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Harnessing nanotopography and integrin–matrix interactions to influence stem cell fate

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Stem cells respond to nanoscale surface features, with changes in cell growth and differentiation mediated by alterations in cell adhesion. The interaction of nanotopographical features with integrin receptors in the cells' focal adhesions alters how the cells adhere to materials surfaces, and defines cell fate through changes in both cell biochemistry and cell morphology. In this Review, we discuss how cell adhesions interact with nanotopography, and we provide insight as to how materials scientists can exploit these interactions to direct stem cell fate and to understand how the behaviour of stem cells in their niche can be controlled. We expect knowledge gained from the study of cell–nanotopography interactions to accelerate the development of next-generation stem cell culture materials and implant interfaces, and to fuel discovery of stem cell therapeutics to support regenerative therapies.

The fact that cells respond to their microenvironment is perhaps obvious. Microscale features are of a comparable size to the cells themselves and typically result in whole-cell contact guidance. Contact guidance, first observed in 1911[1] and subsequently defined[2,3], describes the alignment of cells with topographical features. In contrast, at the nanoscale, where surface features are several orders of magnitude below that of the cells, the response is more complex. At this scale, where features are a similar size to individual cell receptors, it may therefore be possible to target receptor-driven pathways and to manage the response of the cells.

More specifically, the receptors involved with cellular adhesion are integrins, and these are recognized as critical communication channels through which the cells interact with adjacent surfaces. This communication is achieved outside the cell by transmembrane, heterodimeric (containing α- and β-integrin subunits) protein receptors binding to peptide ligands (for example the well-characterized and widely used arginine, glycine, aspartic acid (RGD) sequence), and within the cell by the cell's signalling machinery and cytoskeleton (Fig. 1b). It is important to note that adhesions (on the microscale) are made from many integrins (on the nanoscale) gathered together[4]. Hence, before we consider the effect of nanotopography on cells, we will first consider integrin interaction and gathering (clustering) with the resulting cell adhesion as a target for biomaterials and stem cell research. Furthermore, integrin–ligand binding results in contraction of the attached cytoskeleton through some of the biochemical pathways that will be discussed in this Review. It is this contraction that pulls integrins together into larger adhesions. The cells might be considered as a tent: the larger the tent, the larger the pegs (integrin clusters) would need to be to cope with the tension applied to the guy ropes to provide adequate integrity.

Nanoscale integrin–ligand interactions

Previous studies from the Spatz group have used nanocolloidal assembly to control the number of integrin-adhesive RGD ligands per unit area[5,6]. Using this method, a density threshold corresponding to a spacing of RGD ligands ~70 nm apart was defined[5,6]. When RGD ligands at higher densities (that is, RGD ligands packed closer than 70 nm to neighbouring RGDs) were presented to cells, the cells formed focal adhesions, and polymerization of contractile actin cytoskeletal stress fibres was observed[5]. Focal adhesions (bundles of gathered integrins) and actin have important functions in various cell-signalling pathways and subsequent cell fate (as will be discussed). When the RGD ligands were presented at a lower density (more than 70 nm separation), however, low cell adhesion was noted (smaller focal adhesions and more rounded cells)[5,6]. This low adhesion could potentially lead to cell quiescence or even apoptosis by anoikis, a type of programmed cell death through 'homelessness'[6].

A different approach considered the importance of minimal adhesion gathering by presenting the RGDs as clusters of controlled ligand numbers separated by larger spaces[7]. This provides a cell with the minimal unit size for adhesion that permits cell spreading — that is, the minimum number of RGDs required in the cluster to allow adhesions to form and cells to spread. To achieve this, a nanotopographical cluster array was made using electron-beam lithography, which is a high-resolution top-down fabrication tool creating features down to 10 nm in the x and y axes with high placement precision[8]. In this approach the technique was used to create clusters (dimers to heptamers) of RGD ligands (individual RGD units <60 nm apart to allow integrin gathering), forming adhesive islands with approximately 200-nm spacing between the clusters (to prevent integrin gathering in between the clusters). This array allowed cells to gather integrins depending on how many close RGD units were present. As a result, it was shown that a tetrameric arrangement of RGD ligands in a cluster allowed full cell spreading (Fig. 2a shows cells spreading on a heptameric arrangement), illustrating that at least four integrins need to be gathered into an adhesion to allow spreading[7].

To find a study that considers both spacing and density, we need to refer back over a decade[9]. This study examined the hypothesis that presentation of an integrin ligand in a clustered format would result in more efficient grouping of the ligand-bound integrins than would the same surface density of ligands presented individually[9]. To examine this, a non-adhesive polyethylene oxide (PEO) hydrogel was used, with the PEO molecules in a 'star' configuration and tethered clusters of one to nine YGRGD (Y = tyrosine) adhesion ligands with defined distances of 6–300 nm between clusters. The results for clustered ligands showed a significant advantage in terms of numbers of adhered cells, percentage of cells with clear stress fibres and enhanced cell motility. Clustered ligands, being RGDs that are very close together, allowed a number of integrin units to bind ligands

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in close proximity. Interestingly, even at the maximal density of 30,000 YGRGD ligands per square micrometre, cell response was observed to be significantly lower for individual YGRGDs than for ligands clustered in groups of nine with a cluster density of 2,300 YGRGD μm^{-2}. Using 30,000 individual YGRGD μm^{-2} gives a spacing of just 6 nm, whereas 2,300 YGRGD μm^{-2} in clusters of nine gives the higher average spacing of 190 nm between clusters.

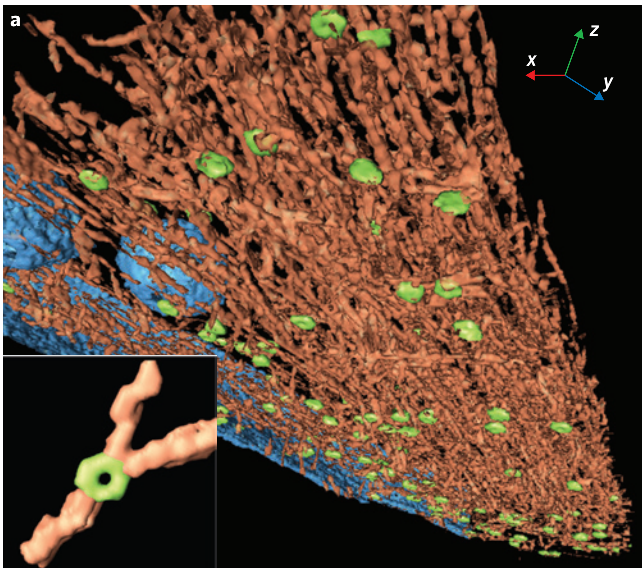
In close agreement with the previously described study[7], the smaller, five-YGRGD clusters were also seen to enhance cell adhesion significantly compared with individual YGRGDs. The data showed that critical distances were required to enable the cells to spread. Calculations indicated that a distance of 60 nm was necessary for clusters of nine, and 9 nm was needed for clusters of five, with <6 nm required for individually presented YGRGD molecules. Although these values seem very low, it was noted that the YGRGD ligand has minimum adhesion (lower integrin affinity) when compared with the higher-affinity cyclic RGD[6,7] or GRGDY used in earlier studies[10]. Thus, more YGRGDs are needed to be presented to cells to achieve the same effect as RGD or GRGDY presentation. A recent study focusing on the response of mesenchymal stem cells (MSCs) to RGD highlights this affinity effect[11]. MSCs cultured on high-affinity cyclic RGD differentiated towards osteoblasts. If cultured on low-affinity linear RGD, however, MSCs expressed myogenic markers at high ligand density and neural markers at low ligand density[11].

Going back to studying ligand density and spacing for minimal adhesive units, a further caveat to note with the low-affinity approach[9] is that the study used a compliant, soft, hydrogel-based system that differed from the rigid surfaces to which the RGD/GRGDY was tethered in previous studies. It is known that stiffness is important in adhesion formation, with cells forming larger adhesions on more rigid surfaces; this provides the cell migration from soft to hard substrates observed in deurotaxis[12]. Recently, it has been shown that cell adhesions are differentially regulated on stiffness gradients compared with adhesions on chemotactic (free chemical gradient, for example changing density of soluble factors) and haptotactic (fixed chemical gradient, for example attached to a surface) gradients, and also compared with maturing adhesions[13]. Furthermore, it is known that stiffness can be used to tune MSC fate[14,15] and that MSCs interpret changes in stiffness as changes in adhesive ligand presentation[16].

Direct comparisons between the RGD presentation systems are challenging as a result of diverse experimental conditions, for example alterations in concentration, spacing or rigidity of the surface, and adhesive affinity of the ligand. A study that systematically varies density and spacing (for example, increasing clusters of RGD units, or increasing their separation) and RGD type (for example, linear, cyclic, placement in peptide group) on a stiff matrix is needed to determine the definitive rules for integrin interactions. It is clear, however, that nanoscale presentation of integrin ligands is critical for adhesion and subsequent cell response. Furthermore, the above studies indicate that surface structuring combined with chemistry provides a powerful tool for studying adhesion at the nanoscale.

Nanoscale structure of cell adhesions

The adhesion of cells to a nanotopographical surface involves nanoscale adhesion-localized structures. These include adhesion-related particles that are reported to change in response to integrin clustering. Adhesion-related particles (doughnut-shaped structures of 25±5 nm diameter) that are located at the cell membrane become aggregated when integrin clustering occurs. This clustering results in the formation of focal adhesions with an average integrin interspacing of about 45 nm. Adhesion-related particles are linked to the actin cytoskeleton and, as a result, chemical inhibition of actin contraction causes a decrease in particle size,



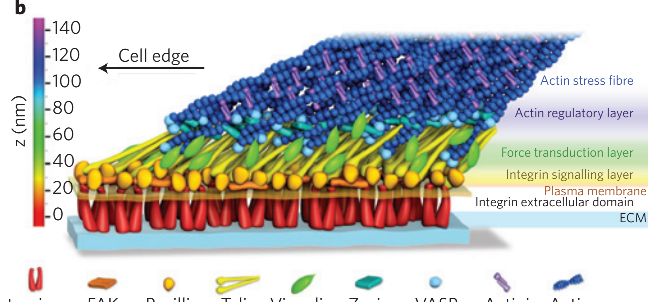


Figure 1 | The nanoscale structure of focal adhesions, and nanoscale connectivity to the cytoskeleton. a, Adhesion-related particles (green) at sites of focal adhesions. These particles can fit between gathered integrins and link nanoscale actin to microscale stress fibres. The inset shows a close-up of a particle. **b**, The nanoscale structure of a focal adhesion showing the integrin/extracellular binding domain, integrin signalling layer, force transduction layer and actin regulatory layer. Figures reproduced with permission from: **a**, ref. 17, 2010 NPG; **b**, ref. 18, 2010 NPG.

suggesting mechanosensitivity of the particles. The relationship of these particles to adhesion is also demonstrated by their increased density at focal adhesion sites: they are found outside focal adhesion sites, but their density there is >20 times lower. It has been hypothesized that the focal-adhesion-related particles are located at the cytoplasmic face of liganded integrins and associate with integrins at the nanoscale. That is, the nanoscale adhesion-related particles are accommodated within the inter-integrin spacing after gathering[17]. The focal-adhesion-related particles then link individual, unbundled, actin fibres to the main contractile (bundled) stress fibres. This allows conversion from nanoscale cell receptor/mechanosensor to microscale signal transducer[17] (Fig. 1a).

When it comes to the anatomy of the adhesions themselves, the signalling and mechanosensory parts of the adhesions are organized in a nanoscale manner. Integrins and actin are separated by a 40-nm-high focal adhesion core-region consisting of strata with specific roles. These strata include, as illustrated in Fig. 1b, (1) a signalling layer consisting of the cytoplasmic tails of the integrins, focal adhesion kinase (FAK) and paxillin (signalling proteins relaying integrin engagement to initiate biochemical signal cascades); (2) an intermediate stratum involved in force transduction containing talin and vinculin; and (3) an actin-regulatory surface containing

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vasodilator-stimulated phosphoprotein (VASP), zyxin and α-actinin. Zyxin and VASP interact to allow actin polymerization and α-actinin to crosslink the growing polymer chains[18] (Fig. 1b).

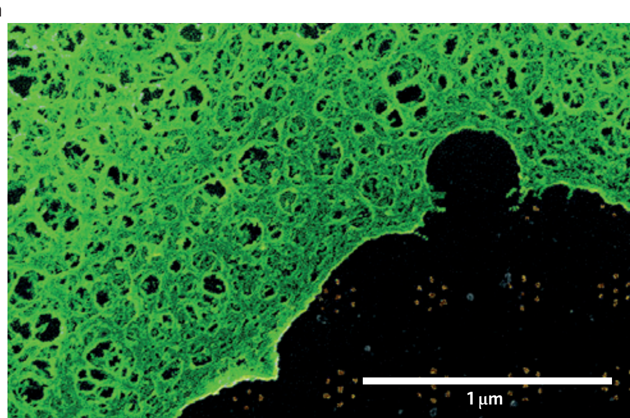
Adhesion guided by nanotopography

Although cells are several orders of magnitude larger than their surrounding nanoscale topographical features, unbundled actin-fibril-driven filopodia allow the cells to probe the surface. Filopodia are fine, integrin-containing, cell membrane projections with a tip diameter on the nanoscale. These cellular projections can experience contact guidance from features just 10 nm in height[19]. We note that filopodia are sometimes referred to as microspikes because of their micrometre-scale length and spiky appearance. Thus, when nanoscale grooves are used to contact guide cells, filopodia are the initial responders aligning along the grooves. Adhesions are subsequently orientated in the groove direction, resulting in cytoskeleton alignment and cell rearrangement along the nanoscale guidance cue[20]. The threshold at which substrate nanogroove dimensions may influence filopodial guidance and subsequent whole-cell guidance (that is, the whole cell body will reorientate and align) seems to be around 35 nm in height[21].

We have recently generated data on the exquisite sensitivity and limits of filopodia sensing. Using a through-mask anodization technique to pattern titania, resulting in features of 8-nm height, we observed a significant change in cell interaction with the nanoscale features[22]. The cells seemed to use filopodia far less than expected, instead using discrete nanolength projections, 'nanopodia' (Fig. 2b). As a result, it would seem, as with changes from submicrometre-scale to nanoscale, that cells can alter their guidance mechanisms at the sub-10-nm range. We might say that the cells are 'hanging on with their fingertips' as they garner the last possible pieces of nanotopographical information from the materials surface. Considering that the diameter of the 8-nm-high features is ~25 nm, coupled with the facts that the extracellular portion of the integrin receptors are ~23 nm wide and that the final 5 nm of the integrin α- and β-chains is the component responsible for ligand binding, it seems that the small features and the receptors are topographical 'reflections' of each other. This is intriguing, as it provides tentative evidence of cell probing at the single-integrin scale (although we note that no other surface modification, for example RGD tethering, was used).

Nanotopography (in the form of grooves) has recently been used to demonstrate that the process of cell orientation requires the downregulation of adhesion scaffolding proteins, such as receptor for activated C kinase 1 (RACK1), that are important in holding large cell adhesions together. Downregulating adhesion scaffolding proteins enables adhesion disassembly and the formation of smaller adhesions with rapid turnover[23], resulting in increased cell motility. Evidence for such a mechanism is provided by the observation of downregulation of α_2-, α_6-, α_V-, β_2-, β_3- and β_4-integrin subunits as cells (more specifically, MSCs) align, with a concomitant reduction in cell stiffness indicating cytoskeletal reorganization[24]. Investigations have demonstrated the direct linkage between nanospatial integrin clustering and cystoskeletal assembly, and thus cell morphology[25]. In a phenomenon termed 'nanoimprinting', spatially defined surface nanotopography is correlated to similar shapes appearing in the cytoskeleton. Studies using antibodies to block integrin binding have shown that the blockade of the β_3-subunit reverses the imprinting effect[25,26].

a



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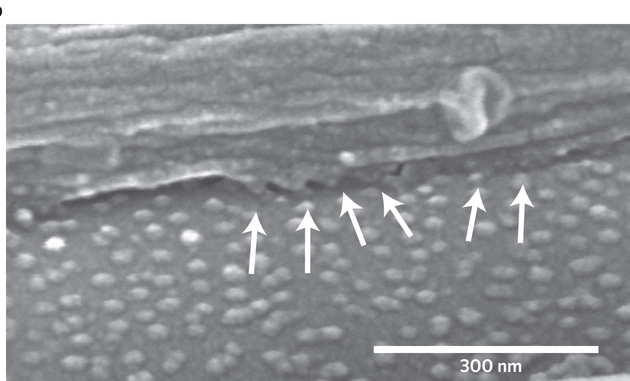

Figure 2 | Interaction of cells with RGD groups and nanoscale topography. **a**, Cell spreading on adhesive clusters spaced 200 nm apart formed from individual RGDs <60 nm apart in a heptomeric arrangement. **b**, Topographical 'sensing' below 10 nm. When cells interact with features of 8 nm in height, filopodial interaction is reduced. Close examination shows that small membrane projections, termed 'nanopodia' (arrows)[22], are used to interact with the 8-nm islands. Figures reproduced with permission from: **a**, ref. 7, © 2011 ACS; **b**, courtesy of L. E. McNamara, Univ. of Glasgow.

