

Micropatterning as a tool to decipher cell morphogenesis and functions

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Summary

In situ, cells are highly sensitive to geometrical and mechanical constraints from their microenvironment. These parameters are, however, uncontrolled under classic culture conditions, which are thus highly artefactual. Micro-engineering techniques provide tools to modify the chemical properties of cell culture substrates at sub-cellular scales. These can be used to restrict the location and shape of the substrate regions, in which cells can attach, so-called micropatterns. Recent progress in micropatterning techniques has enabled the control of most of the crucial parameters of the cell microenvironment. Engineered micropatterns can provide a micrometer-scale, soft, 3-dimensional, complex and dynamic microenvironment for individual cells or for multi-cellular arrangements. Although artificial, micropatterned substrates allow the reconstitution of physiological in situ conditions for controlled in vitro cell culture and have been used to reveal fundamental cell morphogenetic processes as highlighted in this review. By manipulating micropattern shapes, cells were shown to precisely adapt their cytoskeleton architecture to the geometry of their microenvironment. Remodelling of actin and microtubule networks participates in the adaptation of the entire cell polarity with respect to external constraints. These modifications further impact cell migration, growth and differentiation.

Key words: Cell architecture, Cell differentiation, Cell growth, Cell polarity, Microenvironment, Micropatterning

Introduction

Cells in situ, within organs or tissues, are embedded into a highly structured microenvironment. The cell microenvironment, i.e. the extracellular matrix (ECM) and neighbouring cells, imposes specific boundary conditions that influence not only cell architecture and mechanics, but also cell polarity and function. The size of the microenvironment limits the cell volume and cell spreading. Its structure, i.e. the positioning of adjacent cells and the location and orientation of ECM fibres, dictates the spatial distributions of cell adhesion and that of unattached cell surfaces. The biochemical composition and stiffness of the microenvironment specify the factors that can engage in cell adhesion, and thereby affect intracellular signalling pathways (Fig. 1). These pathways subsequently dictate the assembly and dynamics of cytoskeleton networks. In addition to having a role in the configuration of intra-cellular organisation, the cell microenvironment also influences gene expression and cell differentiation. Therefore, the properties of the microenvironment are crucial for the regulation of cellular functions.

However, these important properties of the cell microenvironment are completely abrogated under classic cell culture conditions. In a Petri dish, cells encounter a homogeneous adhesion substrate that is flat, rigid and vast, and thus has little in common with the characteristics of the in situ microenvironment (Fig. 1). Such conditions have been used for decades to stimulate cell growth and to keep cells alive out of the context of their native tissue but, nevertheless, they remain highly artefactual. By contrast, micropatterning methods allow the reconstitution of tissue-like conditions for in vitro cell culture. Cell micropatterning comprises the fabrication and use of a culture substrate with microscopic features that impose a defined cell adhesion pattern. It is a highly efficient method to investigate the sensitivity and response of a cell to specific

microenvironmental cues. Here, some of the classic micropatterning methods are briefly presented, with particular emphasis of those that are now accessible to all cell biology laboratories (see Box 1). A few promising technological developments that may allow the artificial in vitro recapitulation of the complex composition of the cell microenvironment, and its mechanics and dynamics in the near future are also discussed (see Box 2).

As micropatterning methods are now becoming increasingly popular in biomedical research, I will review some of the groundbreaking studies that have used micro-patterning methods to investigate cell physiology. From early signal sensation to multi-cellular morphogenesis, these studies highlight the crucial role of the mechanical and geometrical properties of the microenvironment in cell physiology. Their discussion below will follow the path by which the microenvironment guides cells. Naturally, cell adhesion is the first cellular functional unit that responds to microenvironmental cues. It then guides actin and microtubule networks assembly and, thereby, further orients the construction of cell internal architecture and establishment of cell polarity. This spatial organisation also regulates cell growth and differentiation. Finally, intercellular coordination propagates spatial information, and impacts the mechanical and functional coherence of the entire tissue.

