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Mechanotransduction and Growth Factor Signalling to Engineer Cellular Microenvironments

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Engineering cellular microenvironments involves biochemical factors, the extracellular matrix (ECM) and the interaction with neighbouring cells. This progress report provides a critical overview of key studies that incorporate growth factor (GF) signalling and mechanotransduction into the design of advanced microenvironments. Materials systems have been developed for surface-bound presentation of GFs, either covalently tethered or sequestered through physico-chemical affinity to the matrix, as an alternative to soluble GFs. Furthermore, some materials contain both GF and integrin binding regions and thereby enable synergistic signalling between the two. Mechanotransduction refers to the ability of the cells to sense physical properties of the ECM and to transduce them into biochemical signals. Various aspects of the physics of the ECM, i.e. stiffness, geometry and ligand spacing, as well as time-dependent properties, such as matrix stiffening, degradability, viscoelasticity, surface mobility as well as spatial patterns and gradients of physical cues are discussed. To conclude, various examples illustrate the potential for cooperative signalling of growth factors and the physical properties of the microenvironment for potential applications in regenerative medicine, cancer research and drug testing.

1. Introduction

The local tissue microenvironment or cell niche includes the extracellular matrix (ECM), biochemical factors (soluble or surface-bound) and neighbouring cells; the interaction between the three of them regulates cell function (**Figure 1**). Biochemical factors have been extensively investigated by the stem cell and organoid communities due to their direct impact in triggering biochemical pathways. In the last decade, great progress has been made in understanding the role of ECM chemical and physical properties in cell response, tissue maintenance, regeneration and disease. Synthetic cell instructive materials allow independent control of biochemical and biophysical properties by combining (i) particular polymers, (ii) crosslinking mechanisms, (iii) degradability, (iv) cell adhesion molecules and (v) biochemical factors – in solution, as well as surface-bound, either covalently tethered or sequestered through physico-chemical affinity with the matrix. Such multi-parametric hydrogels have enabled groundbreaking studies on how cells sense biochemical and biophysical stimuli, as well as synergistic effects between them. Lutolf, Clevers and colleagues have recently shown the great potential of fully-defined, degradable poly(ehtylene glycol)-based (PEG) hydrogels, enriched with fibronectin or laminin-111, as designer 3D microenvironment for the organoid community,[1] so far predominantly limited to Matrigel, albeit lack of control in composition, chemical and physical properties. In this progress report we summarize key works in the fields of GF signalling and mechanotransduction, and discuss synergistic effects between the two. Such understanding of cell-matrix interaction is central in the development of new biomedical devices for regenerative medicine. For a more comprehensive understanding of the role of neighbouring cells we refer to several other reviews.[2–5]

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2. Material-Based Systems for Efficient Presentation of Growth Factors

2.1. Solid-Phase Presentation of Growth Factors

Growth factors (GF) are key biochemical stimuli that promote cell proliferation, migration and differentiation. GFs play a fundamental role in embryonic development and are also involved in a range of physiological and pathological processes, including tissue repair and maintenance.[6,7]

Both in vitro and in vivo it is still common to use GFs in solution, either directly added to the culture media or released from a biomaterial carrier.[8–11] This is rather inefficient and unsafe as it uses high concentrations of GFs. Conceptually, it ignores that GFs in the body are bound to the ECM, as both proteins and glycosaminoglycans (GAGs) have GFs binding sites.[12] It was soon realized that the administration of GFs bound to a

surface, i.e. solid-phase presentation of GFs, resulted in higher efficiency compared to soluble release, improved biological functions and reduced concentration and dose.[13–15] **Figure 2** shows a sketch of classical (soluble) vs. surface-bound presentation of GFs.

Insulin was one of the first GFs immobilized on material surfaces but with no obvious correlation with biological activity.[17] It was more than 20 years later when it was shown that low doses of insulin immobilized on polymers had higher mitogenic effect than free insulin.[18] Simultaneously, it was shown that epidermal GF (EGF) could be tethered to surfaces retaining its biological activity and with control of temporal and spatial availability.[19] However, besides these early successes it took another decade to investigate systematically the effect of GFs bound to surfaces.

Most relevant studies involved different GFs and strategies to present them from surfaces: Insulin-like GF-1 (IGF-1) tethered on self-assembled peptides increased the expression of troponin-I in cardiomyocytes in vitro, and improved systolic function after infarction in vivo.[20] Vascular endothelial GF (VEGF) retained within collagen gels (through an ECM binding domain) prolonged activation of its receptor VEGFR2 and activated β_1 integrin, which had not been observed with soluble VEGF.[21] One of the first studies using stem cells revealed that surface-bound EGF promoted both cell spreading and survival more strongly than saturating concentrations of soluble EGF.[22]

Cavalcanti-Adam et al. investigated the influence of the distribution of bone morphogenetic protein (BMP)-2 using block copolymer micellar nanolithography to fabricate substrates with precisely spaced and tunable gold nanoparticle arrays carrying single BMP-2 molecules. They showed that surface presentation of BMP-2 promoted enhanced Smad signaling compared to soluble administration of the GF.[23] Segura et al. used nanoparticles functionalized with VEGF into a fibrin matrix to show that once GFs are bound to a surface, there are still some degrees of freedom in GF presentation (e.g. the density and organisation on the surface) that can influence GF activity.[24]

2.2. Affinity-Based Systems for the Presentation of Growth Factors

Some of the pioneering studies on 'solid-phase' presentation of GFs used covalent tethering of GFs to surfaces.[19,25] However, exploiting the natural affinity of ECM components (GAGs and structural proteins) towards GFs has resulted in the development of GF sequestering biomaterials, which incorporate defined sequences of amino acids to promote GF binding. This approach has been called in some papers affinity-based systems.[26–28] In addition, these systems allow presentation of GFs that can be further internalized following receptor binding.[29,30] Seeking to engineer localized GFs reservoirs, layer-by-layer (LbL) assemblies of polyelectrolytes, which alternate positive and negative charged macromolecules have been used.[31] Picart et al. incorporated BMP-2 into poly(L-lysine)/hyaluronan (PLL/HA) LbLs and showed that the amount of BMP-2 loaded in the system could be controlled by varying the number of layers assembled.[32] BMP-2 was afterwards slowly released from the system, still bioactive, to drive osteogenic cell differentiation.[32]

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2.3. Systems that Promote Growth Factor Receptor – Integrin Crosstalk

The solid-phase presentation of GFs allows better control of their spatial distribution to target GF receptors. Early works revealed the bidirectional cross-modulation of integrins and GF receptors, e.g. β_1 integrin and epithelial growth factor receptor in epithelial cells.[33] Then, and more specifically, the simultaneous excitation of integrin and GF receptors was described in biology to promote synergistic GF signalling.[34] Here we describe materials engineered to promote GF receptor – integrin crosstalk (**Figure 3**a) seeking to achieve high efficiency with low doses of GFs.