Nanotopographical control of stem cell phenotype

The assembly/disassembly of adhesions related to cell culture on nanotopography has been linked to control of cell phenotype, most notably with human MSC differentiation. This result was driven by research with surfaces fabricated by electron-beam lithography, coupled with a desire to improve orthopaedic materials — more specifically, to drive MSC osteogenesis, or bone formation, rather than allow implant encapsulation with soft tissue, as often happens with implant surfaces.

For some years, lithographic studies that investigated cell interactions with nanoscale features focused on highly ordered patterns, as this suited the primary purpose of electron-beam lithography: microelectronic design with precision fabrication of, for example, transistors. But nanotopographical patterns with precise order typically resulted in lower cell adhesion[27,28]. A different school of thought worked in parallel, attempting to use nanoscale roughness (randomness created by, for example, polishing, etching, blasting and anodizing) as a way of increasing cell adhesion and response to implant materials[29]. Evidence in the literature, however, remains conflicted in terms of the intrinsic properties of random submicrometre and nanometre topographies, with positive[30] and negative[31–33] reports on *in vitro* adhesion and *in vivo* implant integration.

In 2007, we reported a third approach, in essence a middle route, that proposed the importance of controlled nanodisorder (that is, not highly ordered, but not random), in inducing rapid osteogenesis from skeletal stem cells, commonly referred to as MSCs. The result was unequivocal and demonstrated that patterns could be used to control MSC osteogenesis with similar efficiency to chemical stimulation[34] (Fig. 3a). This observation was followed by complementary reports demonstrating that feature diameters of 100 nm are optimal for osteogenesis with disordered surfaces[35]; this is close to the 120-nm diameter used in our study.

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To help to understand adhesion-related mechanisms, examination of reports on stable cell lines or terminally differentiated cell types is informative[36,37]. Examination of focal adhesions in fibroblasts on highly ordered surfaces demonstrated that the adhesions could not form directly over the features (for example nanoscale pits in square or hexagonal patterns). Instead, adhesions were forced to form within the reduced areas between pits, as shown by immuno-gold-labelled vinculin in adhesions using scanning[37] and transmission electron microscopy[36], in a process akin to nanoscale contact guidance (that is, the cell adhesions disassemble). This was not the case with the disordered patterns where large, super-mature (>5 μm long) adhesions could form[34].

To put these findings in context, it is interesting to note that similar effects were observed in other reports[15,16,38,39]. These studies focused on altering matrix rigidity or controlling cell size by printing adhesive (RGD-containing fibronectin) shapes. They described rigid surfaces and adhesion-promoting fibronectin microscale shapes as osteogenic promoters driving differentiation of MSCs towards osteoblasts. Particularly revealing in this context are reports detailing printing of small (cell-confining) and large (promoting cell spreading) fibronectin shapes. It was shown that if MSCs were morphologically constrained then smaller adhesions resulted and adipogenesis (fat-cell formation) was noted[38]. If MSCs were encouraged to spread, however, larger adhesions were observed and osteogenesis was promoted[38,39] (these concepts will be explored in more detail later). The commonality with the disordered nanoscale topography is that all the surfaces seem to induce adhesion assembly as a critical step towards osteogenesis.

It is tempting to draw biomimetic parallels to this topographical, disorder-driven phenomenon through comparison to type X collagen structure, found at sites of endochondral ossification and large fractures. Endochrondral ossification is the process by which the embryonic cartilaginous model of most bones contributes to longitudinal growth and is gradually replaced by bone. Type X collagen contains a disordered (although not random) hexagonal morphology on a similar length/disorder scale to the disordered square-arranged pits used above[40,41] (Fig. 4a). Furthermore, we have recently shown, using human embryonic stem cells, that a disordered surface topography is capable of influencing the stem cells to differentiate along the mesodermal lineage towards a stromal osteoblast phenotype[42]. This speculatively indicates a role for nanoscale microarchitecture or environment in development.

At the nanoscale, an integrin-related explanation for the role of disorder can potentially be extrapolated from data in the literature, albeit in an inverse manner. Data have been published showing that if a geometrical array had individual RGD ligands presented at a density just over 70 nm apart, MSCs failed to gather individual integrins into mature adhesions[43]. When disorder was increased and the average centre-to-centre distance was kept constant, however, large areas where integrins could cluster together became available to the cells, enabling adhesion formation and cytoskeletal contraction[43]. This concept is illustrated in Fig. 3b whereby disorder places some RGD ligands in a denser arrangement and others in a less dense arrangement. When using nanotopographies with disordered pit arrays, we postulate the reverse, where pits represent low-adhesion features (as opposed to high-adhesion RGD ligands) and the inter-pit spaces represent higher-adhesion areas. If this is the case, it would explain how larger adhesions are a prerequisite for osteogenesis[44]. This is interesting as it may indicate a critical seed size of adhesion for maturation and also indicate that 'seed areas' are more of an adhesion stimulant than wide open, planar surfaces.

Supporting evidence for our hypothesis can be found in the literature, where studies have used nanocolloidal-mask patterning to create controlled cell-adhesive areas (for example, by presenting fibronectin or RGD) surrounded by areas of low adhesion (by using polyethylene glycol, to which cells fail to adhere). One study from the Sutherland group considers a different phenomenon from integrin gathering, namely adhesion bridging. Bridging determines adhesion-size limits before joining with another adhesion to make a larger adhesion. Results demonstrated that with circles of fibronectin (which predominantly uses the $\alpha_5\beta_1$-integrin pair in mesenchymal-lineage cells) and a polyethylene glycol background,

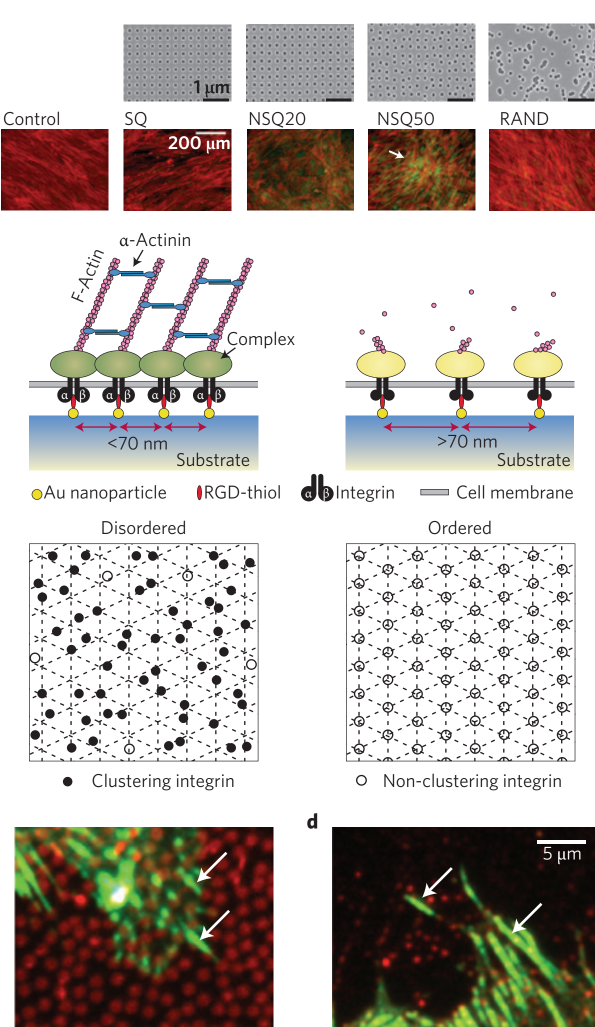

Figure 3 | Nanoscale disorder and adhesion bridging. a, Electron-beam lithography was used to demonstrate that neither order nor randomness successfully led to osteoinduction of MSCs. SQ, square (within the square array the individual pits are 120 nm in diameter, 100-nm deep and have a 300-nm centre–centre spacing); RAND, random. However, controlled disorder (NSQ20 and NSQ50, same as SQ but with ±20 nm and ±50 nm offset from the 300 nm centre–centre position) produced abundant, spontaneous, osteogenesis in basal media. Cells are shown in red (actin) and osteogenesis is shown in green (osteopontin). **b**, At the adhesion level, adding a level of disorder to RGDs placed 70 nm apart allowed much greater integrin clustering in MSCs. Bottom panels show an ordered lattice (right) with RGDs placed >70 nm apart with little integrin clustering possible (open circles). However, if a level of disorder (left) is added while there are areas with gaps where adhesion does not occur, more RGDs are moved within gathering distance (closed circles). **c**,**d**, Fluorescence microscopy images showing 800-nm-diameter fibronectin circles (red, **c**) and 200-nm-diameter vitronectin circles (red, **d**) with adhesions (vinculin in green) seen bridging between the circles (arrows). Figures reproduced with permission from: **a**, ref. 34, 2007 NPG; **b**, ref. 43 © 2009 ACS; **c**,**d**, ref. 46, © 2011 ACS.

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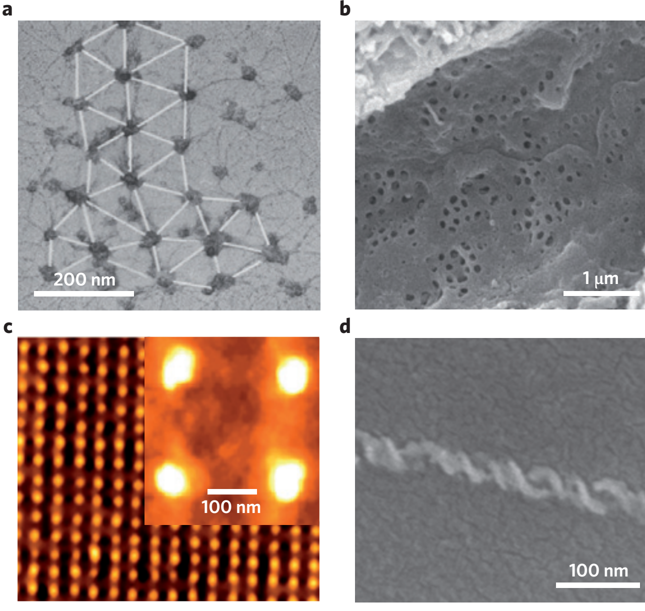

Figure 4 | Natural and synthetic nanopatterns that offer templates for MSC differentiation and self-renewal. a,**b**, Natural. Type X collagen (**a**) and fenestrated sinusoidal capillaries (**b**). **c**,**d**, Synthetic. Dip-pen patterned nanoislands (70-nm diameter with 28-nm centre–centre spacing) that retain MSC multipotency (**c**) and self-assembled helical amphiphile with 63-nm periodicity that stimulates MSC osteogenesis (**d**). Figures reproduced with permission: **a**, ref. 40, © 1991 Rockefeller Univ. Press; **b**, courtesy of R. Fraser and A. Warren, Univ. Sydney; **c**, ref. 51, © 2010 RSC; **d**, ref. 48, © 2013 ACS.

circle diameters larger than 500 nm allowed normal cell spreading[45] by means of adhesion bridging[46]. Critically, it was observed that integrins did not bridge, but rather the structural proteins vinculin and zyxin spanned the gaps. If vitronectin was used instead (RGD-containing but predominantly using the $\alpha_v\beta_3$-integrin receptor), the adhesions could span the gaps when seed points with a 200-nm diameter were used[46] (Fig. 3d).

Moving back a step, although we have discussed the need for large, super-mature adhesions to stabilize intracellular tension in osteogenesis, it seems that an excess of adhesions per se is not required[44,47]. Disordered surfaces create both areas where large adhesions can form through increased integrin gathering (potential 'seeding') and areas where it is harder for adhesions to develop. We postulate that this allows for the formation of fewer but larger adhesions in osteoblasts than in, for example, fibroblasts, which typically contain numerous, smaller, adhesions.

A recent observation of a further nature-inspired nanotopographical parallel is that nanoscale banding (periodicity) is important in MSC differentiation. For example, helical self-assembling amphiphiles with a 63-nm periodicity, or banding pattern, have been shown to promote osteogenic differentiation of MSCs (Fig. 4d). This periodicity is very close to type I collagen (the main collagen found in bone), which displays a banding periodicity of ~67 nm. Interestingly, if the periodicity was increased to a less biomimetic 100 nm (significantly different from collagen) the osteoinductive effect decreased[48].

If we reconsider precise geometric patterns, our recent findings have demonstrated nanotopographically controlled human MSC phenotype retention — self-renewal without loss of phenotype — on highly ordered nanostructures. These surfaces and patterns reduce, but do not completely inhibit, MSC adhesion[49,50]. This is important, as MSCs rapidly and spontaneously differentiate *in vitro* on traditional culture plastic surfaces, limiting scaling up of appropriate high-quality stem and progenitor populations for clinical evaluation and application[50]. A striking parallel can be found in the literature on surface-chemistry-defined control of MSC multipotency, where dip-pen nanolithography was used to print methyl-terminated groups in a highly ordered, square array of features approximately 70 nm in diameter with a centre–centre spacing of 280 nm (ref. 51). This is remarkably close in size and spacing to our aforementioned topographical square array using 120-nm-diameter pits with 300-nm centre–centre spacing. It is further worth noting that the islands fabricated by dip-pen nanolithography will have topography (height) (Fig. 4c). Dip-pen nanolithography uses atomic force microscopy technology, with cantilevers 'dipped', or loaded, with the chemical group or protein that is desired to be patterned at nanoscale resolution.

The observation that a reduction in adhesion is required to achieve retained MSC multipotency[49] allows us to postulate that to limit cell differentiation, cell adhesions need to be reduced to a size that is permissive for cell proliferation as opposed to a level where the cells become rounded and quiescent (for example adipocytes). Thus, adhesions need to be small enough to repress key metabolic and biochemical pathways below a critical threshold required for active differentiation[49].

Such control of adhesion is comparable to adult stem cells in their natural niches, where the cells are observed to be slow-growing and metabolically quiescent[52–56]. Stem cell niches are formed from cellular and non-cellular environments surrounding the stem cells and are likely to comprise both chemical (soluble) and physical (non-soluble or topographical) features of micro- and nanoscale proportions that help to regulate the stem cells through cell–matrix and cell–cell interactions. Thus, a stem cell niche holds distinct regions of quiescent and self-renewing populations. Quiescence affords protection against DNA damage when the stem cells are not required. This is in contrast to maintenance and expansion of stem cell numbers (self-renewal) and the capacity to respond to tissue-regenerative need through differentiation. It is thought that stem cells respond to tissue demand by producing progenitor (or 'transit amplifying') cells, which rapidly proliferate before homing to the area of need for regeneration and (terminally) committing to a functional phenotype.

Within the bone marrow, MSCs typically reside on fenestrated sinusoidal capillaries (perivascular niche). Critically, the endothelial fenestrations of the cells of the sinusoid walls are nanoholes typically 100 nm in diameter (Fig. 4b), indicating that MSCs may naturally reside on 100-nm pitted features that are very similar to those on our nanotopographies. The sinusoidal 'home' for the skeletal MSCs is further suggestive of their potential perivascular origin and of the importance of elucidating the pericyte lineage of MSCs[57].

Nanotopography has also been used to enable retention of embryonic stem cell phenotype. In this case, highly ordered surfaces were produced using colloidal crystal microspheres of 120-nm diameter in a highly organized arrangement[58] to maintain phenotype, whereas a more random arrangement was presented to induce differentiation (nano-roughness produced by reactive ion etch)[59]. This is confirmed by an investigation of the capacity of human embryonic stem cells cultured on disordered nanotopographical substrates to express higher levels of stromal markers than are detected in cells on planar substrates[42].

Furthermore, it has been further demonstrated that nanoscale lattice arrangements had strong effects on embryonic stem cell responsiveness to basic fibroblast growth factor (bFGF)[60]. In serum-free culture, human embryonic stem cells require bFGF to maintain expression of Oct4 (octamer-binding transcription factor 4), a transcription factor required for pluripotency. The authors observed that when the embryonic stem cells were cultured on a nanoscale hexagonal lattice, Oct4 expression was maintained without bFGF.

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On a honeycomb lattice, however, Oct4 expression was reduced, and it was postulated that disruption of adhesion is required to maintain embryonic self-renewal[60]. It is noteworthy that hexagonal patterns have been shown to reduce fibroblast adhesion[61] and that this result concurs with the above discussion on MSC self-renewal. In further agreement, a recent study with embryonic stem cells shows that there were changes in patterns of adhesion formation in self-renewing ($Oct4^{+}$) and differentiating ($Oct4^{-}$) states[59]. This is suggestive that nanotopographical tools for embryonic stem cell growth and differentiation may prove to be as important as observed for MSC studies and, interestingly, is illustrative of possible commonalities of mechanism in renewal and differentiation status. Although the potential for embryonic control is beyond the remit of this Review, a recent article[62] provides further detail. Note that transcription factors are proteins required to aid in transcription of genes in the production of messenger RNA. Function-specific transcription factors help to regulate growth and differentiation, for example Oct4 in embryonic stem cell growth.