Cell architecture

The physiological cell microenvironment consists of extracellular matrix (ECM) fibres, adjacent cells and extracellular fluids, and the cell adhesion machinery is the first cellular component to encounter it. Upon binding of extracellular ligands, localised signalling is induced with subsequent assembly of the cytoskeleton. These localised events will affect the entire cell architecture, because the intracellular space is physically connected and

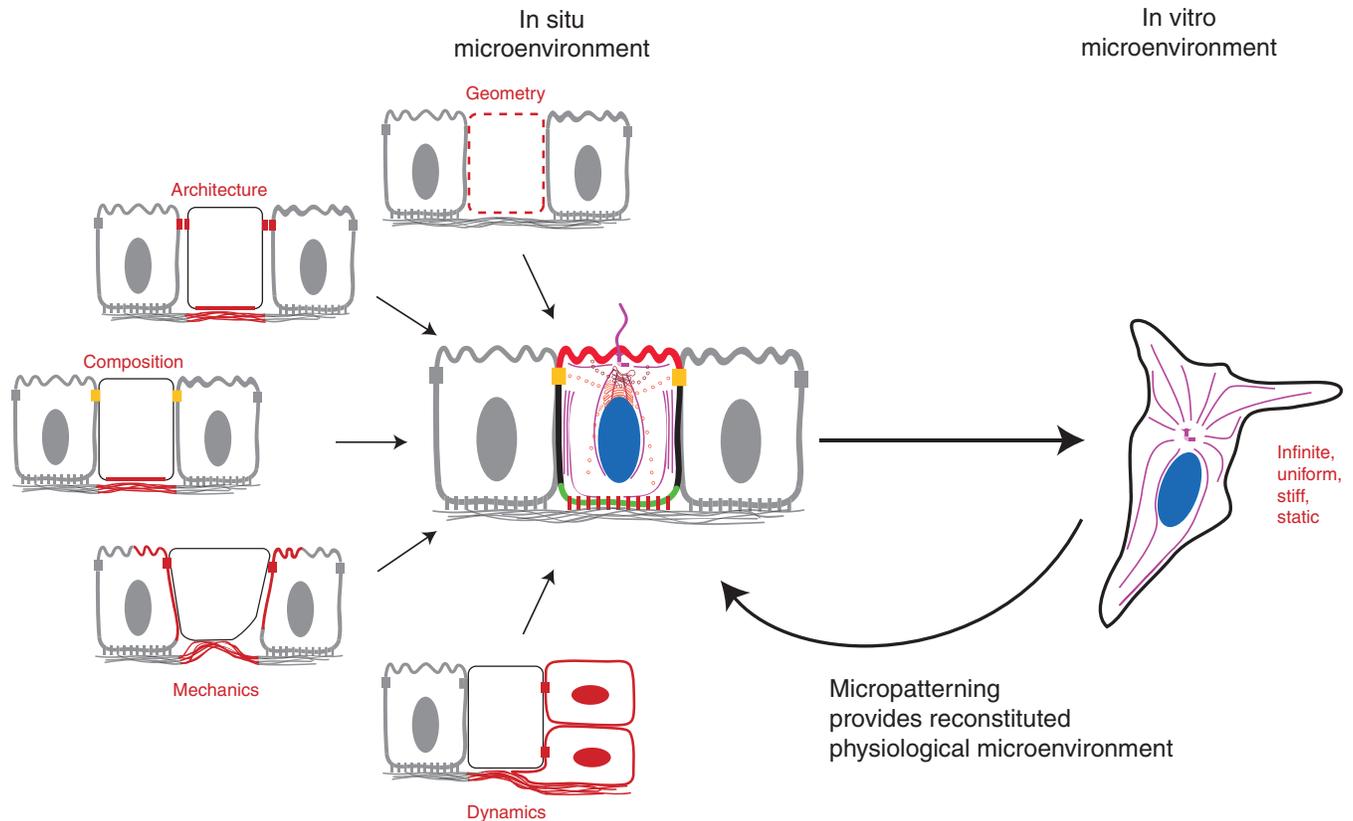


Fig. 1. The cell microenvironment in situ and in vitro. In situ (left), the cell microenvironment, i.e. adjacent cells and the ECM (grey), provide a variety of cues (red) for cell morphogenesis, ranging from geometrical constraint to biochemical signalling and mechanical resistance. Under classic culture conditions (in vitro microenvironment), the entirety of this spatial, chemical and mechanical information is lost as the cell microenvironment is a flat plastic or glass surface. Micropatterning methods can be used to modify the microenvironment of cells in culture and restore – to some extent – the external guiding information. These artificial surface treatments render cell culture conditions less artefactual and resemble more closely physiological conditions.

mechanically supported by a dynamic equilibrium (Ingber, 2003; Ingber, 2006).

Integrin-based cell adhesion

Integrins are transmembrane receptors that bind to ECM proteins and to intracellular actin filaments. When cells contact the ECM, they change their shape and spread in a multi-step process that includes cell attachment, formation of membrane protrusion, extension of cell membrane, and formation and contraction of stress fibers, which further stimulate cell attachment, membrane protrusion and cell shape extension.