Fibronectin and its fragments have been used to engineer synergistic GF microenvironments. Fibronectin contains three kinds of domains which mediate interactions with other fibronectin molecules, other ECM molecules and cells (Figure 3b).[35] Sobel et al. identified the heparin II binding region of fibronectin ($FNIII_{12\text{-}14}$) as a VEGF binding site.[36] They reported that fibronectin fragments including $FNIII_{9\text{-}10}$ (integrin binding region) and $FNIII_{12\text{-}14}$ promoted enhanced

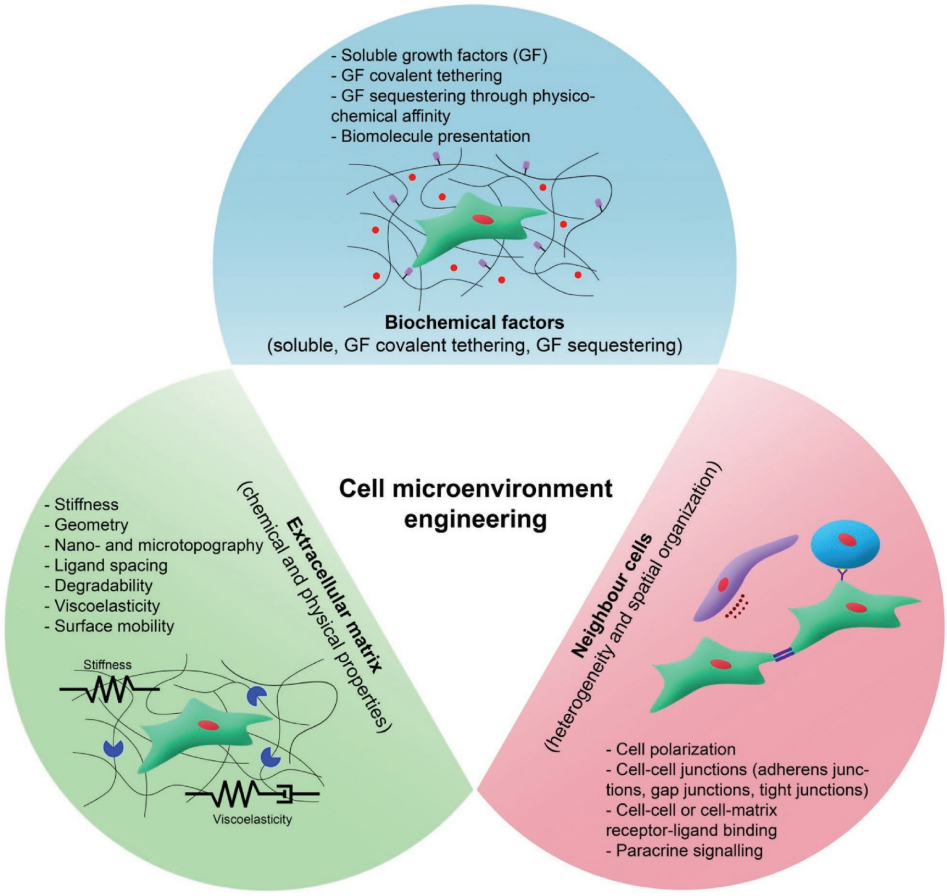

Figure 1. Cell microenvironment engineering targets soluble and surface-bound biochemical factors, chemical and physical properties of the extracellular matrix and the interaction with neighbouring cells.

endothelial cell migration, proliferation and signalling.[36] Martino and Hubbell generalized this result to show that $FNIII_{12\text{-}14}$ not only bound VEGF but was actually a highly promiscuous region with affinity towards GFs from different families.[37]

Based on this ability of fibronectin to bind GFs, material systems that presented GFs by promoting the crosstalk between integrins and GF receptors have been engineered.[16] For example, Martino and colleagues used a fibrin matrix

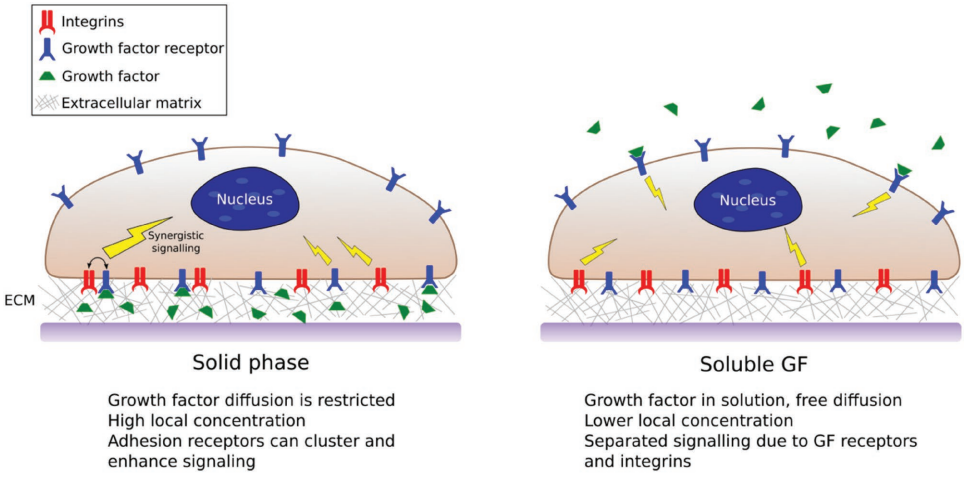

Figure 2. Solid-phase vs. soluble presentation of GFs. GFs bound to material systems are spatially confined and their diffusion is limited. Even if the overall concentration of GFs is low, these are presented in high local concentrations and have the potential to crosstalk with adhesion receptors. Reproduced with permission.[16] Copyright 2016, Royal Society of Chemistry.