Biochemical adhesion-related signalling

Focal adhesions have been discussed as the initiation point of cell–surface interaction. The use of physical cues such as topography and rigidity to control adhesion will alter biochemical signalling, which will in turn affect cell phenotype; this conversion of biochemical signals derived from changes in intracellular tension to phenotypical effect can be described as indirect mechanotransduction (mechanotransduction referring to how cells process mechanical information from change of shape into response). But although functional groups can be tethered to surfaces to control hydrophobicity, for example, such alterations will affect material–protein interactions and thus in turn influence cell adhesion and ultimately phenotype in a rational manner[51,63–65]. It is well known that stiffer surfaces will promote larger adhesions than softer surfaces and, again, govern phenotype in a rational manner[15]. But the rules are less straightforward for nanotopography.

Understanding of nanotopographical control of adhesion and subsequent cell response has been approached in a somewhat haphazard manner, and it has been difficult to predict discrete nanotopographies that will define cell spreading. As a result of recent advances in nanotopography research, however, there are some emerging guidelines for cell morphological changes initiated by nanotopography: first, very small nanoisland shapes (raised features above 10 nm but below 20 nm high) increase cell adhesion size and spreading (of, for example, endothelia[66], fibroblasts[67] and MSCs[68,69]); and second, larger nanoscale features (approximately 100 nm high) typically inhibit cell spreading, cytoskeletal organization and functional differentiation[66–69]. Furthermore, a recent report indicates that these guides could be similar for depth as well as height. Shallow pits of 14 and 29 nm depth increased osteoblast attachment, spreading, selective integrin subunit expression and active, phosphorylated FAK expression when compared with deeper features (45-nm depth)[70]. If our disordered, osteogenic surface described previously is fabricated using 15-nm-high pillars rather than the original 100-nm-deep pits, the raised topography confers further enhanced osteogenic potential[71], complying with both the aforementioned guidelines. In this section, we will strive to reflect the current state of understanding on cell-adhesion-mediated signalling specifically with respect to nanotopography.

It is self-evident that cell adhesion plays a number of roles in discrete cell functions, and it is further clear that adhesion-derived tension is critical for processes, including cell survival[72] and MSC differentiation[15,38,39]. If we consider that surfaces that reduce MSC adhesion and spreading have been implicated in soft-tissue (fat/adipose, nerve/neural) phenotypes and, conversely, surfaces that induce adhesion and spreading have been implicated in hard-tissue (bone/osteoblast) phenotypes, we start to understand that function may follow form, in line with theories on tissue architecture and cell function[73]. In other words, if we manipulate cell morphology via adhesions to encourage an MSC to resemble an adipocyte (round) or an osteoblast (polygonal), the cells will differentiate, or change, to that particular cell lineage. The question then becomes, how do adhesions control cell behaviour?

Nanotopography provides a non-invasive tool to examine this point further. As has been noted, embedded in focal adhesions is FAK, a tyrosine kinase. FAK distribution has been shown to be responsive to different nanoscale topographies in terms of morphology. It appears as punctate dots on nanoislands and also aligns along (and is differentially regulated by) nanogrooves. Furthermore, phosphorylated, active, FAK has been observed to be up- or down-regulated by different heights of nanoislands as cells are guided along aligned extracellular matrix[70,74,75]. Another signalling mediator that is linked to FAK, Src (also a tyrosine kinase), is believed to be important in contact guidance of cells, and its inhibition results in increased sensitivity to micro-[76] or nanogrooves[23].

Both FAK and Src act to regulate G-proteins involved in filopodia (Cdc42, nanoscale sensing), lamellipodia (Rac, cell spreading) and contraction (Rho, stress fibre formation), all targeting actin microfilaments. Of particular interest, bringing together microcontact-printed MSC confinement and MSC response to stiffness, has been the study of Rho A kinase (ROCK). These studies showed the contraction of stress fibres through Rho-driven myosin activation and thus mediation of intracellular tension[15,38,39,77] (Fig. 5). With topography, the importance of ROCK/Rho in nanotopographical guidance has been illustrated through the inhibition of Cdc42 and Rac that failed to significantly alter alignment of cells to 130-nm-deep nanogrooves. Inhibition of Rho was, however, shown to prevent cell alignment[78]. Moreover, Rho and ROCK have been highlighted as differentially regulated in microarray-based experiments testing MSC response to osteogenic nanotopographies[68]. Furthermore, ROCK inhibition has been shown to inhibit MSC growth and differentiation on nanotopographies[49,50].

At this point, it is tempting to link some of these adhesion-driven responses to evidence that FAK can shuttle from focal adhesions to operate within the nucleus, where it targets ubiquitination (degradation) of the cell-cycle mediator p53 (tumour protein 53) and can act as a transcription co-regulator with the GATA4 zinc-finger transcription factor linked to embryogenesis[79–81]; that is, FAK can have a direct role in gene regulation. But it remains speculative to make the link between materials-driven adhesion regulation and roles for FAK within the nucleus.

Rho has also been linked in nanoscale mechanical (vibration) stimulation of MSC osteogenesis[82]. In this study, cells were 'nanokicked' using rapid expansion/contraction of piezoactive ceramics attached to culture plates. A vertical excursion of 15 nm was noted to promote MSC osteogenesis efficiently. This is a striking parallel to the emerging topographical guidelines outlined earlier and further suggests 15 nm as a 'unit' that stimulates cells.

Adhesions and niche regulation

Following the challenge of designing a cohesive approach to nanotopographical research, we now illustrate a unique advantage in adopting a topographical perspective. Nanotopography alone can be used to control both targeted differentiation of MSCs to osteoblasts[34] and bone-stem-cell self-renewal[50]. In each case, the desired objective can be achieved without changing media formulation (cells are cultured in the same conditions), surface chemistry (the nanotopographies have identical contact angles) or material mechanical properties: that is, paired nanotopographical control surfaces exist for self-renewal and targeted differentiation. Thus, a system is present that affords the most artefact-free *in vitro* approach

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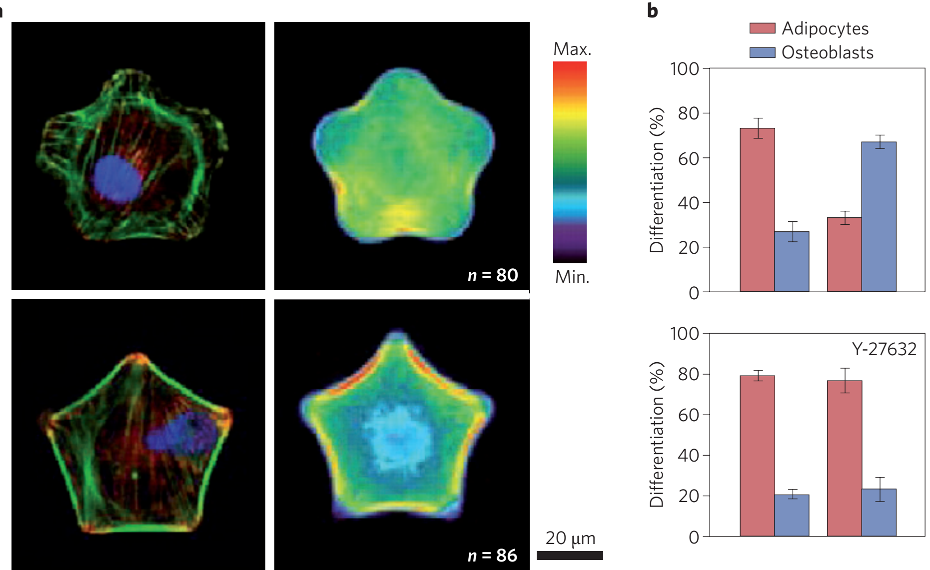

Figure 5 | Cytoskeletal contraction and intracellular tension is important for MSC differentiation. Fibronectin 'flowers' (soft curves) and stars (sharp corners) of otherwise similar dimensions have been used to produce low-tension (flowers) and high-tension (stars) states in MSCs as shown by myosin heat maps. **a**, The left-hand images show cytoskeleton of MSCs on a flower and a star; the right-hand images show low tension in MSCs on flowers and high tension in MSCs on stars (red, high tension; black, no tension). **b**, Top: On the low-tension flowers, mainly adipocytes are formed (left); on the high-tension stars, mainly osteoblasts are formed (right). Bottom: If a Rho A kinase (ROCK; controls actin/myosin contraction and thus intracellular tension) inhibitor, Y-27632, is added to the medium, however, cells cultured on the stars also form adipocytes (right). Figure reproduced with permission from ref. 39, © 2010 NAS.

to understanding MSC behaviour. This section will consider how such controls could be applied to start understanding MSC regulation in more detail.

In a recent report, use of these controls has permitted the study of MSC adhesions and phenotypical response to both the self-renewing and osteogenic nanotopographies. This allows the study of multipotency, differentiation and de-differentiation[49] (or return to a stem cell state after initial differentiation commitment). Stem cell niches are located throughout our bodies in tissues that require constant renewal or that have high regenerative demand, for example in intestinal crypts, hair follicle bulges in the skin, and bone marrow (Fig. 6a).

The use of a nanotopographical system has allowed confirmation that differentiation from the stem cells' multipotent state, and de-differentiation back, can be controlled by cell adhesion. In this context we are not using the term de-differentiation to describe cellular reprogramming, but rather to describe the ability to start to commit MSCs to cells that no longer express MSC markers but express functional markers and subsequently recover MSC-marker expression over a short time course. Adhesion has been hypothesized to be important in the cell niche[54], helping to control self-renewal and migration of cells from/to the niche through effects on cell-cycle control of cell growth (refer back to the potential direct effects of FAK shuttling on cell cycle). This adhesion-related control is possible as most MSCs in the bone marrow rely on extracellular interactions. Although we largely resort to the use of 'free' MSCs in suspension in the marrow harvested by aspiration for research purposes (note the vanishingly small numbers of free MSCs therein), the majority of MSCs adhere to the endosteal niche (bone-lining surface) among the osteoblast population or are located in a perivascular niche on fine sinusoidal capillaries, as has been described.

As well as regulating adhesion size to control growth and differentiation, the nanotopographical system provides evidence of biochemical regulation of indirect targets of adhesion-related signalling in plasticity through ERK 1/2 and c-Jun N-terminal kinase (Jnk). The involvement in signalling of ERK and Jnk is perhaps not unexpected as both are mitogen-activated protein kinases with defined roles in cell growth and differentiation[39,83–85]. The nanotopographical approach reflects these roles by implying that integrin-regulated signalling through ERK 1/2 and Jnk is important in differential regulation of PRC1 (protein regulation of cytokinesis 1), Aurora A kinase (AURKA), S-phase kinase-associated protein 2 (SKP2), serine/threonine-related kinase (WEE1), targeting protein for Xklp2 (TPX2), chromosome-associated kinase 4A (KIF4A) and serine/ threonine polo-like kinase 1 (PLK1), which are involved in cell-cycle progression[86–89]. The study further implicated the importance for biological-molecule (metabolite) signalling in MSC growth and differentiation[49] (Fig. 6b). A role for biological molecules is implicit, given that carbohydrate, lipid and nucleotide metabolism are all involved in energy production. Differentiation requires a switch from a slow-growth, metabolically quiescent state to a high-activity state where energy would be required (carbohydrate, lipid, nucleotide) and also protein demand would be increased to help the more functional cells produce extracellular new matrix (effecting tissue repair) during regeneration.

Thus, nanotopography has been exploited to confirm the likelihood of focal adhesions being central to niche regulation of MSC growth. Furthermore, topography has been used to indicate that adhesion-related signalling may act to reduce unwanted differentiation while targeting growth-related signalling to allow cell turnover and proliferation. This potentially suggests a more complex, environmental control for adult stem cell regulation as compared with the well-established Oct4, SOX2 (sex-determining region Y-Box 2) and Nanog regulation of embryonic stem cell pluripotency. Excitingly, nanotopography may prove key to dissecting niche regulation of stem cells through this kind of *in vitro*, reductionist approach.

Direct adhesion-related signalling

There is a wealth of evidence that focal adhesions and the cytoskeleton are linked to nanotopography-related (and other materials-related) signal-transduction events. As discussed above, evidence

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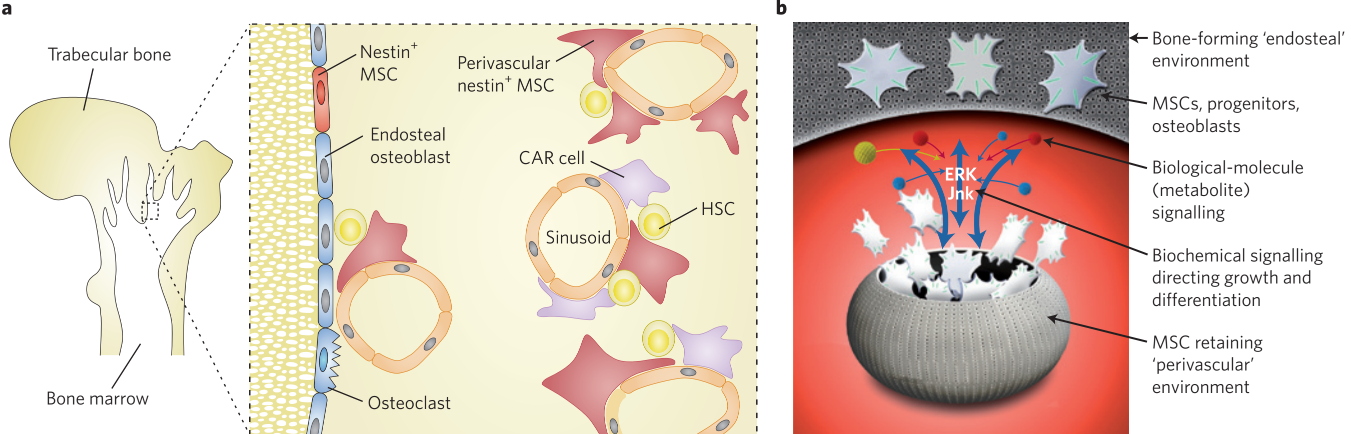

Figure 6 | Image showing marrow-niche architecture and use of paired nanotopographical controls to aid study of MSC growth and differentiation, and to aid understanding of niche regulation. a, MSC niche in the bone marrow with MSCs residing on the endosteal (bone) and sinusoidal (capillary) surfaces and interacting with haematopoietic stem cells (HSCs) and CXCL12 abundant reticular (CAR) cells. **b**, Differentiation (bottom to top) and de-differentiation (top to bottom) from the multipotency-retaining 'nanotopographical niche' (bottom) to osteodifferentiation-programming nanotopography (top) is controlled by ERK, Jnk and small molecules such as lysine, serine and oleic acid. The image illustrates the MSC niche in the marrow (the sinusoidal capillaries) and the bony, endosteal surface where the cells differentiate to form functional bone tissue during regeneration and remodelling. Panel **a** reproduced with permission from ref. 55, © 2011 Rockefeller Univ. Press.

has also been found that change in adhesion and cytoskeletal morphology are necessary for alterations in biochemical (indirect) mechanotransduction. It is likely, however, that such changes will be key to direct mechanotransductive processes that may also be important in the control of phenotype. Direct mechanotransduction describes the cell as a mechanical rather than biochemical unit. Here, changes in spreading and cytoskeletal tension will alter the shape of the nucleus, chromosomal arrangement and gene transcription. Hence, changes in cell mechanics directly affect phenotype.

Ingber's cellular tensegrity theory (Fig. 7b) proposes explanations for a wide range of cellular properties[90–93]. Cellular tensegrity describes how the material characteristics of the individual components of the cytoskeleton are such that mechanical signals could be transduced from adhesions to the nucleus (resulting in distortion of the nucleus) by alterations in cytoskeletal tension. Thus the cytoskeleton is important as it provides the cytoplasmic inhomogeneity that is required for long-distance force propagation[94].

If we therefore consider the nucleus as being connected to the cytoskeleton, this potentially becomes very interesting, as adhesion-driven alterations in cell shape and consequent reorganization of the cytoskeleton could rapidly affect nuclear morphology. This change in nuclear morphology could have effects on the genome as the interphase chromosomes are anchored to the nuclear lamins at matrix-attachment regions[95]. Lamins are intermediate filaments that form the nucleoskeleton and are connected to the cytoskeleton through linkers of nucleoskeleton and cytoskeleton (LINC) complexes[96,97]. Chromosomal organization is hierarchical, with chromosome territories[98] occupying largely distinct sites in the nucleus, and sorted by size or gene richness[99,100] (Fig. 7c,d). For many years, chromosomes in interphase were considered unorganized, until relative consistency of chromosomal positioning was demonstrated in the 1980s[101], and then the existence of chromosome territories was established[94,98]. In general, the peripheral DNA at the edge of the nucleus (close to the lamina, a lamin-rich nuclear 'shell') and bounding the nucleoli is typically transcriptionally silent heterochromatin, whereas the more frequently expressed, euchromatic sequences are typically more centrally located within the nucleus[102].

Much of the pre-eminent work on tensegrity and cytoskeletal force conveyance has used RGD-coated micro/nanoparticle integrin-targeting-and-manipulating techniques such as magnetic twisting cytometry[103] and pulling of beads[104]. Although these studies have been critical in delineating the role of cytoskeleton in force transduction and illuminating with regard to cellular tensegrity, they require substantial cell manipulation. In contrast, we believe that nanoscale topography affords a non-invasive methodology to study direct mechanotransduction.