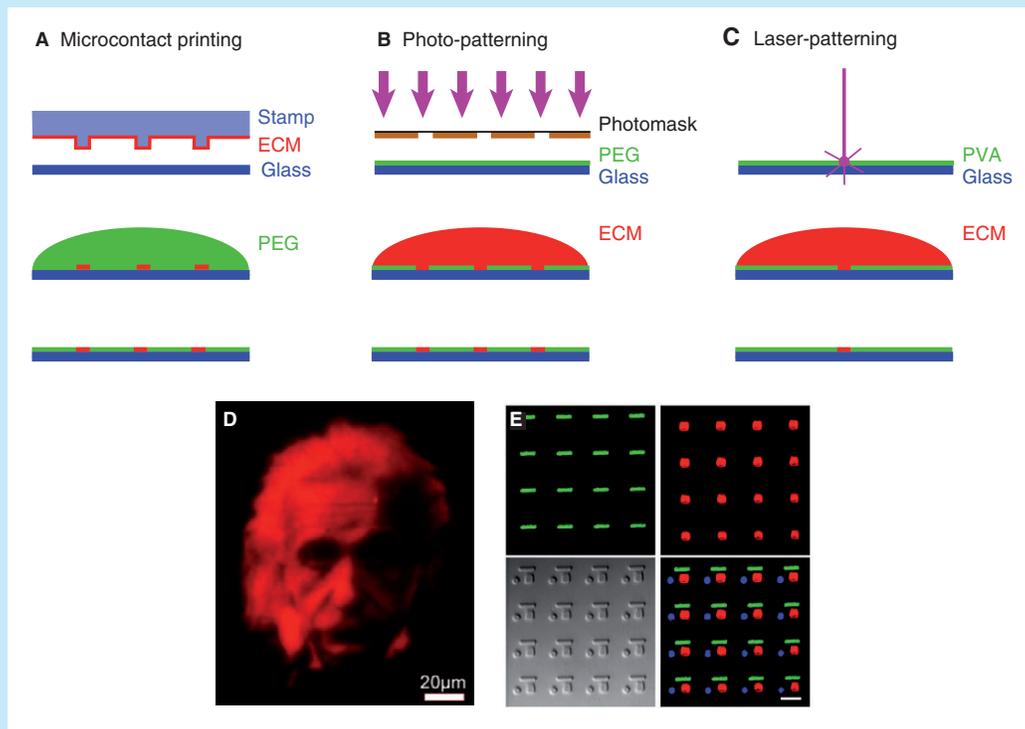
The attachment of the actin cytoskeleton to cell adhesions requires integrin clustering. Nanopatterning methods have led to determine the maximum distance of 60 nm between integrin molecules – a distance that still allows intracellular recruitment of actin filaments and signalling molecules (Arnold et al., 2004). Arrays of adhesive dots have been used to study the formation of filopodia and subsequent spreading steps. Depending on the cell type and the level of Rac activation, cells need a minimal distance between adhesion sites, so that filopodia can bridge them and promote cell spreading (Guillou et al., 2008; Lehnert et al., 2004). Therefore, when dot spacing is non-homogeneous, cells align with the higher linear density of adhesion sites (Xia et al., 2008). Above a critical length of cell spreading, cells will form stress fibers between adhesion sites, which will grow and mature into focal adhesions (Bershadsky et al., 2003). Manipulating the size of

adhesion sites has shown that, as the size of focal adhesions increases, their composition and phosphorylation status changes. Zyxin, a zinc-binding phosphoprotein that concentrates at focal adhesions and along the actin cytoskeleton, is recruited onto actin bundles, thereby promoting the production of traction forces (Goffin et al., 2006). As a cell spreads, it develops larger focal adhesions, forms stress fibres and pulls on the substrate (Fig. 2) (Tan et al., 2003). The shape of micropatterned islands can be used to limit cell spreading and mimic the physiological spatial confinement (Singhvi et al., 1994), which keeps cells in a more relaxed state (Pitaval et al., 2010; Polte et al., 2004; Roca-Cusachs et al., 2008; Tan et al., 2003).