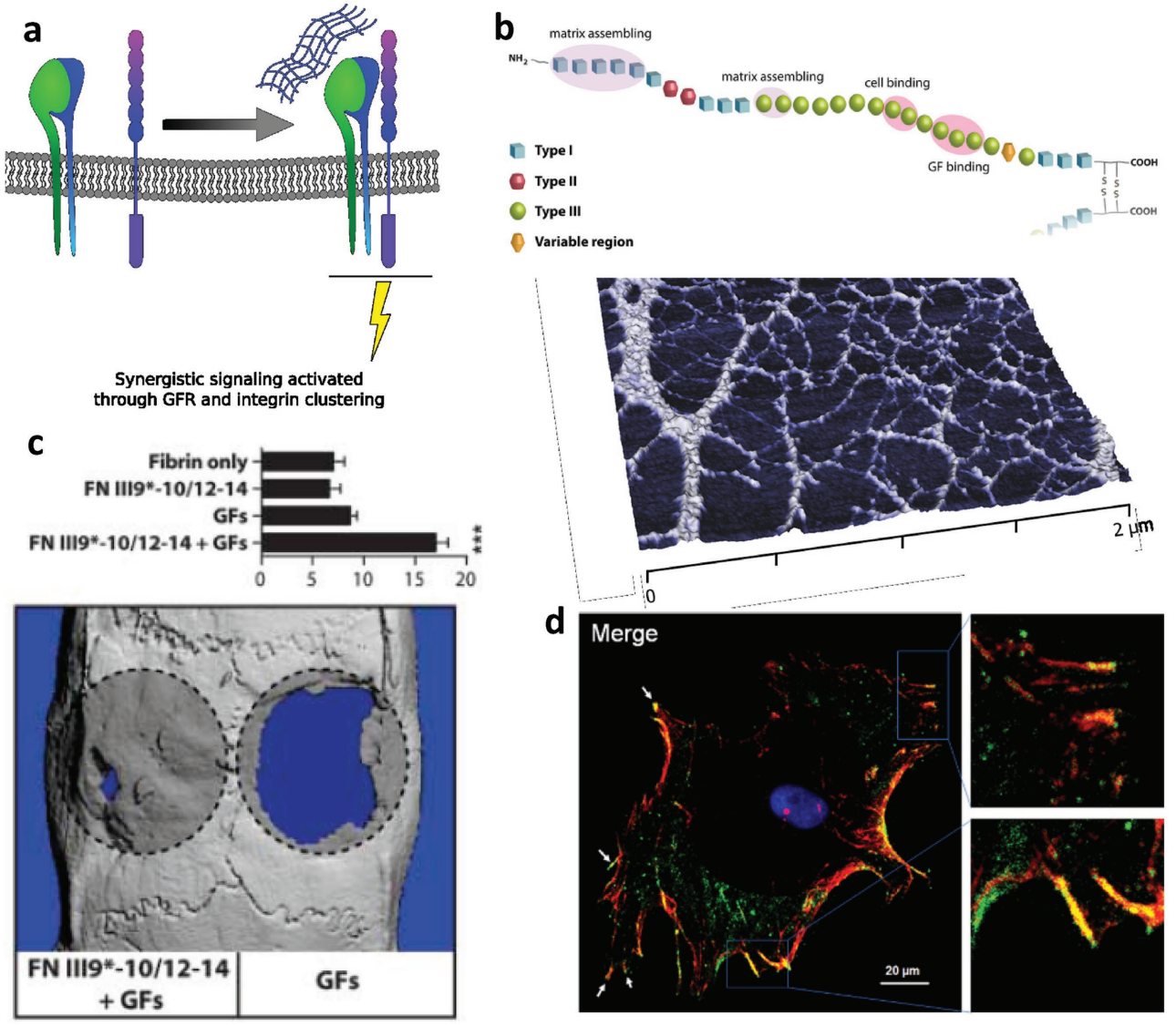

Figure 3. Synergistic growth factor microenvironments based on integrin-GF receptor synergistic signalling. a) Simultaneous targeting of integrins and GF receptors leads to synergistic signalling which maximizes the effect of the GF. b) Fibronectin contains different kinds of domains, in particular $FNIII_{9\text{-}10}$ promotes integrin binding whereas $FNIII_{12\text{-}14}$ is a promiscuous GF binding region. Fibronectin is organized into nanonetworks on poly(ethyl acrylate) (PEA) surfaces with both domains available for interaction. c) The incorporation of a recombinant fragment of fibronectin than encompasses FNIII9-10/12-14 into a fibrin matrix promotes bone regeneration in vivo with low doses of GFs (≈150 ng). d) The addition of BMP-2 to fibronectin nanonetworks on PEA surfaces promotes co-localization of integrin β_1 (green) and BMPRI (red). a) Reproduced with permission.[16] Copyright 2016, Royal Society of Chemistry. b,d) Reproduced with permission.[39] Copyright 2016, American Association for the Advancement of Science. c) Reproduced with permission.[38] Copyright 2011, American Association for the Advancement of Science.

functionalized with two recombinant fragments of fibronectin joined together, $FNIII_{9\text{-}10}$, to promote integrin binding and cell adhesion,[35] and $FNIII_{12\text{-}14}$, to bind GFs.[37] They showed that the system enhanced the formation of tube-like structures in endothelial cells (with VEGF-A), sprouting of smooth muscle cells (with platelet-derived growth factor (PDGF)-BB) and differentiation of mesenchymal stem cells (MSC) (with BMP-2).[38] In addition, the system promoted tissue repair in vivo: wound healing in a diabetic mice model (with VEGF-A165 and PDGF-BB) and bone regeneration in a critical size skull defect (with BMP-2 and PDGF-BB) (Figure 3c).[38] Remarkably, this was done using low doses of GFs (<150 ng) which is relevant for a safe use of GFs in clinical applications. This work represented a major landmark in GF presentation and put synergistic integrin-GF signalling in the map as a way to use GFs efficiently and safely.

Similarly, in the context of bone repair, placenta growth factor (PlGF)-$2_{123\text{-}144}$ fused to BMP-2 and PDGF-BB led to full regeneration in a critical-size skull defect using low concentrations of GFs (≈200 ng).[40] This study shows that the sole delivery of GFs without a biomaterial carrier might work effectively by engineering GFs to bind to the ECM and, paradoxically, reveals the importance of GF presentation to maximise efficiency, even if in this case GFs were delivered topically and in solution, without biomaterials carriers.[40]

A different way to exploit the ability of fibronectin to bind GFs is by inducing the assembly of fibrillar structures, recapitulating the physiological process of cell-mediated fibronectin fibrillogenesis, to allow exposure of domains otherwise unavailable in the globular conformation of the protein.[41–44] We noted that $FNIII_{12\text{-}14}$ (GF binding region) is located next to the integrin binding region ($FNIII_{9\text{-}10}$) with potential for synergistic integrin/GF signaling. However, for this to be exploited, fibronectin must be open, unfolded, to have the relevant regions available for interaction (Figure 3b).[39]

We showed that some polymers, such as poly(alkyl acrylates), with length of the side chain above two (e.g. ethyl – PEA, butyl – PBA, hexyl – PHA) promoted fibronectin assembly into nanonetworks (Figure 3b).[45–47] This assembled fibronectin structure has the ability to present GFs in synergy with $\alpha_5\beta_1$ integrins, i.e., the molecule is open and $FNIII_{9\text{-}10}$ and $III_{12\text{-}14}$ regions are simultaneously available for interaction. Indeed, using atomic force microscopy we showed that BMP-2 binds fibronectin nanonetworks previously assembled on PEA and then promotes co-localization of integrins and GF receptors resulting in enhanced canonical Smad signaling (Figure 3c).[39] This system used very low doses of BMP-2 (25 ng ml^{-1}) to promote MSC differentiation in vitro, as well as bone regeneration in a critical size defect in the mouse radius.[39] The assembly of fibronectin on PEA has been also effective in promoting integrin – VEGF signalling to enhance vascularisation.[48] One of the main advantages of using acrylates to promote high efficiency presentation of GFs is that the material can be applied through several technologies as coatings on biomedical devices, including scaffolds of complex anatomical 3D shapes.[49]

3. Physics of the Microenvironment and Mechanotransduction

The concept of "dynamic reciprocity" was postulated by Bissell and colleagues in 1981, whereby the cell nucleus and cytoskeleton, on the one hand, and the ECM, on the other, influence each other through physical and chemical interactions.[50] One decade later, Ingber and colleagues showed that cells can sense physical signals of the microenvironment using integrins, and then transmit this information throughout the cytoskeleton up to the nuclear structure, and thereby influence cell response.[51,52] He proposed a tensegrity-based transduction system and opened a new field of research, mechanotransduction.[53,54] Since then multiple features of the physics of the ECM been investigated.

3.1. Stiffness

The effect of substrate stiffness on cell migration and focal adhesions (FA) was first described two decades ago by Pelham and Wang.[55] Epithelial and fibroblastic cells exhibited reduced spreading, irregular-shaped and highly dynamic FAs, increased motility and lamellipodial activity on soft (5 kPa) compared to stiffer (70 kPa) collagen-coated polyacrylamide substrates. A vast number of publications followed this study, illustrating the relevance of the matrix mechanical properties on cell response.[56–60] A hallmark study by Engler and colleagues showed that matrix stiffness directs MSC fate, with soft (0.1–1 kPa), middle (8–17 kPa) or stiff (25–40 kPa) collagen-coated polyacrylamide substrates favouring neurogenic, myogenic or osteogenic differentiation, respectively (**Figure 4**a).[61]