Preliminary studies using nanotopography to drive changes in fibroblast and MSC cytoskeletal and nucleoskeletal morphology have indicated that chromosomes can become repositioned relative to each other as the cytoskeleton and nucleoskeleton rearrange in response to the topography[36,105,106]. Such changes in chromosomal arrangement could, speculatively, have implications on chromosomal positioning in transcriptionally active and quiet parts of the nucleus[107]. Thus, it is possible that nanotopography may affect accessibility of genes to transcription factors through changes in nuclear organization.

The application of tensile force to integrins, resulting in altered nuclear morphology and reorientated nucleoli, is further evidence to support the mechanosensitive nature of the nucleus[108]. It is thus intriguing to consider the chromosomes themselves as part of a network connected to the lamina, cytoskeleton and adhesions, and hence to nanoscale cues within the extracellular matrix. Chromosomes, perhaps, could then respond to alterations in the extracellular environment through transcription-factor activation/deactivation and territory movements, allowing key genes involved in growth and function either access to or shielding from the transcription factors.

It is evident from the above discussions that changes in cell shape, integrity and cytoskeleton (including alterations in adhesion and motility) are able to modulate cell tension that is subsequently propagated to the nucleus resulting in chromatin remodelling, and, critically, activation or repression of gene expression. This indicates a possible role for epigenetic mechanisms. Indeed, in our previously discussed study using disordered nanotopography to provide enhanced directed mesodermal differentiation of embryonic stem cells, changes in methylation status of POU domain class 5, transcription factor 1 (*POU5F1*), the gene encoding Oct4, were noted[42]. Thus, incubation on a specific nanotopography resulted in a significant increase in methylation compared with self-renewing embryonic stem cells that correlated with a loss of Oct4 expression[42].

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A recent study further implicates a role for lamins in lineage commitment of MSCs in response to the extracellular environment. In soft tissue environments (low stiffness), adipogenic differentiation was enhanced by low lamin-A levels. In environments reflecting a more bone-like stiffness, however, osteogenesis was seen to be enhanced by increased lamin-A levels. This suggests a role for lamins as a 'mechanostat', not only stabilizing the lamina and DNA when needed (for example, in conditions where cells are under high stress), but also taking a direct role in MSC differentiation[109].

Together, these observations demonstrate the need to consider cells not only as biochemical units but also as mechanical units. Materials provide a powerful tool kit for probing cell adhesion and mechanics and, ultimately, epigenetic function. Nanoscale materials specifically offer a unique opportunity to unravel these mechanisms, as a consequence of their similarity in scale to cell receptors such as integrins (Fig. 7a). The use of materials and nanotopography as tools in helping us to understand direct mechanotransduction is still at an exploratory stage, and their full potential is yet to be realized.

Outlook

Over the past decade, nanoscale topography has played a central role in research studies to understand cell-adhesion mechanisms, resultant biochemical signalling and possible direct mechanotransductive signalling in cells. These studies have been instrumental in understanding skeletal MSC growth and differentiation, and have expanded to consider a range of other cell types, including embryonic stem cells.

We predict that nanotopography will have an important role in understanding regulation and, potentially, the bioengineering of adult-stem-cell niches. It is already being used to elucidate mechanisms of MSC self-renewal, differentiation and, we propose, gene regulatory mechanisms therein. Studying the bone marrow niche reveals that MSC behaviour in the sinusoidal (fenestrae) and endosteal (type X collagen) regions of the niche has parallels to the effects observed on different nanotopographies.

We propose that the use of specifically designed nanotopographical surfaces (as opposed to general roughening or randomness) is important in analysing how cells process nanoscale information. This is, in part, a result of the reproducibility of manufacture: that is, the nanofeatures will be in the same place, retain the same shape and be the same size for every replicate, allowing the reproducibility of cellular response. This is in line with the concept of third-generation biomaterials where reproducible molecular control is desirable — that is, the same response each time[110].

Looking forward, there are key therapeutic and biological goals to be achieved, with demands on both fabrication and biological analysis.

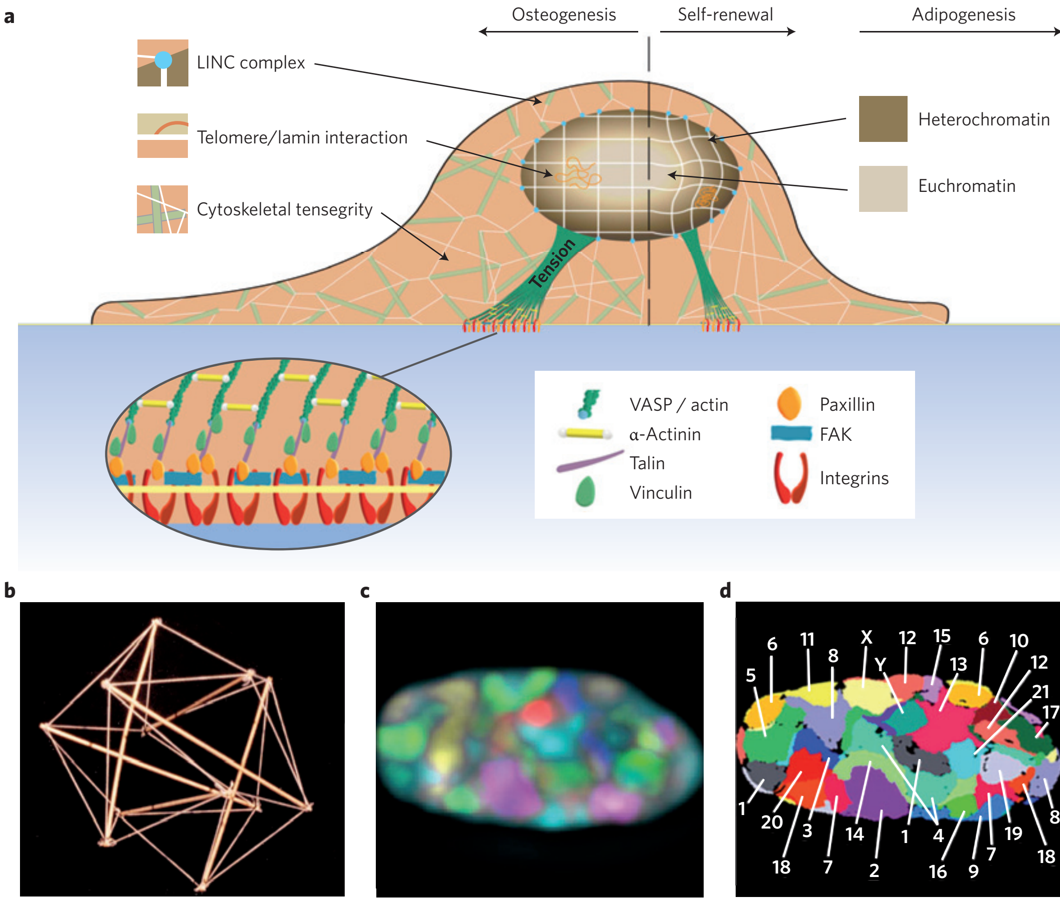

Figure 7 | A model of direct mechanotransduction considering the MSC as a mechanical unit. a, The focal adhesions of the cell link to the nucleus through cytoskeletal tensegrity (background structure) and the LINC complexes. This alters organization of the lamin nucleoskeleton and leads to chromosome-territory repositioning that will, in turn, affect transcription of the genome. **b**, Tensegrity model showing that the balance of compressive struts and tensile wires provides stabilization through tension. In a cell, the thick rods represent compressed cytoskeletal elements (for example, microfilaments) and the thin wires represent cytoskeletal components under tension (for example, microtubules). **c**,**d**, Fluorescent *in situ* hybridization images (**c**) and schematic (**d**) for the chromosome territories in the nucleus, showing positioning of human chromosomes 1–22, X and Y. Figures reproduced with permssion from: **b**, ref. 90, © 2003 The Company of Biologists; **c**,**d**, ref. 100, under Creative Commons licence.

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Much of the work described here has been performed on model polymers such as polycaprolactone, polymethylmethacrylate and polycarbonate, selected for ease of embossing, handling and biocompatibility. There is a pressing need to move to materials with an applied (engineering) purpose, for example titanium and related alloys for use as orthopaedic implants where MSC differentiation to bone-forming osteoblasts is required. Titanium is complicated to emboss as it is a hard material. Moreover, there are biological problems as the surface is 'overly' adhesive to cells, resulting in uncontrolled adhesion. This means that although surface treatments, such as topography, may encourage the MSCs in the bone marrow to form large, osteogenic adhesions on contact with the implant, the native chemistry of titania will compete with these cues to encourage the cells to form many smaller adhesions, more typically associated with fibrogenesis. This quantity rather than quality of adhesion tends to lead to soft tissue encapsulation of implants, micromotion and eventual failure, thus limiting the practical lifetime. That said, approaches such as anodization have been used to achieve nanoscale surface texture, and positive results have been demonstrated in terms of osteogenic differentiation[111,112]. Furthermore, approaches using anodization masks fabricated by block-copolymer phase separation have been used to tune achievable feature order. These approaches have further improved osteogenic differentiation[113].

In our experience, however, it has been difficult to recreate as large a magnitude of MSC osteogenic response as seen on model polymers, possibly as a consequence of the rapid fibrogenic response to titania. Clinically, this gives rise to problematic fibrous encapsulation of implants rather than direct bone bonding. When such problems are addressed more fully, nanotopography will rapidly find a range of applications in orthopaedics.

The main barrier to topography application for delivery of large quantities of high-quality stem cells is the current restriction on fabrication area. Electron-beam lithography can be used to provide an area of square centimetres of pattern. But to expand and provide many millions of cells, areas of the order of square metres would be more clinically useful. As a result, the scale-up of patterning and conversion to three-dimensional culture are urgent requirements.

Biologically, over the next decade it is likely that key areas will centre on our understanding of cell mechanisms (for example tensegrity and nucleus organization, and metabolite use), and on the role of microRNAs and the interrelationship to the epigenome as cells move between environments and energy states (quiescent versus metabolically active). In this context, we note a recent report that presents the ability of microtopography to enhance cell reprogramming[114]. Furthermore, it will be important to expand into other stem cell types (for example, induced pluripotent stem cells, embryonic, neural or epithelial) and to establish approaches to deconstruct and subsequently reconstruct the stem cell niche. Using a nanotopographical approach, we predict that it will be possible to identify specific signalling hubs or mediators of therapeutic potential. At present, we identify ERK 1/2, Jnk; although these are clearly too broad-acting, as they are central to many cell processes, we note that this research is in its infancy. Furthermore, identification of biological small molecules that can address, for example, the conversion of MSCs to osteoblasts in the marrow niche with high specificity could be used to address problems such as osteoporosis. When the potential for this approach is considered for other stem cell types, for example neuronal, where niche response to regenerative demand in conditions such as Alzheimer's, Parkinson's and multiple sclerosis falls far short of that required, the future role for materials in discovery of biologically active metabolites becomes exciting. Of course, this then presents other fabrication challenges, for example the requirement for patterned 96- and 384-well plates to assist in high-throughput testing.

To provide such a step change in nanotopography application and science, new discovery becomes all-important. High-throughput materials-discovery approaches are likely to be instrumental in driving the discovery of nanotopographical (substrate, pattern, organization) control of cells. These approaches describe the testing of many iterations of materials at the same time; such technology has been used for some years in the form of chemical libraries[115,116] and more recently microtopographical libraries for stem cells[117], as well as in generating libraries of stiffness[118] for evaluation of cell response.

In summary, the application of materials science to modulate the extracellular environment at the nanoscale, with exquisite precision, will be fundamental to developing an understanding of stem cell and cell function. We are only starting to unravel the potential of nanotopography to inform our understanding of cell signalling, epigenome interaction and niche-nanoenvironment regulation. Thus, harnessing nanoscale and nanotopographical design and integrin–matrix interactions provides new vistas for stem cell biology and applications within the regenerative-medicine arena.

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Additional information

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Chapter 2
Matrix Stiffness: A Regulator of Cellular Behavior and Tissue Formation

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Abstract The extracellular environment is an essential mediator of cell health and provides both chemical and mechanical stimuli to influence single and collective cell behaviors. While historically there has been significant emphasis placed on chemical regulators within the extracellular matrix, the role of the mechanical environment is less well known. Here, we review the role of matrix mechanics on cell function and tissue integrity. Cellular responses to mechanical signals include differentiation, migration, proliferation, and alterations in cell–cell and cell–matrix adhesion. Interestingly, the mechanical properties of tissues are altered in many disease states, leading to cellular dysfunction and further disease progression. Successful regenerative medicine strategies must consider the native mechanical environment so that they are able to elicit a favorable cellular response and integrate into the native tissue structure.

Matrix Mechanics Are Essential Design Parameters for Regenerative Medicine

Tissue engineering (TE) was defined in the late 1980s as a field concerned with "the application of the principles and methods of engineering and life sciences toward. . .the development of biological substitutes to restore, maintain, or improve functions" [111]. Motivated by a clinical need to restore normal physiologic function to tissues and organs that malfunction due to injury and disease, TE approaches may provide an avenue of treatment for patients with organ and tissue failure additionally plagued by increasing costs of care and donor shortages [63].

Significant numbers of investigations into biomaterials have confirmed that surface chemistry is a critical parameter contributing to the clinical success of

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implanted devices or TE constructs [118]. Surgery and implantation of biomaterial or TE constructs induces biochemical cascades that mediate the normal wound healing and foreign body responses that ultimately result in the success (functional integration into the tissue) or failure (rejection from the tissue, mechanical failure) of the implant. While the need to tailor the surface chemistry of an implant has been given significant attention for decades, the need to also consider the mechanical properties of an implant and its effects on cells has only been gaining momentum in recent years. Similar to surface chemistry, the mechanical properties affect the local behaviors of tissues and cells and contribute to the success of biomaterial and tissue-engineered implants.

While TE and regenerative medicine have recently focused on the micromechanical properties of a construct and its effects on cells, the notion that mechanical forces act as critical regulators of physiological processes at the cell and tissue level is not a new paradigm. Physical forces were known to contribute to the development of brain morphology [46] and bone remodeling [103, 128] as early as the late nineteenth century. Since then, elucidating the relationship between force and biological responses has spanned a variety of mechanical settings and length scales from probing the role of weightlessness on the musculoskeletal system during spaceflight [56] to understanding how shear stress in the vascular tree specifies endothelial cell phenotype [22]. These studies drew attention to the role of the physical environment as an important regulator of biological responses in living systems.

This chapter describes the role of the mechanical properties of the extracellular matrix (ECM) as a mediator of cellular responses and tissue formation. An overview of the nature of the mechanical properties of the cellular microenvironment and how it affects cellular function and tissue formation are discussed. Lastly, the role of matrix mechanics in disease states is presented.

The Cellular Response to Matrix Mechanics: Cellular Function Is Modulated by Local Matrix Stiffness

The Mechanical Environment of Cells

Cells *in vivo* are organized into tissues and organs that reside in complex mechanical environments. At the cellular level, the mechanical environment consists of endogenous (generated *by* cells) and exogenous (applied *to* cells) forces. Endogenous forces generated by cells on their ECM and neighboring cells largely result from cytoskeletal contractility (discussed below; [13, 76]). Examples of exogenous forces include gravity and tissue-specific interactions; for example, endothelial cells in the vasculature are subjected to pulsatile shear forces from blood flow [6] as well as migratory traction forces during leukocyte transmigration [94].

In addition to these actively imposed forces, the local stiffness of the ECM that serves as a biological scaffold is an important mechanical effector of cell function.

Stiffness is a measure of the ability of a material to resist deformation. In the body, tissue stiffness ranges several orders of magnitude, from adipose tissue (Young's Modulus $E \sim$ several kPa) [106] to bone ($E \sim$ GPa) [99]. In addition, tissue stiffness is not static, but changes during physiological processes including embryonic development, tissue remodeling during wound healing, and in pathological responses like tumorigenesis. Since there is an intimate association between cells and the ECM within tissues, and cells function in a variety of mechanical environments, many studies have investigated the mechanisms that cells use to sense and respond to their mechanical environment.

Biological Force Transducers

Tissue cells have an ability to sense and probe the stiffness of their surroundings as they adhere to and interact with the local ECM [28]. Mechanotransduction, where cells convert mechanical stimuli into chemical signals that affect cellular responses, occurs through a variety of mechanisms. Well-described mechanotransducers include stretch-mediated ion channels [74], primary cilia [8], and integrins [36, 100]. Additional mechanosensors, including G-protein receptors [70], cell–cell adhesions [57, 86], and the cytoskeleton [126] have been suggested. While these transducers sense the mechanical environment through a variety of mechanisms, they all share the ability to convert mechanical input into complex intracellular signaling cascades that ultimately regulate cellular responses including adhesion, spreading, migration, and proliferation [54]. The number and variety of mechanosensors identified in cells suggests that cells have a robust capacity to interact with their mechanical environment. This robustness is particularly important when considering that in addition to regulating normal physiological responses, abnormal mechanotransduction at the cellular level has been implicated in mediating a wide variety of prominent disease states including asthma [127], osteoporosis [2, 19], and cancer [51, 52, 115].