Non-migrating cells, in particular when confined on micropatterned islands, develop specific and dynamic actin structures on non-moving focal adhesion. Zyxin and the vasodilator-stimulated phosphoprotein (VASP) localise to actin filaments bundles and display a retrograde flux, particularly on those actin bundles that are formed at cell apices and thus experience higher traction forces (Guo and Wang, 2007) (see below for more details). Focal adhesions accumulate in the most distal regions of cell periphery, such as the apices of a triangle, where they grow and promote the formation of lamellipodia, filopodia and other membrane protrusions (Fig. 2) (Brock et al., 2003; Parker et al., 2002; Théry et al., 2005).

Another important aspect of the physiological ECM network is that it is fibrillar and heterogeneous. Therefore, it does not always

Box 1. Micropatterning of glass or plastic culture substrates



Although the first micropatterning techniques for manipulating cell adhesion pattern have been developed more than 40 years ago (Carter, 1967; Harris, 1973), they only recently became accessible to all cell biology laboratories and are now commercially available. Among the numerous micropatterning techniques (Folch and Toner, 2000; Whitesides et al., 2001), microcontact printing (A) has become the most popular and is widely used [see Ruiz and Chen (Ruiz and Chen, 2007) for a detailed review]. A polydimethylsiloxane (PDMS) stamp with desired micro-features, is used to print ECM proteins onto the culture substrate (Mrksich, 2009; Théry and Piel, 2009). Despite its popularity, microcontact printing has several drawbacks limiting its routine use in cell biology laboratories, for example the requirement of an initial etching step to microfabricate the stamp and variations in the quality of protein transfer.

Alternative, non-etching, methods have been developed on the basis of direct photo-patterning (B). Photosensitisers and fluorophores have been used to produce reactive oxygen species upon light excitation, which induce protein grafting on polyethylene glycol (PEG) surfaces (Balakirev et al., 2005; Belisle et al., 2009). UV light can also be used to excite any photosensitive chemical groups and to detach the protein-repellent part of a molecule that has been grafted on the substrate to allow further binding of ligands (Dillmore et al., 2004; Kim et al., 2010; Kikuchi et al., 2008a). Spatially controlled UV exposure can be achieved either with a photomask in contact with the substrate (see B) (Fink et al., 2007) or by placing a photomask in the object plane of the microscope objective (Belisle et al., 2009). The photomask can also be used as a density filter to control UV exposure onto the sample to finely control the local protein concentration on the substrate resulting in remarkable contrast and detail [panel D, published with permission from Belisle et al. (Belisle et al., 2009)].

However, these photo-patterning methods require either dedicated chemistry to engineer photosensitive materials or the use of photosensitisers, which are generally not very biocompatible. High-energy light, such as deep UV light below 200nm (Azioune et al., 2010) or concentrated light from pulsed lasers (see panel C) (Doyle et al., 2009), have also proved useful to create local plasma and directly oxidise culture substrates without the need for dedicated photo-chemistry. Exposure to plasma renders hydrophobic polystyrene culture substrates hydrophilic (Welle and Gottwald, 2002; Welle et al., 2005) and also destroys the protein and cell repellent properties of PEG (Azioune et al., 2009), thus allowing for further protein grafting.

Most of these methods can be repeated to micropattern several distinct proteins at specific locations (multi-patterning), as long as each step preserves the preceding protein coating. Repeated micro-contact printing steps are difficult to perform, as they require to align all printing steps. Methods in which the substrate is held onto the motorised stage of the microscope are more convenient for the repetition of sequential exposure-grafting steps [see sequential photopatterning in panel E: fibrinogen (green), vitronectin (red) and fibronectin (blue); published with permission from Doyle et al. (Doyle et al., 2009)] (Kim et al., 2010; Nakanishi et al., 2006). In addition, they can be performed in the presence of cells and thus can be used to micropattern multiple cell types using simple successive light exposures (Kikuchi et al., 2008b).

completely surround the cells, leaving free contact surfaces on cells, which are not attached to the ECM or to adjacent cells. Micropatterns of various shapes, such as V or T shapes, have been used to force the cell to spread over non-adhesive regions that aim to mimic this heterogeneity of the ECM (Fig. 2). The resulting cells systematically reinforce their peripheral actin bundles and

form large, RhoA-dependent stress fibers over non-adhesive regions (James et al., 2008; Rossier et al., 2010; Théry et al., 2006a). Taken together, these experiments illustrate how the geometry of the cell-adhesive microenvironment affects adhesion growth and dynamics and, thereby, establishes the early steps in the construction of the cytoskeleton network. They also stress that cells are able to translate