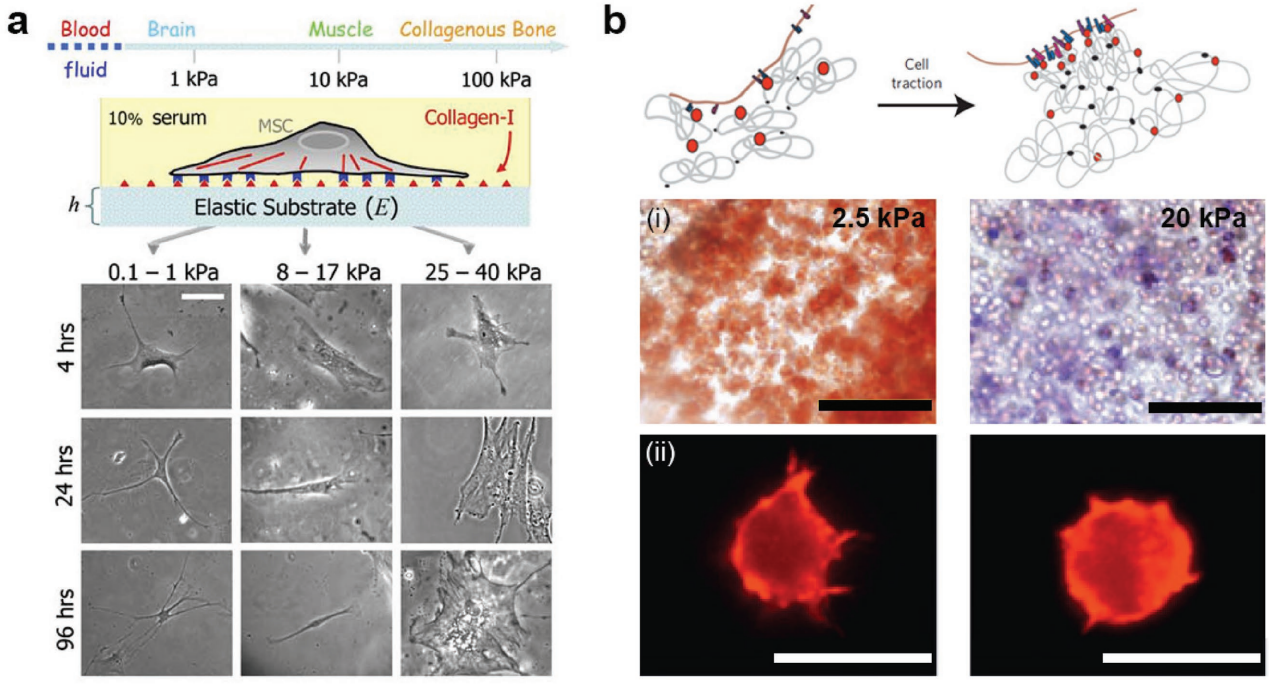

Figure 4. a) Hydrogel stiffness directs MSC fate on collagen-coated polyacrylamide 2D substrates and in (b) RGD-modified, non-degradable, ionically crosslinked alginate 3D matrices. Scale bars: (a) 20 µm, (b-i) 100 µm, (b-ii) 10 µm. a) Reproduced with permission.[61] Copyright 2006, Elsevier. b) Reproduced with permission.[62] Copyright 2010, Macmillan Publishers Limited.

This work was extended to a three dimensional (3D) matrix by Huebsch and colleagues, who showed that MSCs differentiated towards adipogenic or osteogenic lineages at soft (2.5–5 kPa) or stiffer (11–30 kPa), RGD-modified, non-degradable, ionically crosslinked alginate hydrogels (Figure 4b).[62] Cell fate was not associated with differences in cell morphology, which remained unchanged, but rather with integrin clustering, which was maximized in stiffer hydrogels (Figure 4b). In a following study, void-forming, ionically crosslinked alginate hydrogels, consisting of (i) a bulk phase containing ex vivo encapsulated MSCs and stiffness optimized for osteogenic differentiation, and (ii) a sacrificial phase with degradable porogens, proved successful in the regeneration of 8 mm skull defects in nude rats.[63]

Some controversy arose with the work by Trappmann and colleagues, who showed that differences in collagen fibre tethering, caused by variations in pore size of polyacrylamide hydrogels with varying stiffness, altered local stiffness and influenced MSC fate.[64] Furthermore, collagen fibre tethering precisely altered by changing the spacing of gold nanoparticles also had an impact on cell differentiation. A follow up study by Engler and colleagues refuted in part these findings by fabricating polyacrylamide substrates with an equivalent stiffness but changes in pore size and showing that stiffness, and not pore size or protein tethering, was more predictive of cell fate on 2D substrates.[65] This discussion, however, highlighted the concept that biological microenvironments are indeed fibrillar and that cell-matrix interactions differ substantially if the matrix is a nanoporous hydrogel, perceived as a continuum at the cell scale, or an engineered fibrillar microenvironment, with fibres of different local stiffness.[66]

3.2. Geometry

Contemporary to the work by Pelham and Wang, Chen and colleagues pioneered the idea that local geometry is a fundamental mechanism that regulates cell response. They controlled endothelial cell proliferation and apoptosis by simply allowing or restricting cell spreading (black), while maintaining the contact area (grey) constant (**Figure 5**a).[67] In a following study, they developed a bed of microneedles to manipulate and measure cell traction forces as a function of the size of adhesion sites and cell spreading (Figure 5b).[68] It was shown that geometry-induced cytoskeletal tension and RhoA signalling could direct MSC fate, with osteogenic (adipogenic) differentiation associated

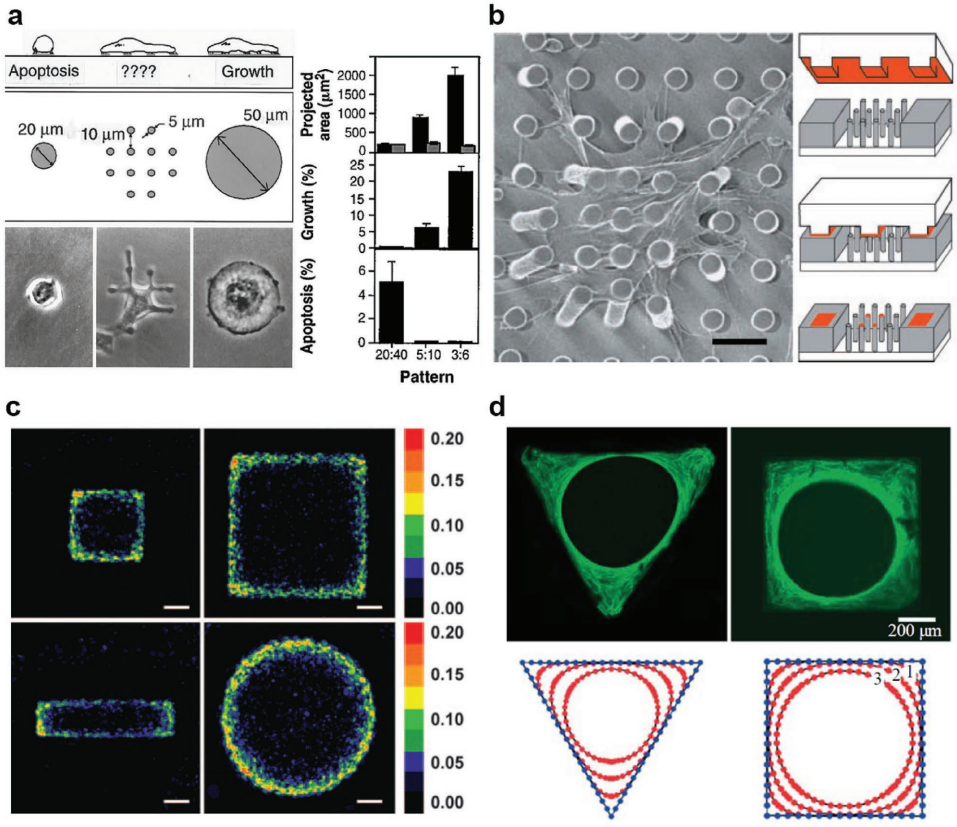

Figure 5. a) Cell spreading can dictate proliferation or apoptosis. b) Cell traction forces can be manipulated as a function of the size of the adhesion sites and cell spreading. (c The geometry of a collective of cells can regulate patterns of cell growth, favoured in regions of high traction stress. (d)Cells on a surface can sense and react to radii of curvature much larger than a single cell, with local tissue growth proportional to the local (concave) curvature. Scale bars: (b) 10 µm, (c) 100 µm. (a) eproduced with permission.[67] Copyright 1997, American Association for the Advancement of Science. b) Reproduced with permission.[68] Copyright 2003, National Academy of Sciences. c) Reproduced with permission.[72] Copyright 2005, National Academy of Sciences. d) Reproduced with permission.[74] Copyright 2008, The Royal Society.

