaction between cells and the ECM is to recapitulate the viscoelasticity of the native microenvironment. Viscoelastic matrices enable cell-mediated reorganization of the matrix nanoarchitecture, which in turn modifies local mechanics. Interestingly, in these 3D matrices, stiffness appears to be less important than the stress relaxation rate and fibre topography and remodelling. These developments have been accompanied by novel characterisation techniques that enable the analysis of biomechanics at the cellular and molecular level (Matellan and del Río Hernández, 2018), which provides new opportunities to study the biomechanical response of cells to their mechanical microenvironment.

Growing evidence suggests that cells mechanosense their microenvironment at the nanoscale and respond to local properties, rather than bulk mechanics. This is particularly evident in nanosheets on liquid–liquid interfaces, which enable cell spreading and FA formation without providing strong bulk mechanical properties. These recent findings raise the question as to whether macroscopic bulk mechanical properties, derived from the framework of continuum mechanics, are adequate to describe mechanobiology at the cellular and molecular level. Indeed, concepts such as stiffness or stress relaxation may not be applicable descriptors of mechanical properties at the level of cellular mechanosensing. In this context, the development of a novel biophysical framework will be required to better interpret the biomechanical interaction between cells and their microenvironment at the nanoscale.

Competing interests
The authors declare no competing or financial interests.

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