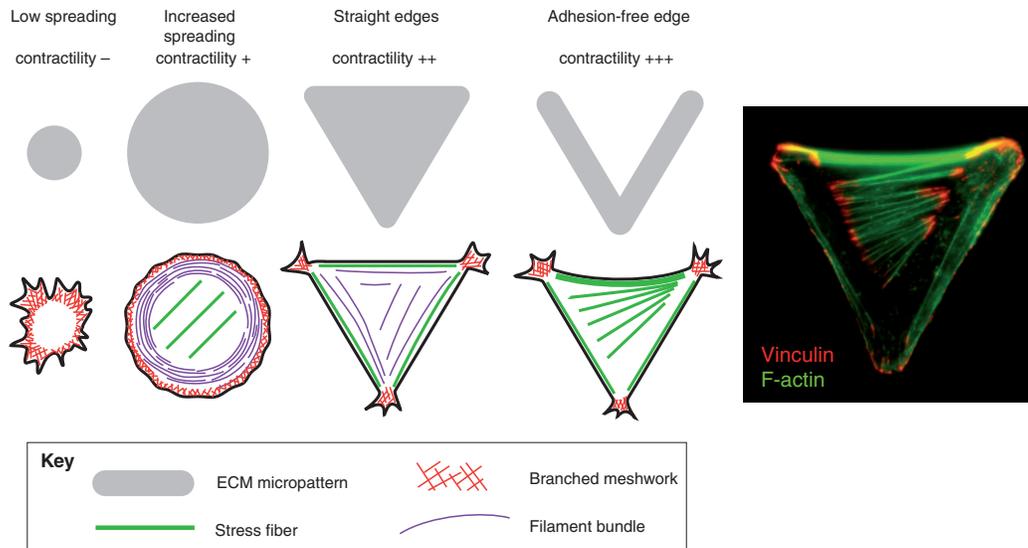


Fig. 2. Effect of cell adhesion patterns on cell shape, architecture and contractility. A cell preferentially assembles branched actin meshworks when confined to a small micropattern (first shape, far left). As the cell spreads, it forms circumferential actin bundles that move inwards, as well as stress fibres within the cell centre (second shape). Straight edges promote the assembly of stress fibres (third shape), which become more pronounced above non adhesive (or concave) edges (fourth shape, far right), e.g. along the edge that connects the two extremities of the V shape where the cell is highly contracted (micrograph); published with permission from Théry et al. (Théry et al., 2006a).

the geometry of their microenvironment into an internal organisation of the cytoskeleton that allows them to bridge distant adhesion site, and ensures a mechanical balance and continuity within tissues.

Cadherin-dependent cell adhesion

Cadherins are another class of transmembrane receptors, which form *trans*-dimers through interactions between the cadherins of contacting cells. Recently, the manipulation of cadherin-coated substrates with a rigidity that can be fine-tuned (see Box 2) has demonstrated that the cadherin-based adhesion network has mechanosensitive properties – with an accumulation of receptors in stiffer substrates and a subsequent increase in tension (Ladoux et al., 2010). Measurement of inter-cellular tension at cell-cell junctions highlights the positive correlation between the size of cell-cell junction and the tension it supports (Liu et al., 2010).

A further step towards the complete reconstitution of a physiological adhesive microenvironment is the co-patterning of integrin and cadherin adhesion sites that will allow to mimic the localised stimulation the cell experiences by different surface receptors *in vivo* (see Box 1). Co-patterning of collagen, to which integrins bind, and cadherin allows to easily and directly investigate their crosstalk. It was found, that focal adhesion maturation on stiff substrates reduces the formation of cadherin complexes between the cell and the patterned cadherin, at least in the early stages of cell adhesion. The formation of cadherin complexes is favoured on more compliant substrates, on which focal adhesion is less (Tsai and Kam, 2009). Interestingly, epithelial cells are not able to migrate on strips that are coated with cadherins, but they are able to migrate on strips coated with collagen (Borghi et al., 2010). On micropatterns with alternating lines of cadherin and collagen, lamellipodial activity was reduced in the presence of cadherin, and traction forces were predominantly found on collagen-coated strips. Thus, cadherins appear to restrict the production of traction forces to collagen and, thereby, seem to orient cell migration. In