to spread, flat morphology (unspread, round).[69] Kilian and colleagues demonstrated that single cells cultured on islands with rectangular or pentagonal symmetry, with varying subcellular curvature but with an equivalent area, exhibited different osteogenesis or adipogenesis, with increased acto-myosin contractility favouring the former.[70] These findings were extended to collective cell behaviour by culturing multicellular islands with different shapes.[71] The spatial gradients of traction forces mirrored MSC differentiation, with osteogenic (adipogenic) differentiation at highly stressed edges (centre of island). Multicellular geometry can also feedback to regulate patterns of cell growth, with regions of high traction stress favouring growth and branching morphogenesis (Figure 5c).[72,73] Cells on a surface can sense and react to radii of curvature much larger than a single cell and regions with high local concave (convex) curvature favour (inhibit) tissue growth (Figure 5d).[74,75]

On the other end of the size scale, surface nano- and microtopography influence size and shape of cell-matrix adhesion points and thus also play an important role in cell mechanotransduction, as well as in stem cell self-renewal and multipotency.[76–82] There are excellent reviews on the effect of nanotopography on (stem) cells.[81,83]

3.3. Ligand Spacing

Cells sense and react to their surrounding environment through focal adhesions.[84] Spatz and colleagues pioneered the idea that spacing of adhesion peptides affects cell attachment, spreading and organisation of focal adhesions, and proposed 58–73 nm as a universal length scale for integrin clustering and activation of cells cultured on 2D flat surfaces.[85] In a following study they showed that the formation of mature, stable focal adhesions and persistent cell spreading, but not cell adhesion, was sensitive to ligand density.[86] With 108 nm spacing, cells exhibited a rapid turnover in focal adhesion formation, delayed spreading and repeated protrusion-retraction cycles compared to cells plated on 58 nm-spaced pattern. For ligand spacing >70 nm, the local order or disorder had an impact on cell adhesion, with increased (reduced) adhesion for disordered (ordered) ligands.[87]

3.4. Time-Dependent Biomaterial Physical Properties

The cell microenvironment is dynamic and undergoes remodelling with time. This is evident in physiological processes such as differentiation, morphogenesis, or maintenance of homeostasis, as well as in progression of disease.[88] In the last years, dynamic biomaterial properties such time-dependent matrix stiffening, degradability, viscoelasticity and surface mobility are beginning to attract attention in the field of regenerative medicine.[89–92]

3.4.1. Time-Dependent Matrix Stiffening

Young and Engler presented one of the first studies describing hydrogel time-dependent stiffening to mimic developing myocardium.[93] Thiolated hyaluronic acid (HA) hydrogels were designed to stiffen from 1.9 ± 0.1 to 8.2 ± 1.1 kPa, imitating the 9-fold increase in elastic modulus measured in developing embryonic chicken heart. A three-fold increase in mature cardiac specific markers and formation of up to 60% more maturing muscle fibres was observed on dynamic HA substrates compared to static polyacrylamide hydrogels. Guvendiren and Burdick showed that the kinetics of light-mediated stiffening (≈3–30 kPa) of HA substrates modulated MSC differentiation.[94] MSCs differentiated into osteoblasts (adipocytes) when stiffening took place within hours (within days-to-weeks) time, maximizing (minimizing) the time cells were on stiff substrates. Stiffening HA substrates were used to mimic fibrosis and consequent myofibroblast activation in hepatic stellate cells.[95] Anseth and colleagues extended this work to cell-laden 3D hydrogels using PEG-based hydrogels to investigate microenvironmental stiffening on valvular interstitial cell (VIC) activation.[96] VIC embedded in soft gels (0.24 kPa) exhibited a myofibroblast phenotype, while upon stiffening (to 1.2 or 13 kPa) via a photoinitiated thiol-ene polymerization, they reverted to a quiescent, fibroblast phenotype, irrespective of cell morphology. This was in contrast to previous 2D studies, where light-mediated hydrogel softening (32 to 7 kPa) induced VIC deactivation, highlighting the importance of matrix dimensionality.[97,98]

3.4.2. Degradability

Biomaterials for tissue regeneration should degrade at a rate matching that of the new tissue growth.[99] Lutolf and colleagues pioneered matrix metalloproteinase (MMP)-sensitive hydrogels using PEG-based system.[100] They used a rat skull defect to show that bone regeneration, induced by BMP-2, was dependent on the sensitivity to MMP degradation. Khetan et al. highlighted the fact that cell response to matrix physical properties is highly dependent on the crosslinking method and degradation properties.[101] In contrast to previous studies showing stiffness-mediated MSC differentiation in non-degradable, ionically crosslinked alginate hydrogels,[62] Khetan and colleagues reported that covalently crosslinked HA hydrogels, with equivalent stiffness (≈4 kPa) and enzymatic degradation (non-degradable), permitted (restricted) cell spreading, high (low) traction forces and favoured osteogenic (adipogenic) differentiation (**Figure 6**a). A very recent study by Gjorevski et al. describes the use of fully-defined, degradable PEG-based gels, enriched with fibronectin or laminin-111, for intestinal stem cell (ISC) and organoid culture.[1] Enzymatically degradable hydrogels did not support ISC expansion and non-degradable matrices were needed. However, for organoid development matrix softening through ester-based hydrolysis of hybrid PEG gels was required.

3.4.3. Viscoelasticity

Covalently-crosslinked hydrogels are typically elastic, while native ECM is viscoelastic. Chaudhuri et al. observed increased cell spreading on soft, viscoelastic substrates compared to elastic gels with the same stiffness, and similar to that on