While it is likely that no single cell feature is responsible for driving all mechanobiological responses, the integrin family of proteins has emerged as a prominent and well-studied force transducer. The concept of a mechanical linkage between the ECM and the intracellular cytoskeleton was postulated in the mid-1970s [49], and the structure of integrins was determined in the next decade [116]. Composed of α and β subunits (18α and 8β subunits combine to form over 20 distinct integrin heterodimers to-date), integrin receptors are a family of transmembrane glycoproteins that serve as mechanical linkages between the ECM and the cytoskeleton [50]. On the exterior of the cell, integrins bind ECM protein ligands including collagen, laminin, and fibronectin [93]. Within the cell, the β subunit of integrin heterodimers binds to the actin cytoskeleton through a variety of adaptor proteins [66]. Integrins cluster into focal adhesions that spatially localize and

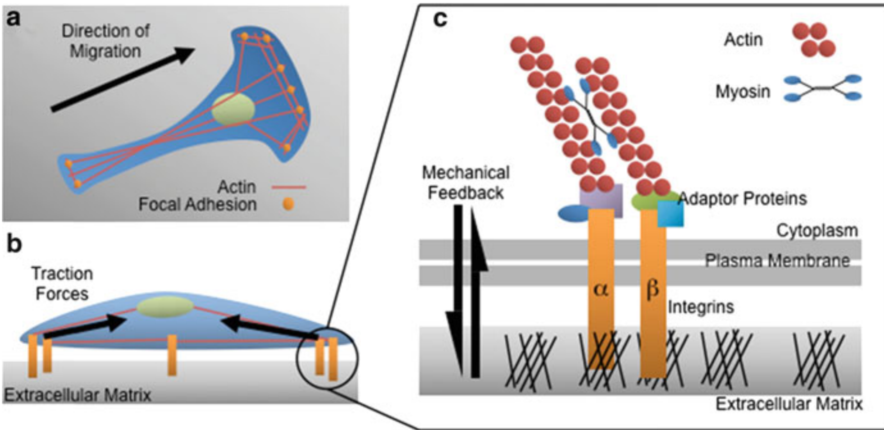

Fig. 2.1 (**a**) A typical cell migrating over a substrate utilizes actin stress fibers anchored to focal adhesions. (**b**) Together with the actin cytoskeleton, focal adhesions composed of integrins facilitate cell–substrate adhesion, contractility, and traction force generation. (**c**) A close-up depiction of a focal adhesion. Actin stress fibers are tensed by myosin motors and attach to integrin receptors via adaptor proteins within the cytoplasm. Integrin transmembrane receptors bind to the extracellular matrix outside the cell and participate in mechanosensing events

anchor actin stress fibers to the plasma membrane thus providing a mechanical linkage between the ECM and the cytoskeleton (Fig. 2.1a) [37]. Moreover, the integrin "adhesome" serves as a scaffold for a host of signaling proteins within the cell [132], suggesting that integrin receptors serve as prominent sensors and integrators of environmental signals.

Cells Sense Matrix Stiffness with Cellular Contractility and Traction Forces

"Stiffness sensing" means that cells have an ability to detect and respond to the mechanical resistivity of the extracellular environment. Stiffness sensing has been demonstrated in a variety of cell types including endothelial cells [17, 26, 96, 98], smooth muscle cells [31, 53], and transformed cells [67, 125]. The ability to sense stiffness is partly dependent on actomyosin-generated contractility that is transmitted to the extracellular environment through transmembrane integrin receptors that, with a number of intracellular signaling and scaffold proteins, organize into focal adhesions. Cells, in turn, respond to the stiffness of their substrate by altering cytoskeletal organization, cell–substrate adhesions, and other processes important for regulating cell behaviors.

Cellular contractility is generated in part by the actomyosin cytoskeleton. Actin stress fibers are tensed by myosin motors [61, 101], and cytoskeletal contractility is

transmitted to the ECM as traction forces (Fig. 2.1b, c) [65]. Cellular traction forces were first observed in landmark experiments as wrinkles or strains in flexible silicone rubber substrates [44]. Since then, methods have been developed to quantify traction forces generated by cells. Prominent techniques include traction force microscopy [25, 72] and the use of microfabricated post-array detectors [113, 117]. Other methods include the use of microfabricated cantilevers [35] and micropatterned silicone elastomeric substrates [4]. These techniques calculate traction forces based on strains created in the substrate by adherent cells. The ability of adherent cells to generate traction forces and cell–substrate adhesions facilitates sensing of the local extracellular environment and is involved in feedback mechanisms where matrix stiffness in turn modulates responses such as adhesion, spreading, and migration.

Matrix Stiffness Modulates Focal Adhesions, Cytoskeletal Assembly, and Traction Forces

The measurement of cell traction forces has helped to describe the role of force and focal adhesions as mediators of cell–substrate attachment and matrix stiffness. Experiments in real-time indicate that focal adhesion size is linearly dependent on the local force exerted by a cell [4]. Mature focal adhesions elongate and orient in the direction of actin stress fibers and applied force. However, the correlation of focal adhesion size with cell-generated forces may only hold for adhesions larger than 1 μm^2, as smaller adhesions are capable of exerting large traction forces that do not correlate with adhesion size [117]. Indeed, small nascent adhesions (focal complexes) at the leading edge of cells are capable of generating strong transient traction forces that drive cell migration [7]. Moreover, when cells on magnetic microposts are deflected by an external magnetic field, changes in traction force generation occur at sites of adhesion peripheral to the site of force application [112]. These data are indicative of a dynamic association between the actin cytoskeleton, cellular traction forces, and focal adhesions that mediates cell adhesion and migration.

Additional work has investigated focal adhesion organization with regard to matrix stiffness. Seminal experiments with fibroblasts and epithelial cells indicate that compliant ($E \sim 1$ kPa) substrates promote focal adhesions that are dynamic and irregular punctate structures [90]. In contrast, an increase in stiffness ($E \sim 30$–100 kPa) promotes the formation of stable arrays of elongated focal adhesions and an increase in tyrosine phosphorylation of focal adhesion kinase (FAK) and paxillin, suggesting that stiffness sensing involves intracellular signaling events. Such changes in focal adhesion organization suggest alterations in cell–substrate adhesivity. Accordingly, an increase in cell–substrate adhesion with increasing substrate stiffness has been demonstrated [32].

In general, stiff substrates increase both focal adhesion and cytoskeletal organization [31, 38, 41, 90, 130]. The formation of stable focal adhesions with increasing substrate stiffness is accompanied by changes in cell shape. For example, fibroblasts plated on compliant substrates are rounded with diffuse actin, while those plated on stiff substrates exhibit an increase in spread area and actin stress fiber organization [39, 130]. Similarly, endothelial cell spread area increases with increasing substrate stiffness [16, 97], where endothelial cells on compliant substrates adopt an elongated spindle-shaped morphology, while those on stiffer substrates exhibit more isotropic spreading [17]. Interestingly, endothelial cell stiffness is also modulated by matrix stiffness in 2D and 3D environments [15]. These data suggest an intimate association between substrate stiffness, cytoskeletal organization and cell shape, focal adhesions, and traction force generation.

The investigation of matrix stiffness as a mediator of cell shape has further elucidated the relationship between stiffness and force generation. It has been shown that matrix stiffness and cell shape help regulate the polarization and alignment of stress fibers within cells [134]. Indeed, matrix stiffness can alter cellular contractility [135]; traction force generation by fibroblasts and endothelial cells increases with increasing substrate stiffness [17, 41, 68]. Moreover, experiments with endothelial cells have demonstrated that both cell area and substrate stiffness are significant predictors of traction force generation [17]. In turn, the orientation and organization of the actin cytoskeleton helps determine cell shape; the ablation of a single stress fiber in a cell results in significant rearrangements in cell shape and cytoskeletal organization [61]. These data provide evidence for feedback mechanisms that relate matrix stiffness to cytoskeletal organization and traction force generation and provide a role for mechanotransduction as a contributor to cell shape.

The sensitivity of cellular traction force generation to matrix stiffness has implications for the organization of the local ECM. For example, the fibrillogenesis of the ECM protein fibronectin is mediated by endogenous cellular contractility [5]. Experiments with fibronectin-based native ECM scaffolds versus scaffolds stiffened by chemical crosslinking indicated differential scaffold remodeling by fibroblasts; native scaffolds were progressively remodeled over several days while cross-linked scaffolds were not [60]. These data indicate that there are feedback mechanisms that relate matrix stiffness to matrix remodeling and suggest that cellular responses to matrix stiffness may regulate ECM homeostasis.

Matrix Stiffness Modulates Cell–Cell Assembly, Migration, and Proliferation

In addition to modulating cellular contractility and force generation, matrix stiffness plays a role in mediating cell–cell interactions. Seminal work by Guo et al. established a relationship between matrix stiffness, cell–matrix, and cell–cell

interactions [43]. When heart tissue explants were plated on stiff matrices, cells from the tissue migrated out of the explant to cover the matrix. In contrast, cells in explants plated on compliant matrices did not migrate out of the explant. Separate studies with endothelial cells also indicate sensitivity of cell–cell interactions to matrix stiffness. On compliant substrates, endothelial cells prefer cell–cell interactions [98] and self-assemble into networks [16]. On stiffer substrates, ECs prefer cell–substrate interactions and fail to form network assemblies. In epithelial cells, cell–cell assembly is anisotropic along directions of stiff substrate and correlates with actin cytoskeletal organization and force generation [104]. These data suggest that matrix stiffness and traction forces modulate cell–cell organization.

Further work has investigated the role of matrix stiffness in mediating cell migration [55, 91]. For example, fibroblasts migrate toward substrates of increasing stiffness, a response termed durotaxis [68]. Smooth muscle cells also exhibit durotaxis with respect to the magnitude of substrate stiffness gradient [53]. These data indicate that substrate stiffness provides important cues that foster traction force organization responsible for cell migration. The sensitivity of cell migration to stiffness gradients may have important implications for disease states such as fibrosis or tumorigenesis that are accompanied in increases in ECM stiffness.

In addition to affecting migration, forces between contacting cells can also influence proliferation. Gray et al. found that the number of cell–cell contacts influences the proliferation of a cell in a bi-phasic manner [42]. Single cells are less proliferative than those with at least one cell–cell contact but increasing the number of neighbors inhibits proliferation. Interestingly, increasing the amount of cell–cell contacts may concurrently decrease the ability of cells to adhere to the ECM, thus decreasing proliferation. This response is essential for healthy tissue function where contractility, spreading, and proliferation are intricately regulated by cell–cell and cell–matrix adhesion and tension.

Collective Cell Responses to Matrix Mechanics: Implications for Tissue Development, Regeneration, and Repair

We have discussed the importance of matrix mechanics on individual cellular behavior and function. However, while single cell studies may be informative of cellular behavior, cells within tissues interact and respond collectively to stimuli. Similar to the influences on individual cells, mechanics are integral to overall tissue and organ physiology and mechanical alterations or disturbances can lead to disease and tissue malformation (discussed below). Interestingly, the earliest stages of embryonic development, tissue patterning, and organ formation are governed, in part, by mechanical interactions with the extracellular environment [21, 82, 110]. Studying these interactions can inform the design of tissue engineered and regenerative therapies.

Mechanical Stimuli Influence Embryonic Development

Throughout embryonic development, all tissues of the body are derived from a single-fertilized cell via a complex process of specification and differentiation. Cellular differentiation is the process whereby a cell with an unspecified fate is influenced by genetic, chemical, and mechanical [14] factors to become a specific cell type. A fully differentiated cell maintains its gene expression patterns through generations of proliferation and has a distinct role within an organized tissue. During embryogenesis, biochemical factors and pre-programmed genetic cues initially dictate the polarity of the embryo as well as the cell lineage specification of its progeny into the three germ layers: ectoderm, endoderm, and mesoderm [34, 92]. Concurrent with these chemical and genetic signals, mechanical stimuli reinforce and further specify cell fate and play a crucial role in the development of the unique tissues and organs of the body [34]. Specifically, mechanical signals such as pressure, fluid flow, shear stress, tension, and stiffness are important regulators of embryogenesis and have been shown to affect the development and tissue patterning of many major organs [71] including the eye [45, 82], heart [48, 89], vasculature [77], and neural tube [136].

Further investigations into developmental processes have indicated that matrix mechanics play a vital role in proper tissue development throughout the entire embryo. Recent work in *Xenopus* has confirmed a temporal and spatial distribution of mechanical stiffness within developing embryos due to the contraction of the actomyosin network [136]. This cytoskeletal contraction not only increases the stiffness of the surrounding tissue structures as much as 50-fold within 8 h, but may also drive the formation of the neural tube and allow for further cell patterning and differentiation [136]. Similarly, repeated and coordinated contractions of the actomyosin cortex in *Drosophila* embryos create tension between cells that facilitate cell invagination and formation of the ventral furrow [73]. These data indicate that intra- and inter-cellular contractility drive tissue morphogenesis.

In addition to the exogenous mechanical stimuli within developing tissues, differential adhesion and repulsion between cells and the surrounding matrix plays an integral role in embryonic tissue morphogenesis [114, 121]. It has been shown that the ectoderm–mesoderm boundary is not only maintained by self-sorting due to preferential adhesion of similar cells to each other, but is also a function of the active repulsion between unlike cells [102]. Interestingly, the development of structures within the retinal epithelium in *Drosophila* embryos mimics the formation of soap bubble aggregates, where the surface tension is minimized during aggregate formation [45]. This patterning occurs due to differential adhesion between cells with the most adhesive cells forming central aggregates surrounded by less-adhesive cells to minimize the "surface energies" of the cell contacts. Similarly, during a phase of embryogenesis known as epiboly, cell adhesion proteins are differentially expressed so that a group of cells can migrate toward the vegetal pole of the embryo and begin gastrulation [110]. These data indicate that tissue formation is influenced by the balance of cell–cell and cell–substrate adhesion.

The mechanical environment is intimately linked with collective cell behavior such as contractility, adhesion, and tissue patterning during embryogenesis. Importantly, matrix mechanics can regulate cellular specification and tissue formation. Regenerative strategies may exploit these responses to mechanical stimuli to produce organized cellular structures that mimic the original, healthy tissues.

Mechanical Control of Cellular Differentiation

In addition to embryogenesis, mechanical cues play an integral role in maintaining and influencing cell fate and tissue maintenance throughout life. While the process of differentiation is most obvious during embryonic development, some cells (e.g., stem cells) remain multipotent even in adult tissue [80]. These stem cells are essential for tissue maintenance and repair, may have important implications for disease progression, and have been the focus of many engineered tissue therapies. Importantly, each of these processes is influenced by the mechanical properties of the surrounding environment.

Although initial tissue engineering strategies were concerned primarily with maintaining the mechanical integrity of the implant, current therapies look to integrate mechanical cues to differentiate and pattern cells into complex tissues. Stem cells have been a popular choice for regenerative medicine research since they are capable of self-renewal and differentiating into multiple cell types [80]. The stem cell niche, the 3D microenvironment surrounding the cells, is a key factor in their maintenance and differentiation [9, 29, 124]. To further understand the factors that influence stem cell differentiation in 2D and 3D, synthetic and natural scaffolds have been used to probe the interactions of the cells with their extracellular environment [27]. Many groups have combined novel materials and chemical cues to encourage stem cell differentiation along a chosen lineage in the hopes of creating regenerative therapies [69].

Endogenous cellular stiffness is predominantly regulated by the actomyosin cytoskeleton and has been shown to change during differentiation [64]. Using AFM, Titushkin and Cho observed that mesenchymal stem cells stimulated with osteogenic medium became less stiff throughout their course of differentiation [119]. In contrast, cells differentiated from mouse embryonic stem cells are tenfold stiffer than their precursors [21]. Similarly, Pajerowski et al. found that the nucleus of human embryonic stem cells becomes sixfold stiffer when terminally differentiated (Fig. 2.2a) [87]. These results suggest that the mechanical properties of cells depend on both the origin and differentiation stage of the stem cells.

Matrix mechanics are also known to be independently capable of dictating stem cell differentiation into different lineages. In a seminal study, Engler and colleagues demonstrated that mesenchymal stem cells can be stimulated to differentiate into neurons and osteoblasts when plated on soft and stiff matrices, respectively, that were chemically similar (Fig. 2.2b) [33]. Recently, scientists have exploited the ability of stem cells to sense and respond to their mechanical environment to create

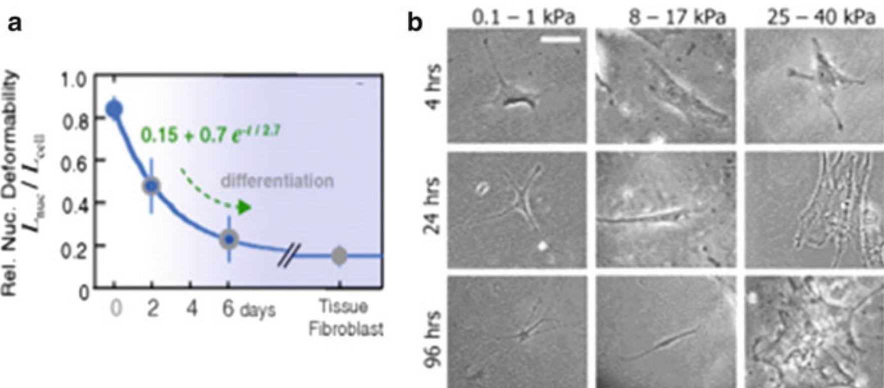

Fig. 2.2 (**a**) During differentiation, the nuclear compliance of human embryonic stem cells decreases (stiffness increases) relative to the cellular cytoplasm. Reprinted with permission from PNAS 104(40): Pajerowski et al.: Physical plasticity of the nucleus in stem cell differentiation, 15619–15624, Copyright 2007 National Academy of Sciences, U.S.A. [87]. (**b**) Mesenchymal stem cells sense and respond to substrate stiffness by changing differentiating to neural cells and myoblasts on soft and stiff substrates, respectively. Reprinted from Cell 126(4): Engler et al.: Matrix Elasticity Directs Stem Cell Lineage Specification, 677–689, Copyright 2006 [33], with permission from Elsevier

scaffolds that vary in stiffness spatially such that an entire tissue might be created by simply seeding the engineered matrix with stem cells [20, 59, 109, 123]. Very recent work indicates that mesenchymal stem cells plated on a stiffness gradient directionally migrate toward the stiffer portions of the substrate and subsequently differentiate [123]. Interestingly, the cells that migrate from soft to stiff regions of the substrate maintain neuronal markers similar to the cells that are plated on uniformly soft substrates [123]. Importantly, these results suggest that even though the cells in a specific lineage may become differentiated, they are able to retain a "memory" of the previous signals they have received. These data suggest that mechanical microenvironmental cues are essential to the promotion and preservation of stem cell lineage specification and, to produce a functional tissue replacement, will be required design parameters for regenerative therapeutics.