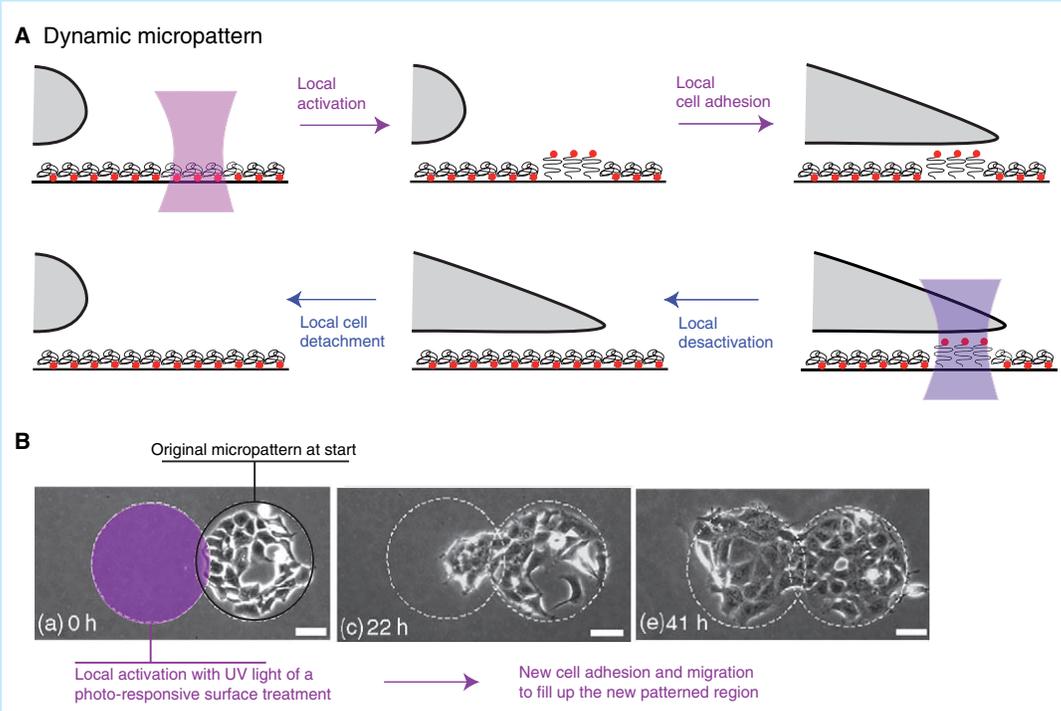
conclusion, manipulating the spatial positioning of integrins and cadherins has revealed how cadherins and integrins influence and oppose each other. The interplay between integrin and cadherin is very likely to have important consequences for the establishment of multicellular structures.

Oriented cell spreading

Many cells within tissues, such as fibroblasts or muscle cells, have elongated shapes. To investigate whether cell shape elongation has any effect on the cell architecture, experiments have been carried out, in which cells have been plated on micropatterns of ellipsoidal or rectangular shape. The resulting anisotropic cell adhesion and shape lead to an anisotropic intra-cellular organisation of actin filaments. Stress fibers are found to align with the long cell axis in fibroblasts, mesenchymal cells, vascular smooth muscle cells and vascular endothelial cells (James et al., 2008; Kilian et al., 2010). Similarly, myofibrils align with the long cell axis in myocytes (Bray et al., 2008; Geisse et al., 2009). As cells elongate, they form more focal adhesions at their extremity, which promotes further elongation. How the final cell length is determined is unknown, but the use of micropatterned lines has revealed that precise regulation mechanisms exist in some cell types. For example, fibroblasts adopt the same length when they are either rounded on substrates without a pattern or elongated on micropatterned lines. However, epithelial cells do not appear to regulate their cell length to this extent and become longer when cultured on lines compared with homogeneous substrates (Levina et al., 2001).

Interestingly, some differences in the regulation of cell contractility in elongated cells have been observed depending on the cell type. In mesenchymal cells, high levels of myosin II decoration on stress fibers are observed in elongated cells compared with round cells, suggesting that elongated cells are more contractile (Kilian et al., 2010). By contrast, in vascular smooth muscle cells and vascular endothelial cells, cell elongation induces a reduction of F-actin content, cytoskeletal stiffness and contractility (Roca-

Box 2. Micropatterning techniques for soft and dynamic 3D micro-environments



The 3D structure of the cell microenvironment can be mimicked by plating cells in either microwells or photo-polymerised gels. Microwells can be obtained in PDMS, agarose or gelled collagen replicates of microfabricated templates (Dusseiller et al., 2005; Nelson and Chen, 2002; Nelson et al., 2006). Pulsed laser can be used to design holes in collagen gels (Liu et al., 2005b) or to photo-polymerise hydrogel for the purpose of guiding cells in 3D (Hahn et al., 2006; Lee et al., 2008; Liu Tsang et al., 2007).