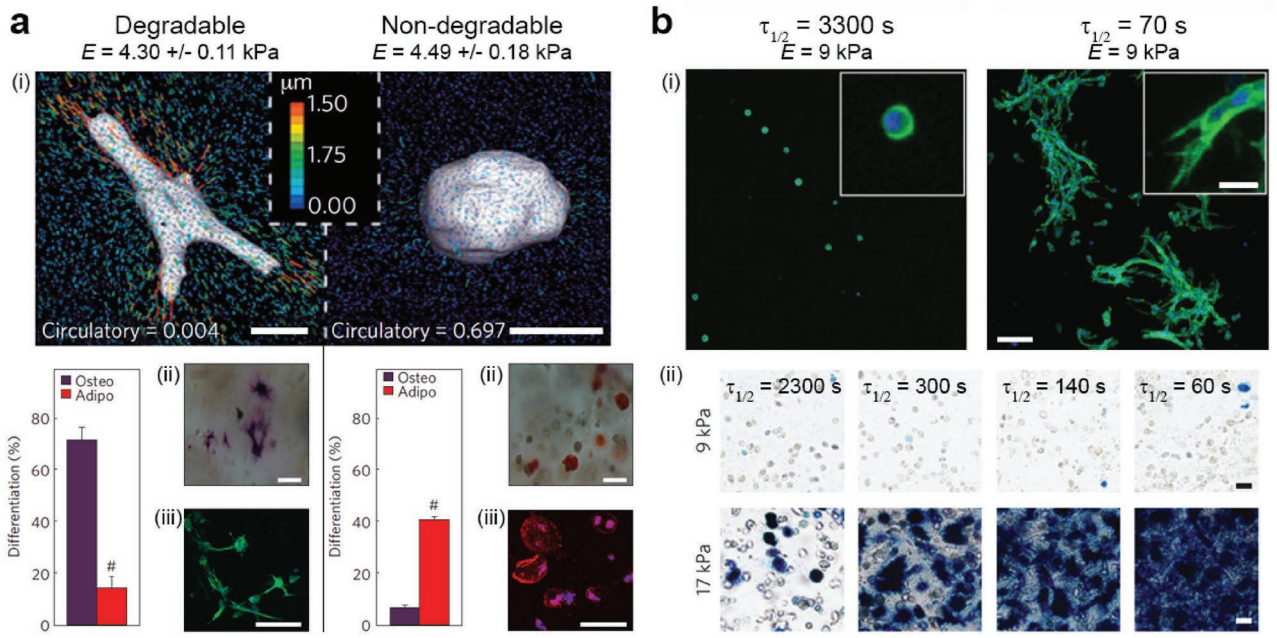

Figure 6. Time-dependent biomaterial physical properties such as degradability (a) or viscoelasticity (b) can also direct osteogenic differentiation, for an equivalent matrix stiffness. Scale bars: (a-i) 10 μm, (a-ii) 25 μm, (a-iii) 20 μm, (b-i) 100 μm for the larger image and 20 μm for the insert, (b-ii) 25 μm. a) Reproduced with permission.[101] Copyright 2013, Macmillan Publishers Limited. b) Reproduced with permission.[103] Copyright 2016, Macmillan Publishers Limited.

stiffer, elastic substrates.[102] This work was extended to 3D alginate-PEG based hydrogels with tunable stress relaxation, independent of initial stiffness, degradability and number of adhesion sites.[103] MSC spreading, proliferation and osteogenic differentiation were enhanced in rapidly relaxing hydrogels ($\tau_{1/2}$ = 70 s) compared to elastic gels ($\tau_{1/2}$ = 3300 s), for a stiffness of 17 kPa (Figure 6b).

3.4.4. Surface Mobility

Surface mobility is another less known and poorly exploited dynamic material property with the potential to alter cell behaviour.[104,105] Surface mobility is related to the dynamic properties of hydrated interfacial polymer chains triggered by their interactions with the surrounding. Examples include model chemistries and polypeptides tethered to surfaces with spacers of different length, as well as PEG-based systems, which modulate cell adhesion, spreading and long term phenotype.[105–107]

Surface mobility is sometimes mistaken with surface stiffness but it is actually a different physical property. Cells transduce stiffness by pulling on the surface with nanoscale forces (5.5 nN μm^{-2}), normally after assembling focal adhesions.[108] Surface mobility only involves single receptor interactions with much lower and fluctuating forces (picoscale). Using model systems based on RGD-functionalized lipid bilayers, which are highly mobile entities, it has been shown that integrin activation and early clustering are independent of lateral forces and that cells on these highly mobile surface fail to form focal adhesions.[109,110] Very recently, the mobility of supported lipid bilayers has been used to control MSC differentiation independently of ligand density.[111]

Polymers chains are mobile elements and their dynamics suffer a quantitative change at the glass transition temperature (T_g). At temperatures below T_g, polymer chains are arrested and their movement is limited to the side groups of the chains. However, at temperatures above T_g, polymer chains are highly mobile within the characteristic distance of a few tens of nanometers. Experiments using thin polymer films with nanometric thicknesses demonstrated the scale of these movements.[112]

Using a family of poly(alkyl acrylates) with different surface mobility at 37 °C, it was shown that the mobility of the polymer surface was indeed translated into the mobility of an interfacial layer of fibronectin adsorbed on their surface. Differences in the mobility of the adsorbed protein layer played a role in cell adhesion, reorganization and differentiation, through a mechanism that involved cell contractility.[113,114] These findings on 2D surfaces have recently been extended to 3D matrices. Novel sliding hydrogels, with stable chemical crosslinks combined with mobile crosslinks and mobile biochemical ligands, were shown to support cellular reorganization of surrounding ligands, changes in cell shape and differentiation.[115]

3.5. Spatial Patterns or Gradients of Physical Cues

3.5.1. Spatial Patterns of Stiffness

Not only the magnitude, but also the spatial distribution or gradients of matrix physical cues can influence cell function. It was shown that gradients in substrate rigidity guide cell migration, with cells preferentially migrating from soft towards

stiffer substrates (durotaxis).[116] Recently, Trepat and colleagues observed that durotaxis can be an emergent property in a collective of epithelial cells, even if isolated cells did not show this effect.[117] Marklein and Burdick used sequential crosslinking of HA hydrogel to create spatial patterns of stiffness ranging from 3–100 kPa and observed higher MSC spreading and proliferation on stiffer regions.[118] In a following study, hepatic stellate cell differentiation into myofibroblasts was observed on large (1 mm diameter) islands with higher stiffness (23.8 ± 4.6 kPa), while they remained quiescent on soft substrates (2.1 ± 0.7 kPa) or on small (50 µm diameter), stiff islands that prevented cell spreading.[119] In a recent study, Yang and colleagues showed that the subcellular, microscale spatial distribution of matrix mechanical properties affect MSC function, with regular (random) distribution of stiff regions resulting in cell spreading (round morphology) and higher (lower) Yes-associated protein (YAP) activation.[120]

3.5.2. Spatial Patterns of Topography

Cells can also sense gradients in topography. Fibroblasts plated on grooves with 1 µm width and 400 nm depth oriented more strongly along the direction of ridges and migrated faster compared to sparser areas with width up to 9.1 µm.[121] Furthermore, in the short term cells migrated along the pattern orientation, while in the long term, they migrated towards denser areas both in unidirectional[121] and 2D rectangular lattices.[122] Sochol and colleagues investigated the combined effect of topography of microposts with varying stiffness and showed that endothelial cells preferentially migrated towards stiffer microposts, with a migration speed proportional to the gradient strength.[123] Recently, Levchenko and colleagues described the guided migration of invasive and non-invasive melanoma cells according to the gradient in matrix nanotopography.[124] They showed that invasive (non-invasive) cells migrated towards sparser (denser) areas and concluded that the topotactic response depends on both the density and structure of the ECM, and the stiffness of the cell itself.

3.5.3. Spatial Patterns of Degradability

Gradients in biomaterial degradation and resulting dynamic biomaterial properties, such as time-dependent topography or stiffness, are beginning to attract attention both in 2D and 3D synthetic environments. Anseth and colleagues employed photodegradable PEG-based hydrogels – tunable in situ by UV light, visible light or irradiation in the presence of encapsulated cells – to create channels that allowed cell spreading and migration in 3D or to release specific functional groups on-demand.[125] This work was extended to click-based hydrogels that allow orthogonal and spatiotemporal control of photocleavage of crosslinks and photoconjugation of functional groups.[126] Heilshorn and colleagues developed protein polymers with tailored degradation rates to create dynamic structures emerging over time through enzymatic degradation in the bulk or on the hydrogel surface, as well as to release biomolecules with distinct spatiotemporal patterns.[127] In another study, spatial patterns of enzymatic degradation in HA hydrogels were used to enable or inhibit cell remodelling required for vascular network formation.[128]