Matrix Mechanobiology Alterations in Disease and Injury

Altered tissue mechanics are a prominent feature of many injured diseased tissue states and are commonly a result of abnormal ECM deposition, matrix cross-linking and/or matrix degradation. Specifically, matrix stiffening accompanies aging [23], cardiovascular disease [105], wound healing [40], and tumor formation [85]. Native ECM mechanics can be modified by changes in protein deposition or cross-linking of preexisting matrix components. These changes in matrix mechanics can lead to aberrant cell behavior that can cause or exacerbate disease states [3, 62].

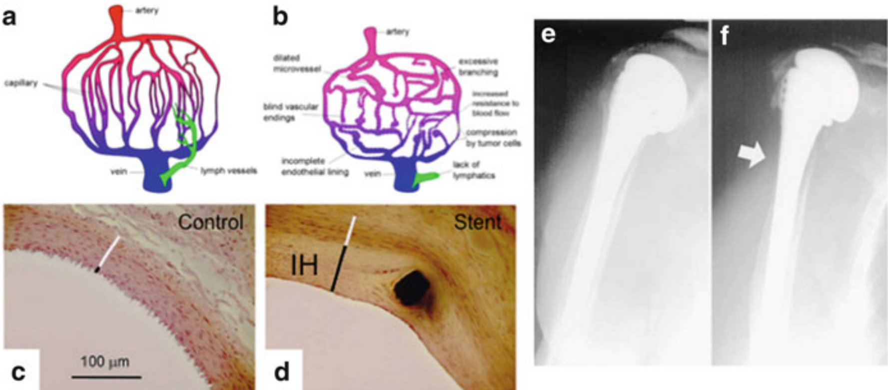

Fig. 2.3 (**a**, **b**) A cartoon depicting the vascular system in a normal tissue (**a**) and in a solid tumor (**b**). Reprinted with permission from Oxford University Press from Trédan et al.: Drug Resistance and the Solid Tumor Microenvironment. Journal of the National Cancer Institute 99(19):1441–54 [122]. (**c**, **d**) Measurements of intima (*black bars*) and media (*white bars*) in control (**c**) and stented (**d**) rabbit carotid arteries. *IH* intimal hyperplasia. Reprinted with permission from Oxford University Press from Alp et al.: Increased intimal hyperplasia in experimental vein graft stenting compared with arterial stenting: comparisons in a new rabbit model of stent injury. Cardiovascular Research 56(1):164–72, 2002 [1]. (**e**, **f**) Clinical radiograph taken immediately after shoulder prosthesis implantation (**e**) and after 7 years of follow-up (**f**). The arrow in (**f**) depicts a region of cortical bone resorption. Reprinted from the Journal of Shoulder and Elbow Surgery 12(1): Nagels et al.: Stress shielding and bone remodeling in shoulder arthroplasty, 35–39, 2003 [81], with permission from Elsevier

In general, tumor tissues have altered mechanical properties as compared to native, healthy tissue [83, 85, 108, 131]. In fact, breast cancer is often first detected by the patient or physician finding a palpable mass or lump that is stiffer than the surrounding tissue. Large tumors are associated with an increase in local ECM stiffness and angiogenesis, an in growth of newly sprouted blood vessels that facilitate increased tumor mass (Fig. 2.3a, b) [122]. The increase in ECM stiffness is primarily due to increased collagen deposition and cross-linking within the tumor stroma [85], but a disruption in the tensional homeostasis of the cells may also contribute [88]. As discussed previously, changes in the stiffness of the ECM can lead to phenotypic cellular changes such as increased proliferation and migration. Indeed, Paszek and colleagues found that increasing substrate stiffness correlated with changes in cytoskeletal tension, integrin expression, cellular proliferation, oncogene activity, and tissue formation in mammary epithelial cells [88]. Additionally, tumor cell migration was found to be modulated by the stiffness of the ECM [133]. These results indicate that the increased mechanical stiffness of the surrounding ECM that accompanies tumor progression may, in fact, drive malignancy.

ECM stiffening is also known to be a critical factor in the progression of cardiovascular disease. Vessel stiffening occurs through a number of mechanisms including glycation, the formation non-enzymatic cross-links (also known as

advanced glycation end products or AGEs) within the ECM [23]. These post-translational biochemical alterations cause tissue stiffening and prevent cellular remodeling of the existing tissue [79]. For example, the greater prevalence of reducing sugars such as glucose and ribose within the blood of diabetic patients leads to increased cross-link density of collagen and elastin, and consequently increased stiffness of the vasculature when compared with non-diabetics [12, 24]. These alterations in the mechanical environment cause changes in cellular behavior and result in an inability to maintain proper vascular tone and regulate blood pressure effectively [58]. Together, these changes contribute to the increased prevalence of cardiovascular disease in diabetic patients. These data indicate that changing the matrix mechanics of a tissue can lead to disease.

Tissue stiffening also accompanies wound healing. Unfortunately, most of the time the body is unable to perfectly replicate the native tissue structure and a scar is formed at the site of an injury. In some areas of the body, such as the skin, a small scar does not typically impair function. However, in other regions of the body such as the central nervous system, scar formation can cause the tissue to severely malfunction [75]. Specifically, within the brain and spinal cord, tissue injury leads to glial scar formation which acts as a mechanical barrier and inhibits signal transduction [47]. In a study that investigated the molecular changes that occur during glial scar maturation, Camand et al. found that fibronectin matrix deposition inhibits axonal growth and healing [18], but promotes astrocyte attachment as a mechanism of physically separating the injured site from the surrounding tissue [95]. To better understand how the mechanical cues from the glial scar affect cellular function, Georges and colleagues investigated the response of astrocytes and cortical neurons to matrix stiffness [40]. Interestingly, they found that while the cortical neurons were able to spread and extend neurites on both soft and stiff surfaces, the soft substrates were not conducive to astrocyte growth. These data suggest that the mechanical properties of the glial scar are promoting astrocyte recruitment and barrier formation, thus limiting axonal regeneration. These results suggest that matrix mechanics play a key role in wound healing and tissue regeneration.

Just as perturbations in native tissue mechanics can lead to disease states, regenerative tissue engineering therapies can also facilitate the formation and progression of disease when the mechanical properties of the native tissue are not recapitulated. One prominent example is intimal hyperplasia (IH), a response characterized by thickening of the blood vessel wall due in part to the proliferation and migration of smooth muscle cells from the medial layer of the vessel wall and increased ECM deposition (Fig. 2.3c, d) [84]. Notably, mechanical differences in the matrix have been shown to induce migration [129] and proliferation [11] of vascular smooth muscle cells, both hallmarks of IH. The causes of IH stem from mechanical damage to the endothelium due to compliance mismatch between synthetic vascular grafts and native vascular tissue at sites of anastomoses [105] and changes in blood flow characteristics or luminal diameter at the anastomosis [107]. IH is ultimately responsible for poor patency after bypass grafting [78, 120] that may require additional surgical intervention. Similarly, mechanical mismatch between implant and native tissue also occurs in orthopedic implants that reduce the

physical loading on nearby bone tissue. This phenomenon, known as stress shielding, results from the difference in stiffness between the orthopedic implant and the host tissue, and results in bone resorption and osteopenia (Fig. 2.3e, f) [30]. Such changes at the bone–implant interface may ultimately allow micromotion that facilitates implant loosening, osteolytic particle debris [10], and implant failure.

These examples demonstrate that matrix mechanobiology plays a significant role in promoting a diseased phenotype. Moreover, they illustrate that the mechanical properties of engineered regenerative therapies are a critical design consideration for implant success.

Conclusions

The mechanical properties of tissues are not only important for maintaining macro-scale mechanical integrity but also essential regulators of cellular function. Cells sense stiffness using structures such as integrins to attach to the ECM and then respond and, oftentimes, remodel their environment by generating traction forces via actomyosin contractility. When alterations are made to the extracellular mechanical environment, cells can react to these mechanical stimuli by influencing tissue development, cellular differentiation, or disease progression. An understanding of how the mechanical properties of the ECM contribute to cell responses and tissue formation will ultimately further the understanding of disease states associated with aberrant mechanosensing and guide the design parameters of successful biomaterials and TE constructs. Future tissue engineering strategies should work to produce biomaterials and implants that are not only chemically favorable, but also integrate mechanical cues that dictate cellular behavior to aid in cellular differentiation and tissue regeneration.

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Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering

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New generations of synthetic biomaterials are being developed at a rapid pace for use as three-dimensional extracellular microenvironments to mimic the regulatory characteristics of natural extracellular matrices (ECMs) and ECM-bound growth factors, both for therapeutic applications and basic biological studies. Recent advances include nanofibrillar networks formed by self-assembly of small building blocks, artificial ECM networks from protein polymers or peptide-conjugated synthetic polymers that present bioactive ligands and respond to cell-secreted signals to enable proteolytic remodeling. These materials have already found application in differentiating stem cells into neurons, repairing bone and inducing angiogenesis. Although modern synthetic biomaterials represent oversimplified mimics of natural ECMs lacking the essential natural temporal and spatial complexity, a growing symbiosis of materials engineering and cell biology may ultimately result in synthetic materials that contain the necessary signals to recapitulate developmental processes in tissue- and organ-specific differentiation and morphogenesis.

Biomaterials play central roles in modern strategies in regenerative medicine and tissue engineering as designable biophysical and biochemical milieus that direct cellular behavior and function[1–3]. The guidance provided by biomaterials may facilitate restoration of structure and function of damaged or dysfunctional tissues, both in cell-based therapies, such as those where carriers deliver transplanted cells or matrices induce morphogenesis in bioengineered tissues constructed *ex vivo*, and in acellular therapies, such as those where materials induce ingrowth and differentiation of cells from healthy residual tissues *in situ*. Such materials should provide a provisional three-dimensional (3-D) support to interact biomolecularly with cells to control their function, guiding the spatially and temporally complex multicellular processes of tissue formation and regeneration.

Both biologically derived and synthetic materials have been extensively explored in regenerative medicine and tissue engineering. In general, materials from natural sources (e.g., purified protein components such as collagens from animal tissues) are advantageous because of their inherent properties of biological recognition, including presentation of receptor-binding ligands and susceptibility to cell-triggered proteolytic degradation and remodeling. Despite these advantages, many issues have spurred the development of synthetic biomaterials as cellular substrates, including complexities associated with purification, immunogenicity and pathogen transmission. Although some of these limitations can be overcome by recombinant protein expression technologies[4], greater control over materials properties and tissue responses could be achieved were synthetic analogs available.

The last few years have marked a substantial paradigm shift in design criteria for modern synthetic biomaterials, fully integrating principles from cell and molecular biology: materials equipped with molecular cues mimicking certain aspects of structure or function of natural extracellular microenvironments are quickly being developed. This review considers the design and application of such synthetic biomaterials originating from a symbiosis of materials engineering and molecular cell biology. We highlight the role of the ECM and its interactions with cells in natural processes of tissue dynamics, examine the basic principles of materials science that could be applied to address mimicry and exploitation of those interactions for tissue engineering, and finally discuss more sophisticated synthetic materials that can interact with their biological environment to a level that allows them to participate actively in pathways of tissue morphogenesis. The materials focus is limited to 3-D applications and is on emerging classes of polymeric biomimetic materials, such as nanofibrillar, supramolecular materials formed by self-assembly processes, and matrices presenting individual or multiple biochemical ECM-derived signals. For a more comprehensive overview of biomaterials, also including nonpolymeric and naturally derived materials, as well as their successful application in biomedicine we refer to several excellent recent reviews[3,5,6].

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Importance of cell-matrix interactions

Tissue dynamics, that is, its formation, function and regeneration after damage, as well as its function in pathology, is the result of an intricate

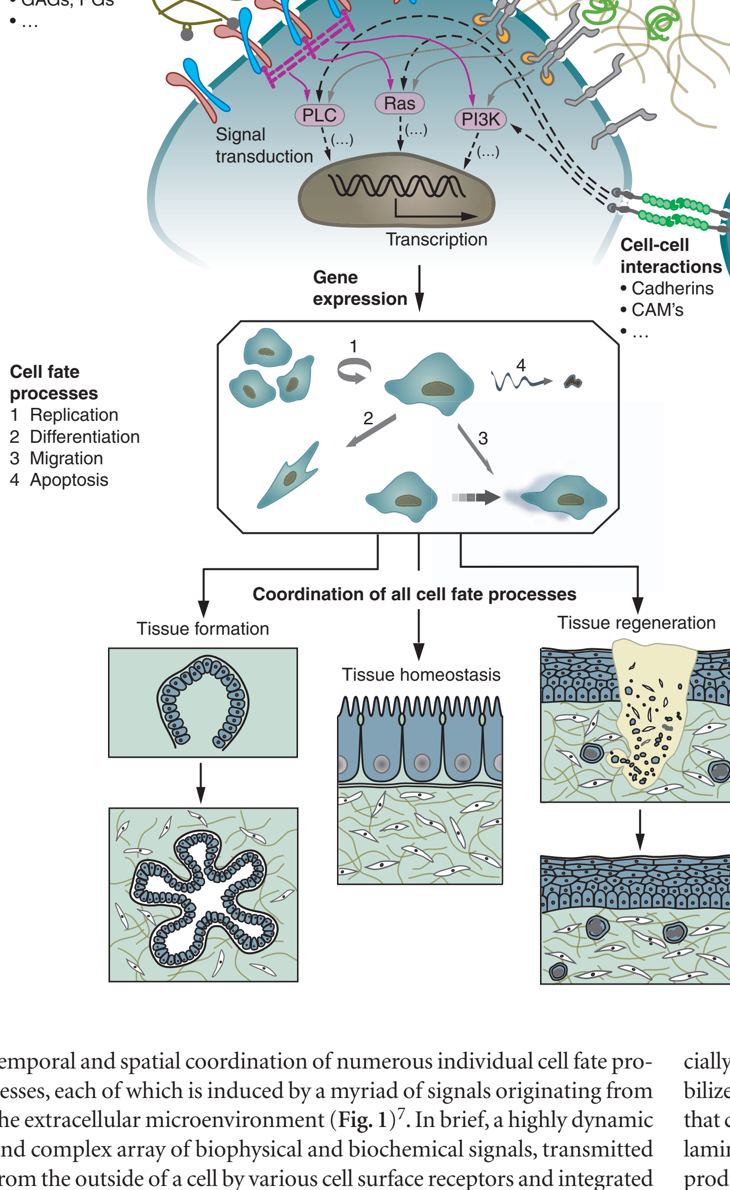

Figure 1 The behavior of individual cells and the dynamic state of multicellular tissues is regulated by intricate reciprocal molecular interactions between cells and their surroundings. This extracellular microenvironment is a hydrated protein- and proteoglycan-based gel network comprising soluble and physically bound signals as well as signals arising from cell-cell interactions. Adapted from ref. 112. Specific binding of these signaling cues with cell-surface receptors induces complex intracellular signaling cascades that converge to regulate gene expression, establish cell phenotype and direct tissue formation, homeostasis and regeneration. Ellipsis (...) indicates that the lists of signals are not intended to be complete. PLC, phospholipase C; GAGs, glycosaminoglycans; PGs, proteoglycans; CAMs, cell adhesion molecules.

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temporal and spatial coordination of numerous individual cell fate processes, each of which is induced by a myriad of signals originating from the extracellular microenvironment (**Fig. 1**)[7]. In brief, a highly dynamic and complex array of biophysical and biochemical signals, transmitted from the outside of a cell by various cell surface receptors and integrated by intracellular signaling pathways, converge to regulate gene expression and ultimately establish cell phenotype. The extracellular microenvironment, which surrounds cells and comprises the molecular signals, is a highly hydrated network hosting three main effectors: (i) insoluble hydrated macromolecules (fibrillar proteins such as collagens, noncollagenous glycoproteins such as elastin, laminin or fibronectin, and hydrophilic proteoglycans with large glycosaminoglycan side chains) called physical signals in **Figure 1**, (ii) soluble macromolecules (growth factors, chemokines and cytokines) and (iii) proteins on the surfaces of neighboring cells. Thus, the ultimate decision of a cell to differentiate, proliferate, migrate, apoptose or perform other specific functions is a coordinated response to the molecular interactions with these ECM effectors. It is noteworthy that the flow of information between cells and their ECM is highly bidirectional, as, for example, observed in processes involving ECM degradation and remodeling.