Soft substrates with controlled stiffness can be obtained with polyacrylamide gels with controlled degree of reticulation (Beningo et al., 2002), which can be micropatterned using microfabricated stencils with square holes (Parker et al., 2002; Wang et al., 2002) or microcontact printing methods (Damjanovic et al., 2005). Alternatively, arrays of micropillars of various aspect ratios have been used (Fu et al., 2010; Ladoux et al., 2010), which can be micropatterned using microcontact printing (Liu et al., 2010; Tan et al., 2003).

The cell microenvironment is also highly dynamic because of movements of adjacent cells and ECM remodelling. Reconstructing these changes over time requires turning localised adhesive regions into non-adhesive regions, and vice versa. To that end, technological efforts have been made to fabricate so-called dynamic or switchable surfaces, whose adhesiveness can be turned on and off at will (see panel A). Several stimuli can be used to regulate the physico-chemical properties of a substrate in a localised manner, including electric potential, temperature, pH or light (reviewed in Liu et al., 2005a; Nakanishi et al., 2008). Electric fields can be used to detach the cell-repellent surfaces (Fan et al., 2008; Yeo and Mrksich, 2006; Yousaf et al., 2001). Once the surface is desorbed, cells are released from their micropattern constraint and are free to move (Jiang et al., 2005). Electric stimulation can be performed on transparent electrodes that are compatible with optical imaging and allows for the sequential addition of different cell types at different locations (Gabi et al., 2010; Shah et al., 2008) and for reversible ligand patterning (Yeo et al., 2003). However, these local electrical desorption requires sophisticated methodologies that use microfabricated electrodes and/or micropatterning of electroresponsive surface coatings (Raghavan et al., 2010a). Photo-patterning methods (see Box 1) allow 'on the fly' micropatterning during cell culture to guide cell movement [see panel B; still images from a time-lapse movie of cell migration towards a photo-activated region, published with permission from Nakanishi et al. (Nakanishi et al., 2006)] (Kikuchi et al., 2008a) or to sequentially add new cell types (Kikuchi et al., 2008b).

An alternative to changing the cell microenvironment without detaching cells is to culture them on a micromechanical device that has moving and removable parts in the shape of comb teeth, which allows to plate different cell types separately from each other, but to bring them into contact, and to subsequently separate them at will (Hui and Bhatia, 2007).

Cusachs et al., 2008; Thakar et al., 2009; Thakar et al., 2003). Considering the stimulating effect of cell contractility on cell growth (discussed below), these results suggest that, depending on the cell type, cell elongation regulates cell growth in different ways.

Cell adhesion on soft and on 3D substrates

3D micropatterning is more appropriate for recapitulating a physiological microenvironment than 2D micropatterning because

cell behaviour is different in 2D and 3D matrices (Johnson et al., 2007; Fraley et al., 2010). For example, non-transformed mammalian breast cancer cells grown in 3D culture systems based on basement membrane components such as Matrigel, can self-assemble to form growth-arrested acini-like structures that closely reproduce the glandular epithelium architecture (O'Brien et al., 2002). When, however, grown in a 2D matrix they form flat and disorganised multicellular islands. For this reason, efforts have been made to generate 3D micropatterns that more closely resemble

in vivo scenarios. A recent study has shown that the level of confinement below which cells have difficulties to form stress fibres is lower in 3D microwells than in 2D micropatterns, supporting the idea that stimulation along the *z*-axis matters (Ochsner et al., 2010). However, in these experiments, the difference in the ability of a cell to form stress fibres is only visible when the microwells are molded in soft polymers and not when rigid polymers are used, indicating a crucial role for the stiffness of the microenvironment in organizing the cellular actin architecture. Indeed, a main difference between classic 2D culture on glass or Petri dishes and 'classic' 3D culture in collagen gels is not only the added third dimension, but also the stiffness of the cell microenvironment. When cells are placed on top of a soft matrix, without being fully immersed into it, they can form acini, suggesting that rigidity is more important than 3D encapsulation (Guo et al., 2008; Lee et al., 2007). In addition, covering the dorsal surface of fibroblasts with an upper layer affects cell shape and the production of force only if this layer is more rigid than the bottom layer on which they are plated (Beningo et al., 2004). Taken together, these data support the conclusion that rigidity is a main regulator of morphogenesis in ECM gels and within tissues.