3.6. Multiple Features of the Physics of the ECM and the Effect on Ligand Clustering

The improved understanding of the role of various aspects of the physics of the ECM on cell response has also led to some controversies, as to which are the key parameters modulating matrix mechanosensing. Various physical cues of the ECM have been shown to induce osteogenic differentiation of MSCs in 3D: (i) the bulk stiffness of non-degradable, ionically crosslinked alginate hydrogels,[62] (ii) the degradability of covalently crosslinked HA hydrogels, for an equivalent stiffness[101] and (iii) the viscoelastic properties of ionically crosslinked alginate hydrogels with a polyethylene glycol spacer, for an equivalent stiffness and in the absence of matrix degradation.[103] These studies utilize different hydrogel systems and behave differently at a molecular level. However, ligand clustering was identified as a common underlying effect in the ionically crosslinked alginates and degradable, covalently crosslinked HA hydrogels.[129] Ionically crosslinked alginates are flexible and cells could reorganize the surrounding RGD ligands bound to the matrix and thereby their focal adhesions, in the absence of changes in cell shape or matrix degradation. The stiffness that allowed maximal α_5-integrin-RGD clustering correlated with MSC osteogenic differentiation. Covalently crosslinked HA gels are more stable and matrix degradation was likely required to reach otherwise unavailable RGD ligands and generate high traction forces and osteogenic differentiation. Chaudhuri and colleagues also observed a correlation between faster relaxing gels and osteogenesis, with enhanced RGD ligand clustering and local hydrogel remodelling.[103] Baker and colleagues showed that in fibrillar microenvironments, soft fibres favoured fibre recruitment by cells, increased local ligand density, focal adhesion assembly, cell spreading and proliferation.[66] Not only ligand density and spatial distribution, but also ligand mobility determines how cells sense the ECM and adapt their shape, motility and fate.[113–115] It seems that various aspect of the physical ECM could relate back to integrin clustering associated to local ligand density and mobility.

4. The Interplay Between Growth Factor Signalling and Mechanotransduction

Cells respond to the mechanical properties of the microenvironment. This progress report has shown examples of how elastic and viscoelastic properties influence cell behaviour, including cell adhesion, cell migration and cell differentiation. For example, MSCs are committed to osteoblasts on rigid substrates (>25 kPa) but to adipocytes on soft ones (<10 kPa).[61] It is remarkable that the mechanical properties of the environment (i.e. the ECM in vivo) play a key role regardless of the biochemical 'soluble' environment: even using osteogenic media, MSCs did not follow the osteogenic lineage unless the stiffness of the substrate was above a certain threshold.[61] This seminal

work revealed how determinant the physical properties of the ECM are and suggested a certain preferential influence of physical over biochemical signalling. However, Picart and colleagues realized that the preponderance in terms of physical vs. biochemical signalling was also related to the way growth factors were presented to cells.[130] Using a model of mouse myoblast C2C12 cells they showed that on stiff LbL-based surfaces cells respond to both solid-phase BMP-2 and soluble BMP-2 in the culture media. However, on softer surfaces cells did not differentiate in the presence of soluble BMP-2 but indeed followed osteogenic commitments using solid-phase presentation of BMP-2, i.e. matrix bound BMP-2. These experiments demonstrated the subtle interplay between integrins and focal adhesions as mechanoreceptors that feel the substrate stiffness[131] and GF receptors that transduce biochemical signals. The combined effect of the stiffness of the environment and BMP-2 was also described in 3D by using gelatin based hydrogels and C2C12 commitment to osteogenic lineages.[132] In this study, authors modulated the crosslinking degree of gelatin gels and investigated the osteogenic differentiation with or without BMP-2 supplemented in the culture medium. The preferential role of stiffer substrates in promoting osteogenesis was shown with and without BMP-2 but the lack of osteogenicity in softer substrates was overcome in the presence of BMP-2.[132]

The importance of matrix stiffness in combination with the spatial distribution of GFs has been investigated using other cell types. Chang et al. showed that hepatocyte GF (HGF) has a stronger effect on endothelial cells on lower stiffness substrates, in terms of cell migration and differentiation.[133] Using LbL-based substrates of different stiffness, they demonstrated that endothelial cell adhesion, migration and proliferation were positively correlated with increasing substrate stiffness and this behaviour was further promoted by HGF. Interestingly, they showed that the effect of HGF on cell migration and proliferation was stronger on soft substrates, suggesting that HGF can profoundly influence the stiffness-dependent endothelial cell response. In addition to this, this paper demonstrated that endothelial cell function (monolayer integrity, nitric oxide production and gene expression of endothelial nitric oxide synthase) displayed a negative correlation with substrate stiffness. An improvement was observed with the addition of HGF but the effect was not strong enough to change the stiffness-dependent endothelial cell response, i.e. functionality on stiff substrates in the presence of HGF was still below functionality on softer substrates in the absence of HGF.[133]

The interplay between stiffness and growth factor signalling has also been investigated in the context of MSC differentiation, with different results depending on the GF and immobilisation technique used. Zouani et al. used surfaces of different stiffness and a BMP-2 mimetic-peptide to identify a minimal threshold of stiffness (≈3.5 kPa) below which the presence of the GF had no effect on MSC differentiation. For stiffness values above this threshold, and in combination with BMP-2, cells were committed to osteogenesis.[134] Note that osteogenic commitment in the absence of immobilized BMP-2 was only obtained on stiffer surfaces (>25 kPa). In addition to the combined effect of mechanical properties and the presentation of GFs, Banks and colleagues developed a platform for independent manipulation of mechanical properties and spatially controlled presentation of BMP-2 and PDGF using collagen-GAGs with controlled crosslinking density. Carbodiimide crosslinking coordinated with benzophenone photo-immobilization allowed orthogonal manipulation of mechanical stiffness and immobilization of GFs to a collagen-GAG biomaterial. They showed that adipose stem cells were responsive to certain combinations of substrate stiffness and PDGF densities, while a lack of correlation was found with BMP-2, which contradicts previous findings.[135] This paper highlights how the bioactivity and lineage commitment of adipose stem cells depend on a variety of biophysical and biochemical cues, whose optimal combinations are difficult to predict and deserve further investigation.[135]

The interplay between physical properties of the matrix and biochemical stimuli (including GF receptor activation and signaling) has also been demonstrated using peptide amphiphiles (PA). PA molecules assemble into supramolecular nanofibres that have been engineered to incorporate sequences that bind GFs.[136] Stupp and colleagues designed structures that promoted differential raft mobility within the cell membrane and incorporated sequences to bind BMP-2 (**Figure 7**a). Two PAs with similar charge and molecular architecture were designed with different propensities for β-sheet hydrogen bonding. Both molecules were similar in amino acid composition with the exception of valine, which has a strong preference to adopt a β-sheet secondary structure (strong PA, Figure 7a) or glycine, which prefers a random coil conformation and then reduces the degree of intermolecular hydrogen bonding (weak PA, Figure 7a).[137] Positively charged lysine residues promote the association of PA nanostructures with the negatively charged cell surface. They showed increased raft mobility in samples treated with weak β-sheet PA nanofibres due to PA molecules intercalating within the cell membrane and lipid-rich microdomains. Importantly, this work showed that these structures that maximise cell membrane mobility (weak PA) also promoted more effective osteogenic differentiation and signalling in the presence of BMP-2 (Figure 7b). Mechanisms involved the electrostatic interaction between the PA and GFs that can localize the ligand at the cell surface in proximity to the signalling receptors that reside in the lipid-rich microdomains. Furthermore, the increased mobility of the lipid rafts after treatment with PA nanofibres might increase the statistical probability of a ligand–receptor interaction. This work offers another very different example of the interplay between physical factors (i.e. membrane mobility) and the presentation of GFs from biomaterials.[137] In relation to this work, we have previously discussed that fibronectin on polymer surfaces with different mobility (a family of polyacrylates with increased length of the side chain that translated different mobility of the polymer backbone into mobility of fibronectin adsorbed onto it) promoted differential cell adhesion and differentiation.[113,114] As fibronectin contains GF binding sites, the role of sequestered GFs from the surrounding media on these surfaces with different mobility might play an additional role on the effect of mobility in combination with integrin clustering.[37]