Naturally derived model ECMs

Cell and matrix biologists have long realized that understanding cell behavior within complex multicellular tissues requires systematically studying cells within the context of specific model microenvironments. These model systems mimic to a certain degree the *in vivo* situation and at the same time may significantly reduce its complexity. Although two-dimensional (2-D) *in vitro* assays are still applied in many cell culture studies, there is increasing agreement that 3-D matrices provide better model systems for physiologic situations[8–13]. Indeed, many physiological (examples exist in morphogenesis and organogenesis) and pathological (e.g., in tumor growth) cellular processes have been demonstrated to occur exclusively when cells are organized in a 3-D fashion.

Cell biologists have a number of experimental model systems at their disposal[13]. These range from multicomponent matrices derived from cells or tissues (e.g., Matrigel, commercially available from BD Biosciences (San Jose, CA, USA), which is solubilized basement membrane preparations extracted from mouse tumors that contains several components of basement membranes enriched with laminin), to matrices composed of individual purified or recombinantly produced ECM proteins, and modified versions of these ECM components, as well as proteolytic or recombinant fragments. These matrices have been used in cell culture studies to recapitulate some aspects of both the organization and multicellular complexity of tissues and to gain insight into functions of the ECMs within diverse tissues and organs.

Therapeutic strategies in regenerative medicine and tissue engineering have greatly benefited from the above studies[14]. Natural ECM-derived biomaterials can be used as carriers for transplanted cells that are subsequently grafted into tissue defects[15,16], and also as cell infiltration matrices to induce regeneration and remodeling *in vivo*[17,18]. For example, collagen and fibrin are clinically well-established and FDA-approved matrices for wound healing to treat burns and chronic wounds, and as tissue sealants, respectively. More pertinent for biomedical and materials engineers, naturally derived materials represent valuable models from which one can derive engineering principles to create artificial materials with similar biological function[19,20].

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Building biomimetic elements into synthetic materials

Although naturally derived biomaterials have proved effective in many basic and clinical applications, the need for custom-made matrices for tissue-specific cell biological investigations[12] drives recapitulation of their key characteristics in synthetic materials. These materials are still being developed to gain more control over the material and thus over the cellular behaviors they induce.

Natural ECMs. From a structural perspective, natural ECMs are gels composed of various protein fibrils and fibers interwoven within a hydrated network of glycosaminoglycan chains. In their most elemental function, ECMs thus provide a structural scaffold that, in combination with interstitial fluid, can resist tensile (via the fibrils) and compressive (via the hydrated network) stresses. In this context it is worth mentioning just how small a proportion of solid material is needed to build mechanically quite robust structures: in many cases less than 1%. Structural ECM proteins include collagens, some of which are long and stiff and thus serve structural functions, whereas others of which serve connecting and recognition functions, and elastin, which forms an extensive crosslinked network of elastic fibers and sheets. The anisotropic fibrillar architecture of natural ECMs has apparent consequences for cell behavior. Because of a tight connection between the cytoskeleton and the ECM through cell-surface receptors, cells sense and respond to the mechanical properties of their environment by converting mechanical signals into chemical signals[21,22]. Consequently, the biophysical properties of ECMs influence various cell functions, including adhesion and migration. Moreover, the fibrillar structure of matrix components brings about adhesion ligand clustering, which has been demonstrated to alter cell behavior[23]. Structural ECM features, such as fibrils and pores, are often of a size compatible with cellular processes involved in migration, which may influence the strategy by which cells migrate through ECMs[24].

Micro- and nanofibrillar synthetic biomaterials. The intricate fibrillar architecture of natural ECM components has inspired several researchers to produce materials with similar structure. Upon fibers that are tens of microns in diameter, cells seem to respond as though to a 2-D substrate, acquiring an unnatural flat shape, leading to a nonphysiological, asymmetrical occupation of adhesion receptors; notwithstanding, such matrices have already shown remarkable success in tissue engineering applications, such as in the reconstruction of a dog urinary bladder[25] or as scaffolds for neural stem cells to facilitate regeneration after brain injury in a mouse stroke model[26]. Polymer processing technologies such as electrospinning[27] allow fiber formation down to the 10 nm scale. One difficulty in nanofiber technology has been in placing cells within a nanofibrillar structure with pore spaces much smaller than a cellular diameter; somehow the network must be formed *in situ*, around the cells, without cellular damage.

Important progress has been made using supramolecular self-assembly to form nanofibrillar matrices *in situ*[28]. Inspired by the understanding of protein self-assembly, these approaches use noncovalent intermolecular interactions to fabricate higher order structures by self-assembly of oligomeric peptide, nucleotide and nonbiological amphiphilic building blocks[29,30]. Whereas many of these systems require self-assembly under conditions that are intolerable to cells, several can gel at near-physiologic conditions. For example, Zhang and coworkers developed a class of nanofibrillar gels with very high water content (>99%) crosslinked by self-assembling of self-complementary amphiphilic peptides in physiological medium. Under appropriate culture conditions, these matrices have been demonstrated to maintain the functions of differentiated neural cells[31] and chondrocytes[32], and to promote the differentiation of liver progenitor cells[33]. Although not equipped with any specific biofunctional ligands, these gels are scaffolds that biomechanically organize cells in a 3-D fashion. Deming and coworkers have presented fibrillar hydrogels from diblock copolypeptide amphiphiles[34]; self-assembly occurs at low solid content and the mild gelation conditions support cell encapsulation[35]. Rational design principles have been put forward to control fiber morphology (e.g., to produce kinked, wavy or branched fibers) and thus scaffold architecture[36]. Stupp and coworkers have presented the next step in such supramolecular gels by synthesizing self-assembling oligomeric-amphiphiles that allow incorporation of specific biomolecular signals[37]; encapsulated neural progenitor cells were observed to differentiate into neurons within scaffolds presenting the laminin-derived peptide IKVAV[38]. This very promising result underscores the potential of incorporating both biomechanical and biomolecular cues.

Nonfibrillar synthetic polymer hydrogels. As the hydrogel character of the natural ECM is one of its key features, it is not surprising that synthetic hydrogels have found important roles in biology and medicine[3,39–41]. Several distinctive features make synthetic hydrogels excellent physicochemical mimetics of natural ECMs. The molecular architecture of crosslinked, hydrophilic polymers can result in tissue-like viscoelastic, diffusive transport, and interstitial flow characteristics. Of critical importance for cell-containing hydrogels, reaction schemes have been developed that are sufficiently gentle to allow formation *in situ*, in the presence of cells, just as gels from ECM components are formed[42]. Such mild chemistries can even be carried out *in vivo*, for example, using minimally invasive surgical techniques, directly within tissue defects. It is possible to incorporate a number of biological characteristics within synthetic hydrogels, including cell adhesion ligands, proteolytic susceptibility and biologically relevant elasticity, as described below.

Materials that present insoluble ligands. The ECM provides bound multifunctional adhesion ligands, including fibronectin, vitronectin and laminin that guide the development and maintenance of cell function. The integrins, a large family of transmembrane, heterodimeric, cell-surface molecules, function as the principle receptors of animal cells for many of these ECM adhesion molecules. Integrins primarily link the macromolecules of the ECM with the cell's cytoskeleton, but are involved as well in cell-cell adhesion and binding to proteases. When bound to ECM ligands, integrins cluster and form associations with various signal transducing molecules to activate specific signaling pathways including those regulated by protein kinase C, the small GTPases Rac and Rho, and MAP kinase. As such, integrins transmit information across the cell membrane and are critical regulators of cell adhesion and migration as well as many other cell functions[43,44].

The pioneering identification of small oligopeptide sequences within ECM adhesion proteins[45] opened an important door to creation of ligand-functionalized materials. Indeed, numerous cell-adhesive ligands have been grafted to materials, as reviewed elsewhere[46,47]. The creation of such highly defined synthetic ECM analogs, in which ligand type, concentration and spatial distribution can be modulated upon a passive background, may help in deciphering the complexity of signaling in cell-ECM interactions. Relevant studies include work on the quantitative information on the ligand density required for a particular cellular response[48]; the influence of adhesion ligand density on cell migration (that is, the discovery of intermediate adhesion strength for optimal cell migration) in 2-D[49,50] and also in 3-D, in modified biopolymer matrices[51–53] and synthetic gels[54,55]; the finding that cells respond to the nanoscale spatial organization of adhesion ligands[23,56]; the relevance of ligand gradients[57] and finally studies on the coregulation of signals[58,59]. These studies provide several examples that well-controlled biomaterial matrices can yield insight into basic cell biological principles.

Materials that enable binding and release of soluble effectors. Natural ECMs modulate tissue dynamics through their ability to locally bind, store and release soluble bioactive ECM effectors such as growth factors to direct them to the right place at the right time[60]. When many growth

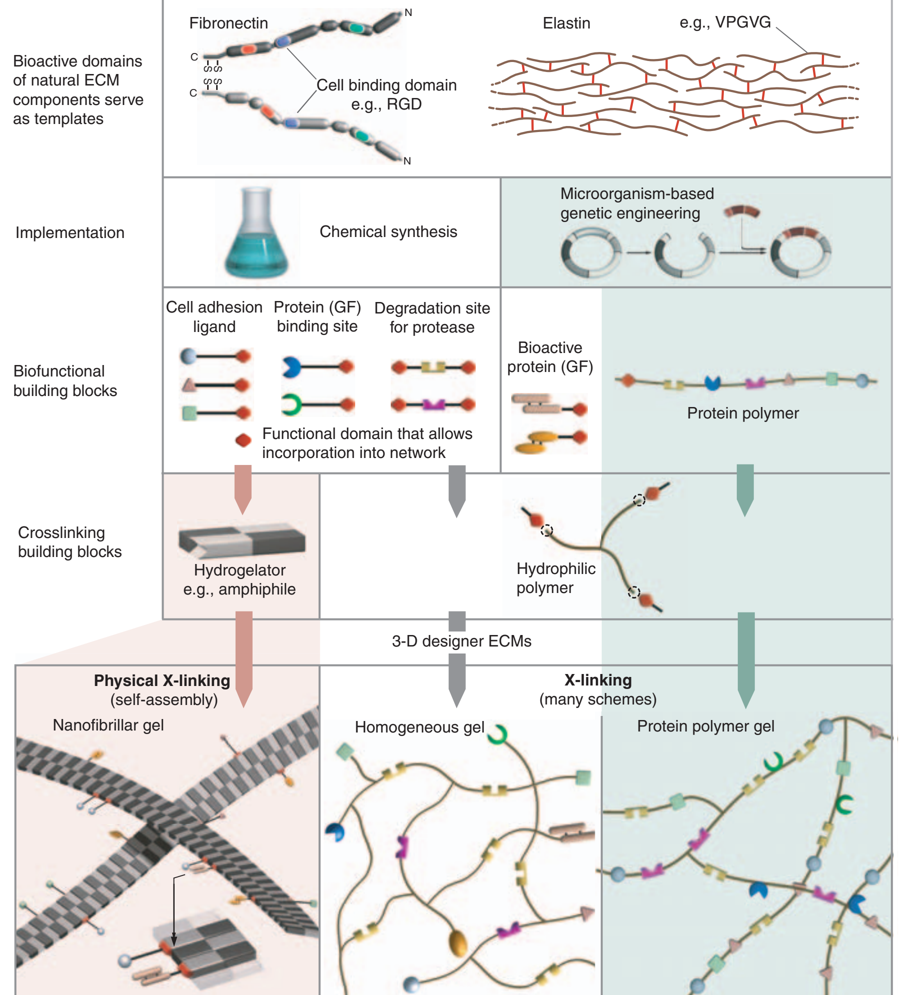

Figure 2 Design strategies for the creation of synthetic biomolecular materials that mimic the complexity of natural ECMs. Bioactive domains of naturally occurring proteins are identified as building blocks (top) and synthesized by either chemical strategies or by protein engineering (recombinant technology). The most important components include cell-adhesive ligands (such as integrin-binding peptides of the prototypical RGD family), binding sites for growth factor (GF) proteins, domains with susceptibility to degradation by cell-secreted or cell-activated proteases to facilitate bidirectional cell-matrix interactions, but also domains with structural function (such as the elastin-derived peptide sequence VPGVG). Synthetic networks can than be obtained by crosslinking of these biofunctional components (from an entire array of building blocks) by distinct crosslinking schemes, involving physical (self-assembly to produce nanofibrillar gels) or chemical mechanisms. The use of such synthetic approaches in ECM design may allow matrices to be tailor-made for a specific cell or tissue.

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factors bind to ECM molecules through, for example, electrostatic interactions to heparan sulfate proteoglycans, it raises their local concentration to levels appropriate for signaling, localizes their morphogenetic activity, protects them from enzymatic degradation and in some cases may increase their biological activity by optimizing receptor-ligand interactions. As growth factors are required in only very tiny quantities to elicit a biological response, the main focus in designing synthetic matrices for growth factor presentation has been to control local growth factor concentration. Several strategies to engineer growth factor release from biomaterials have been presented over the past years and some initial success has been reported in animal models for the regeneration of bone and skin as well as the induction of vascularization, as reviewed elsewhere[61–63]. As many cellular processes involved in morphogenesis require a complex network of several signaling pathways and usually more than one growth factor, recent research efforts have focused on schemes for sequential delivery of multiple growth factors[64]. The use of biological feedback mechanisms in growth factor delivery has also been explored[65]. In this case, a growth factor is bound to the matrix and released upon cellular demand through cell-mediated localized proteolytic cleavage from the matrix[66,67]; this approach substantially mimics the mechanism by which these factors are released *in vivo* from stores in the natural ECM by invading cells in tissue repair.

Stimulus-sensitive materials. Whereas many synthetic biomaterials have been designed to degrade by ester hydrolysis, such nonenzymatic hydrolysis of matrices is uncommon *in vivo*. Rather, the macromolecular components of natural ECMs are degraded by cell-secreted and cell-activated proteases, mainly by matrix metalloproteinases (MMP) and serine proteases. This creates a dynamic reciprocal response, with the ECM stimulating the cells within it and cellular proteases remodeling the ECM and releasing bioactive components from it. As discussed later in more detail, cell-induced proteolysis is often required for 3-D cell migration and invasion, because the porosity of the ECM may lead to barrier function and thus impede migration[68].

Exciting progress has been made in mimicking the proteolytic recognition of natural ECMs in synthetic polymer gels, as examples of a larger class of biomolecule-sensitive networks[69]. Pioneered by early work of Kopecek and coworkers[70], several novel methodologies to create synthetic hydrogels with sensitivity to proteases, including plasmin[71,72], MMPs[55,73] or both of these protease families[54,74,75] have been developed. Proteolytic sensitivity in these materials has been achieved either by stepwise copolymerization of hydrophilic polymers and proteolytically sensitive oligopeptide or protein building blocks or by photo-crosslinking of telechelic peptide-flanked PEG copolymers.

Principles of morphogenesis applied to tissue engineering

Much of the preceding discussion has focused on the structure and function of natural ECMs and how it can be mimicked in synthetic materials to control distinct and relatively simple cell fate processes. As the dynamic state of a tissue is regulated by a highly complex temporal and spatial coordination of many different cell-matrix and cell-cell interactions, such reductionist materials approaches may fail in imitating the complexity of natural ECMs. Therefore, more complex biomaterial systems may be required that contain molecular cues to recapitulate or induce developmental processes in tissue- and organ-specific differentiation and morphogenesis.

Morphogenesis during tissue development is regulated by a number of protein families including hedgehog proteins (Hhgs), Wnt proteins,

Notch ligands, members of the transforming growth factor beta (TGFβ) superfamily such as bone morphogenetic proteins (BMPs), or fibroblast growth factors (FGFs) and fibronectin. These morphogens control self-renewal, migration, differentiation as well as other cell fate processes of uncommitted stem or progenitor cells. The question of how the right quantity of a signaling molecule is detected by the right cells at the right time is the subject of extensive investigation. Once differentiation by the interpretation of morphogenetic signals and gradients has occurred, local cell-cell interactions establish boundaries between different populations of cells.

Intriguingly, morphogenetic processes similar to the ones observed during embryonic development also occur during regeneration of some adult human tissues and therefore regeneration may be regarded as a postembryonic recapitulation of fundamental developmental processes[76,77]. Tissues that have the capacity to regenerate include epithelia, liver, blood, and to a limited extent bone and muscle. In many other tissues, however, the response to damage is the formation of collagen-rich scar tissue following an acute inflammatory response. Although rapid scar tissue formation represents a powerful defense mechanism against infection, it may severely compromise tissue function (e.g., in spinal cord injuries or myocardial infarction). In very general terms, functional tissue recovery can only occur when (i) regeneration-competent progenitor and stem cells are present (e.g., satellite cells in the muscle), and (ii) when these cells are conducted into a regeneration pathway by the presence of relevant morphogenetic signals, or alternatively (iii) when the regenerative process is not suppressed by signals that give way to rapid scar formation. An increased understanding of the cellular and molecular foundations of tissue development and regeneration has paved the way towards more effective therapies for regenerative medicine and tissue engineering[78,79]. A potential lack of regeneration-competent progenitor or stem cells in a particular tissue defect can be overcome by transplanting such cells, either isolated or after integration (and eventual differentiation) into bioengineered tissues constructed from cells and matrices *in vitro*. Several sources of adult stem cells are now readily available and significant progress has been made in controlling their differentiation into multiple lineage pathways. Alternatively, tissue regeneration may be promoted by the application of appropriate biomaterial-based microenvironments to stimulate regeneration *in vivo*. Such matrices should contain signals to attract regeneration-competent cells and to stimulate their proliferation and differentiation into tissue-specific cells, or to block regeneration-suppressing signals.