Mechanisms by which matrix elasticity influence GF signalling are only starting to be elucidated. Ingber and colleagues showed that angiogenesis – a process that is controlled by physical interactions between cells and the ECM as well as GFs such as VEGF – is also controlled by a signalling pathway that involves the Rho inhibitor p190RhoGAP. This is

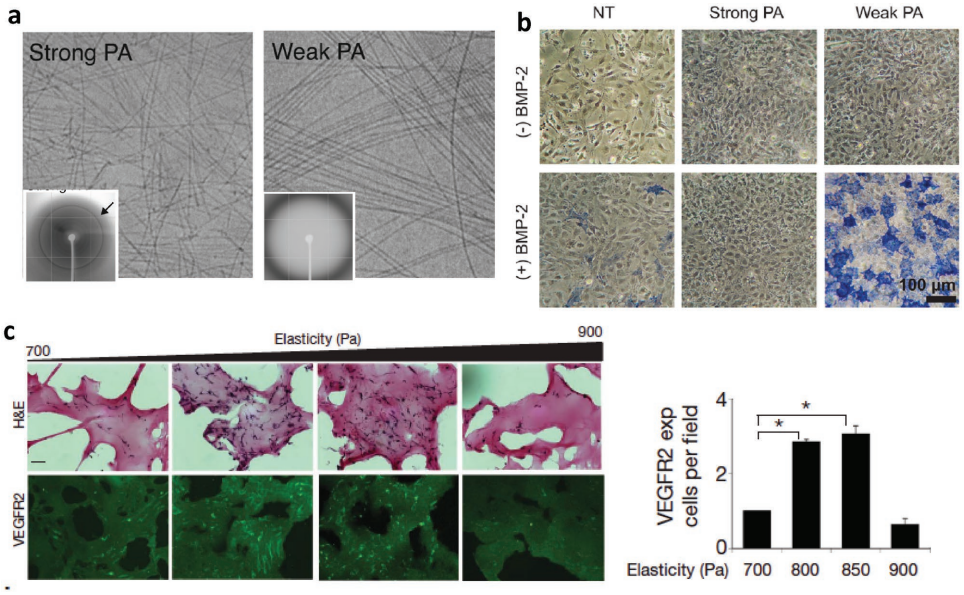

Figure 7. Combination of physical properties and growth factors. a) Peptide amphiphiles (PA) molecules assembled into supramolecular nanofibres that have been engineered to show either strong or weak intermolecular hydrogen bonding which promote lipid raft mobility differently. The inserts show wide-angle X-ray scattering from solutions of both assemblies (the arrow shows spacing for β-sheet hydrogen bonding). b) Fast blue staining to visualize ALP shows the differential effect of both PAs in the presence of BMP-2. Only weak PA and BMP-2 promotes osteogenesis. c) Matrix elasticity controls the expression of VEGFR2 in endothelial cells and then vascular development in vitro and in vivo. a,b) Reproduced with permission.[137] Copyright 2009, American Chemical Society. c) Reproduced with permission.[138] Copyright 2009, McMillan Publishers Limited.

a mechanosensitive transcriptional factor that controls VEGF receptor 2 (VEGFR2) promoter activity and expression. Importantly, this novel angiogenesis signalling pathway is sensitive to ECM elasticity as well as VEGF (Figure 7c).[138] This work suggested that an appropriate level of ECM stiffness might be required for optimal VEGFR2 expression and vascular development in vitro and in vivo. More recently, Picart and colleagues investigated mechanisms by which BMP-2 presented from surfaces is internalized by cells, and found this process to be also dependent on the stiffness of the substrate from which the GF was presented, with higher internalisation rates on soft substrates.[30] They showed that internalization is mediated by both clathrin and caveolin-dependent pathways. While inhibiting clathrin-dependent endocytosis affected only non-canonical (ALP) signalling, blocking caveolin-1-dependent endocytosis reduced both canonical and non-canonical BMP signalling. The signalling pathways found for matrix-bound BMP-2 were similar to those found for soluble BMP-2.[30]

In translational research, the complementary effect of scaffold architecture and growth factor stimulation was shown in a 30 mm critical-sized segmental bone defect in sheep tibiae.[139] It was shown that the scaffold geometry guides in vivo soft tissue formation and following mineralization.[140] The delivery of BMP complemented the guiding effect of the scaffold architecture, without altering the microstructure of the newly formed bone at different length scales.[139]

5. Conclusions and Outlook

Engineering material systems to control cell fate is at the cornerstone of a broad range of applications in regenerative medicine, cancer research and drug testing. There is strong evidence that material physical properties influence cell behaviour, including stem cell differentiation. Seminal papers have shown that stiffness, geometry, ligand spacing, time-dependent properties, such as matrix stiffening, degradability, viscoelasticity or surface mobility, and spatial patterns or gradients of physical cues can trigger specific signalling pathways. On a parallel note, growth factors have been used mainly as soluble molecules to induce particular cell responses (e.g. growth or differentiation). However, there is increasing evidence that the presentation of GFs from a solid phase, recapitulating the way this is done in the ECM, is more efficient and can trigger cell response more specifically. This has been shown not only by engineering surfaces that promote GF binding but also, and importantly, by doing protein engineering and modifying GFs with key domains that bind the ECM.

Integrins and focal adhesions are mechanosensors, which transduce physical stimuli into biochemical reactions, whereas GF receptors initiate specific GF-related signalling cascades. Basic biological experiments revealed that integrin-GF receptor crosstalk was a way to produce synergistic signalling and, thus, some efforts have been put since then to engineer material systems that target simultaneously integrins and GF receptors. This strategy might have translational consequences, as this is a way to dramatically reduce the dose of GFs to produce similar biological effects. Therefore, it has the potential to revolutionize the use of GFs in the clinic, which is currently undermined by the side effects mainly related to the high doses applied with conventional delivery systems. For example, it is well known that BMP-2 has been widely used in clinical applications, in particular in spinal fusion. A collagen sponge loaded with a high dose of BMP-2 (1.5 mg ml^{-1}) is effective in promoting

bone formation but also has involved a high number of off-target unwanted effects, including ectopic bone formation, neurological problems, difficulty in breathing, and high risk of cancer.[141] This led the U.S. Food and Drug Administration (FDA) to issue a public health notification of life-threatening complications.[142] High dose and uncontrolled delivery of GFs which end up having systemic rather than local effects are the key issues to overcome to translate GFs into safe clinical applications.

In parallel, there is increasing evidence that the combination of physical properties (e.g. stiffness) and GF presentation also plays a key role in the efficiency of the system to trigger cellular responses. It is especially important to further investigate and understand this crosstalk between physical properties (of carriers) and biochemical effects (of GFs) to design advanced systems that maximize the cellular response by using a superposition of effects. Advances in this field have shown, e.g., how osteogenesis can be achieved on soft substrates as long as cells are simultaneously stimulated with BMP-2. But there is a long road ahead to design systems that make use of synergies between relevant physical properties and GFs, seeking to reduce the dose used in clinical applications.

The future is cooperative and we envisage the development of advanced systems which will use GFs in very low and efficient doses in combination with key (dynamic) material properties. This will allow to engineer a broad range of material-based systems to support fundamental (stem) cell studies, develope devices for tissue engineering and engineer organoids to model diseases and develop treatments.

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Conflict of Interest

The authors declare no conflict of interest.

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