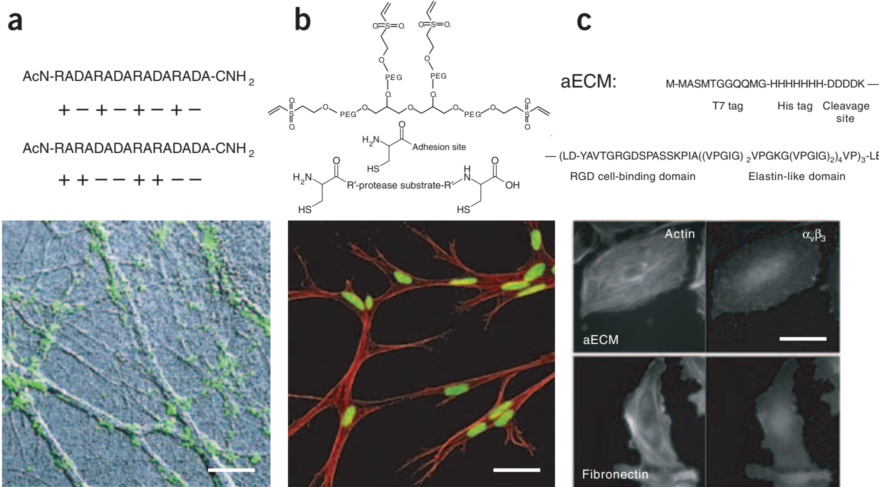

Figure 3 Examples of complex synthetic ECM mimetics proposed in **Figure 2**. (**a**) Nanofibrillar hydrogels formed under physiological conditions from ionic self-assembling peptides (top). These networks support neuronal cell differentiation and extensive neurite outgrowth (bottom). Scale bar, 10 μm. Adapted and reprinted with permission from T.C. Holmes *et al*[31]. © 2003 National Academy of Sciences, USA. (**b**) Hybrid gels formed from cysteine-bearing cell-adhesive and proteolytically degradable peptide building blocks and vinylsulfone-functionalized PEG macromers (top). These gels enable extensive 3-D migration of primary fibroblasts by matrix metalloproteinase- and integrin-dependent mechanisms and, because of localized matrix proteolysis, the morphogenesis of single cells into multicellular structures (bottom). Scale bar, 40 μm. Adapted and reprinted with permission from M.P. Lutolf *et al*[80]. © 2004 Wiley-VCH. (**c**) Creation of synthetic ECMs from artificial protein polymers (aECMs, represented here by one example of a broader family) containing bioactive domains derived from elastin and fibronectin (top). Sequence-specific adhesion of human umbilical vein endothelial cells to bioactive proteins can be achieved by this approach. A similar adhesion behavior of the aECM compared to fibronectin can be observed (bottom). Responses to an artifical ECM (aECM, upper panels) are remarkably similar to responses to a natural extracellular matrix molecule, fibronectin (lower panels). This is true at both the level of the cytoskeleton (left panels) and at the level of the adhesion receptors (right panels). Scale bar, 25 μm. Adapted and reprinted with permission from Liu J.C. *et al*[85]. © 2004 American Chemical Society.

Recapitulating natural ECMs in synthetics: biological cues

Mimicking natural ECMs that regulate complex morphogenetic processes in tissue formation and regeneration necessitates novel design strategies for synthetic biomaterials (**Fig. 2**). These synthetic materials should be biologically multifunctional hydrogel networks, synthesized under physiological conditions, that both biochemically and biophysically mimic natural ECMs. Their functionality should be adjustable to a particular biological environment to obtain cell- and tissue-specificity. Ideally, one would create them from an array of biologically functional building blocks, in some form of a modular design. The precursor building blocks could be crosslinked into solid networks by several means (**Fig. 2** and **Fig. 3**): (i) Small organic gel-formers, such as peptides or peptide-amphiphiles, containing binding sites for biologically functional ligands[38], can be designed to self-assemble into supramolecular structures, allowing the creation of heterogeneous nanofibrillar ECM mimetics[28] (**Fig. 2**, bottom left; **Fig. 3**, left). (ii) Hybrid gels can be formed from bioactive building blocks bearing chemically reactive functional groups (such as amines or thiols)[80] or physically interactive groups[81] and end-functionalized hydrophilic polymers such as N-(2-hydroxypropyl)-methacrylamide (HPMA) or PEG that act as chemical or physical crosslinkers (**Fig. 2**, bottom middle; **Fig. 3**, middle). (iii) Recombinant DNA technology can be used to create artificial protein polymers with desired bioactive domains *de novo*[82–85] (**Fig. 2**, bottom right; **Fig. 3**, right). Genes corresponding to structural and functional elements found in natural ECMs can be synthesized, cloned and expressed in a convenient production host. Such protein polymers can be covalently crosslinked into a network, for example, by reaction with functionalized hydrophilic polymers[71] or other chemical crosslinkers[86] targeting amines or thiols on the protein polymer, by radiation crosslinking[87] or through self-assembly by protein-protein interactions[88].

Synthetic ECMs that enable 3-D cell migration. Cell migration through extracellular matrices is fundamental to morphogenetic

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Morphogenetic steps	Cellular processes and ECM regulators	Synthetic ECM engineering	Building block
① Endothelial progenitors	Endothelial progenitor recruitment and differentiation VEGF, placental growth factor (PlGF), angiopoietin (Ang)-1, cytokines, Notch, Shh, …	Matrix that releases signals to recruit and differentiate progenitors	
② Endothelial cells (ECs)	EC quiescence ECM proteins: collagens, fibronectin, … (integrins: $\alpha_2\beta_1$, $\alpha_1\beta_1$, $\alpha_5\beta_1$, $\alpha_t\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, …)	Matrix that presents multiple adhesion ligands	
③ Vacuole and lumen formation	EC activation, proliferation, migration Growth factors: VEGFs,FGFs, … Cytokines Proteases: MMPs, plasmin HIF	Growth factor delivery (ECM tethered and released upon cellular demand) Protease sensitivity to enable remodeling	
④ Branching and sprouting	EC differentiation and morphogenesis VEGF, Notch, angiopoietins, ephrin, … Cell-cell interactions: cadherins, connexins, …	Induced	
⑤ Capillary tube networks		Induced	
⑥ SMC recruitment Tube stabilization and maintenance	SMC recruitment PDGF, Ang-1, TGF-β, PlGF, … Vessel stabilization Protease inhibitors Cell-cell interactions Flow	Partially induced Matrix-presenting signals for SMC recruitment (ev.)	

Figure 4 Morphogenetic steps and underlying regulatory molecules involved in endothelial cell assembly into capillary tube structures, and subsequent stabilization of tubes into mature blood vessels. Highly complex, multifunctional synthetic matrices (with hypothetical building blocks corresponding to the one in **Figure 2**) will be required to recapitulate these processes in the future.

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processes in tissue development, homeostasis and regeneration[89]. Migration occurs, for example, in response to gradients of soluble or insoluble signals and changes in cell-cell contacts. On planar surfaces, many key aspects of the underlying regulatory pathways have been identified[90]. 2-D migration can be regarded as a cyclic process involving multiple steps: polarization of the cell and extension of protrusions in the direction of migration, stabilization of protrusions by adhesion to the ECM, forward movement by contraction and cell detachment at the rear. Not surprisingly, integrins play an important role by providing traction for the forward movement and also by transmitting ECM guidance signals. In 3-D, migration is further complicated by the need to overcome the biophysical resistance of the surrounding ECM. Depending on cell and ECM type, migration of single cells in 3-D can involve proteolytic (most commonly) and nonproteolytic (e.g., in leukocytes) strategies[24]; recent studies on tumor cell migration provide evidence for a cellular plasticity in selecting a particular migration strategy[91]. During proteolytic migration, cells clear a path by secreting and activating proteases, including MMPs, serine proteases and hyaluronidase, that specifically degrade protein or proteoglycan components of the pericellular matrix. Degradation is highly localized because of the involvement of membrane-bound proteases (e.g., MT-MMPs), complexation of soluble proteases to cell surface receptors (e.g., MMP-2 interaction with integrin $\alpha_v\beta_3$, and binding of urokinase plasminogen activator to urokinase plasminogen activator receptor), and a tightly regulated balance between active proteases and their natural inhibitors (such as inhibition of MMPs by TIMPs). On the other hand, amoeboid migration is driven by cell shape adaptation (that is, squeezing through preexisting matrix pores) and deformation of the ECM network.

Synthetic materials must contain cell-adhesive ligands for traction, as well as space for forward movement. Nonproteolytic migration is thus enabled by cell-adhesive matrices with preformed macroscopic pores (e.g., ref. 92), however, only when the minimal pore size is larger than the cell diameter. Nonproteolytic migration may also occur in soft (physically crosslinked) fibrillar networks[31,38], probably by a combination of squeezing through pores and fiber deformation or even rupture.

In perhaps the most biomimetic concept for 3-D cell migration in synthetic ECMs, proteolytic pathways have been exploited. In one approach, photopolymerized hydrogels have been synthesized by polymerization of acrylated PEG derivatives containing peptide substrates for plasmin and MMPs in their backbone, and cell adhesive peptides grafted on one end in a pendant fashion[54,74,75]. A related system has been described based on conjugate addition reactions[72,80]. Crosslinking occurs upon stepwise copolymerization of two building blocks, namely biologically active peptide sequences as protease substrates (for MMPs or plasmin) that contain flanking cysteine residues with the reactive thiols, and end-functionalized multiarm polyethylene glycol (PEG) macromers serving

as a crosslinking entity responsible for the networks biophysical characteristics. The simultaneous incorporation of adhesion ligands as pendant functionalities into the networks has been demonstrated to enable integrin-dependent proteolytic (as demonstrated independently for MMPs and plasmin) 3-D migration of fibroblasts and endothelial cells. The cell migration rate in these networks can be controlled almost independently by several characteristics of the matrix[55], including its physicochemical characteristics, adhesion ligand density or proteolytic sensitivity of the cysteine-containing peptide. Synthetic materials that support proteolytic migration have also been engineered from protein polymers, by grafting PEG diacrylate onto artificial, cell adhesive and plasmin-sensitive protein polymers containing multiple cysteine residues[71].

Synthetic ECMs that can control stem cell fate: engineering stem cell niches. Tissue formation, homeostasis and regeneration are critically dependent on stem cells and their commitment to differentiated lineages. Although knowledge about signals and the underlying pathways regulating stem cell fate are being identified rapidly, significant technical obstacles need to be overcome before stem cells can be used efficiently and safely in the clinic[93,94]. One of the main challenges appears to be the control of stem cell fate outside of the cells' natural environment. Adult stem cells normally reside within specific extracellular regulatory microenvironments—stem cell niches—consisting of a complex mixture of soluble and insoluble, short- and long-range ECM signals, which regulate their behavior[95,96]. These multiple, local environmental cues are integrated by cells that respond by choosing self-renewal or a pathway of differentiation. Outside of their niche, adult stem cells lose their developmental potential quickly[97]. The design of synthetic materials that mimic natural stem cell microenvironments may be a potentially powerful tool to both understand and control stem cell function.

A variety of artificial stem cell microenvironments are being explored in the context of neural stem cell fate control. Mahoney and Saltzman have designed a synthetic microenvironment useful as a transplantation vehicle based on polylysine-coated poly(lactide-co-glycolide) microparticles loaded with nerve growth factor-beta[98]. The combination of a cell-adhesive matrix and a controlled-release scheme for a morphogenetic factor allowed them to control fetal brain cell survival and differentiation in a rat model. Liu and colleagues have pioneered the concept of engineered stem cell niches by developing a family of artificial ECM proteins to control neural stem cell function[99,100]. Protein polymers were expressed in *Escherichia coli*, consisting of an elastin backbone along with two ligands to the Notch receptor, namely the active domain of hJagged1 and hDelta1. Synthetic biomaterials with less biological functionality may also be beneficial because they function as a mechanical scaffold for stem cells to support cell growth and to bridge large tissue defects[26,101]. Combinatorial and microarray approaches have been adopted recently in high-throughput screening of combinatorial materials[102,103] to control embryonic stem cell fate on polymer surfaces[104]. Such high-throughput approaches may also hold much promise in 3-D screening of libraries[105] of biofunctional groups such as morphogenetic proteins incorporated within synthetic 3-D materials.

Synthetic ECMs permit cellular remodeling and tissue regeneration *in vivo*. Synthetic biomimetic materials have been developed to serve as provisional matrices for tissue regeneration *in vivo*. Based upon the paradigm of fibrin's function as a temporary matrix in tissue repair, biomimetic characteristics for synthetic materials needed to induce regeneration *in vivo* are the presence of (i) ligands for cell adhesion (ii) a mechanism of relatively rapid and localized matrix dissolution, ideally in temporal and spatial synchrony with cell invasion, and (iii) the delivery of morphogenetic signals to attract endogenous progenitor cells and induce their differentiation to a tissue-specific pathway. When bioactive materials were designed, based on this rationale, to be sensitive to MMPs or plasmin, and also contained an integrin-binding adhesion ligand and the bone-inducing growth factor BMP-2, complete matrix remodeling into bone was observed when the materials were implanted in bone defects[72,106]. This design of a cell-adhesive and cell-responsive (that is, provisional) matrix may be extended to other applications in regeneration.

Blood vessel growth represents another major challenge. The lack of a functional vasculature is the cause of numerous pathologies and is also a major stumbling block for successful cell-transplantation tissue engineering therapies[107]. Three distinct mechanisms of vessel growth are known: vasculogenesis (*de novo* formation of blood vessels by endothelial progenitors), angiogenesis (sprouting of vessels from preexisting ones) and arteriogenesis (stabilization of blood vessels by mural cells). Many regulating soluble signals and their receptors have been identified. The major signals include vascular endothelial growth factors (VEGFs), FGFs, TGFs, angiopoietins, ephrins, placental growth factors and various chemokines[108]. As mentioned above, synthetic biomaterials have been developed that are able to deliver such factors with controlled pharmacokinetics. However, an entire cascade of morphogenetic processes is required for blood vessel formation within a biomaterial matrix, with many signals being provided by the extracellular milieu[109] (**Fig. 4**). Recent progress has been made by our group with biomimetic matrices toward this end as well: MMP-sensitive, integrin-binding and VEGF-containing matrices have been replaced by well-formed new blood vessels in animal models[65]. In this case, an important relationship between local growth factor dose and blood vessel morphology seems to be apparent, with only low local concentrations leading to well-formed structures and higher concentrations leading to hyperpermeable vasculature[110,111]. Thus in this application the concept of the cell directing the release of growth factor seems particularly valuable since the tissues responded in a physiological manner, forming healthy vessels in response to the matrix-bound VEGF.

Future challenges

Although considerable progress has been made already, much work remains to develop biomaterial and biomolecular approaches that recapitulate the elaborate biological recognition and signaling functions of the extracellular milieu for application in tissue engineering, repair and regeneration. Ongoing challenges remain in controlling the dynamics and spatial organization of presentation of multiple signals.

Toward modulation of dynamics, the use of stimulus-sensitive linkers, protecting groups and exposing mechanisms may provide paths forward. Combination of display approaches using both bioresponsive and time-sensitive mechanisms may be particularly powerful. It may be possible to exploit biomechanical and biochemical stimuli to expose cryptic biomolecular signals in synthetic biomaterials, as also occurs in some natural ECM molecules.

Toward modulation of spatial arrangement, hierarchically complex tissues may require the establishment of boundaries between different tissue and cell types. Accomplishing this could be imagined by incorporating cell-type-specific chemotactic factors in a spatially controlled manner, by using or upregulating self-segregating molecules, such as cadherins, or by incorporating potentially boundary-forming signals, such as the ephrins. Coculture and cotransplantation of different cell types may also enable hierarchical segmentation.

As to the signals themselves, the number of molecule families and even entire biomolecule classes remain underexploited in tissue engineering and regeneration. Many signals involved in embryonic development, such as Wnts, hedgehog proteins or Notch ligands, may be important to control adult stem cell self-renewal. Likewise, it may be particularly interesting to manipulate transcription directly, for example,

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by biomaterial matrix-controlled delivery of molecules that manipulate expression of transcription factors regulating development or morphogen expression.

As to more basic investigations, until recently, cell and matrix biologists have almost exclusively used natural ECM-derived materials as 3-D model systems. Precise control over the extracellular microenvironment may be useful not only in tissue engineering and regeneration, but also in more basic studies of development and pathogenesis and it is likely that we will see many payoffs of such strategies in the near future.

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