The development of micropatterned substrates with adjustable softness would, therefore, constitute a major step towards the fabrication of a controlled microenvironment that is highly similar to in situ conditions. However only few studies have combined the geometrical control provided by micropatterning techniques and the mechanical control provided by the soft, deformable substrates that constitute a considerable technological challenge (see Box 2). Myocyte fusion and their differentiation into myotubes that occurs in vivo can be recapitulated when myocytes are aligned on micropatterned soft substrates over long time periods (Griffin et al., 2004). Here it was shown that substrate stiffness of around 10 kPa, which mimics muscle tissue stiffness, favours myotube differentiation and the formation of striated muscles (Engler et al., 2004). A parallel orientation of myotubes over long distances appears necessary for the collective production of the forces that are necessary for the deformation of the underlying substrate (Feinberg et al., 2007). In addition, micropatterning on soft substrates has been used to measure the spatial distribution of traction forces within cells. It appears that cell spreading does not result from a type of liquefaction as would be the case for a passive visco-elastic material but that, on the contrary, the magnitude of cell traction forces increases with cell spreading and cells pulling stronger at their apices (Parker et al., 2002; Tan et al., 2003; Wang et al., 2002).

Cell polarity

Most internal cell structures and compartments, such as the cytoskeleton or endo-membrane networks, are highly dynamic, and assemble and disassemble permanently. Through mutual interactions, these compartments are nevertheless, with respect to each other organised, in a specific spatial manner (Bornens, 2008). The oriented assembly of the actin cytoskeleton in response to the geometry and architecture of external adhesive conditions, therefore, impacts on the intra-cellular organisation and directs cellular processes, such as cell polarity, migration and division.

Intracellular organisation

When spatially confined, quiescent cells assemble a branched actin meshwork along their dorsal surface, at which they form a primary cilium. By contrast, highly spread cells mostly assemble stress

fibres, whose contractility perturbs centrosome positioning and prevents the growth of the primary cilium (Fig. 3A) (Pitaval et al., 2010). The microtubule network is polarised by the cortical actin network. Controlling the actin cytoskeleton architecture with micropatterns demonstrated that microtubules adopt different behaviours depending on the local actin architecture. They grow along contractile stress fibres and stop growing in regions where actin filament polymerisation induces membrane protrusions (Fig. 3B) (Théry et al., 2006b). Thus the microtubule network is not isotropic and microtubule plus ends accumulate close to focal adhesions and regions where strong traction forces develop. Despite this anisotropic organisation of microtubules, the centrosome, which nucleates the microtubules that form the astral array, tends to sit at the cell centre (Fig. 3B) (Théry et al., 2006b). This central positioning was shown to depend on the microtubule network (Dupin et al., 2009). But the centrosome position is the result of a balance between multiple contributory factors, notably its interaction with the nucleus. Therefore, although the centring mechanism appears to be the same for most cell types (Zhu et al., 2010), the centrosome can sometimes be found away from the cell centre – particularly in large cells – if the nucleus is in contact with cell edges (Dupin et al., 2009). The Golgi complex is tightly associated to the centrosome and packed around it (Bornens, 2008). The use of micropatterns of crossbow shapes revealed that intracellular trafficking from and towards the Golgi is oriented towards cell adhesive regions (Schauer et al., 2010). The nucleus is generally off-centred, away from cell-ECM adhesions and, thus, directed either towards regions deprived of ECM (Théry et al., 2006b) or towards cell-cell junctions (Fig. 3B) (Desai et al., 2009; Dupin et al., 2009). This mechanism does not depend on microtubules and might instead rely on interactions between the nucleus and actin (Desai et al., 2009; Dupin et al., 2009; Wang et al., 2009). As a consequence, the nucleus-centrosome-Golgi axis, which reveals the global orientation of cell polarity, is oriented from regions that lack ECM adhesions towards regions where they are abundant (James et al., 2008; Théry et al., 2006a).

Neurons are highly polarised cells, whose axon position will determine the connectivity and signal propagation within a neuronal network. Micropatterning methods have been proven useful to control the presentation of specific ligands to each dendrite, and have allowed the investigation of mechanism(s) that determine axon specification and neuronal polarity. For example, when neurons were cultured on micropatterned lines coated with the cell adhesion molecule L1 [Shi et al. and Oliva et al. (Shi et al., 2007; Oliva et al., 2003) and references therein], a mixture of laminin and polylysine (Kam et al., 2001; Wheeler et al., 1999) or with cAMP (Shelly et al., 2010), they formed axons. By contrast, neurons formed dendrites only on lines coated with polylysine or cGMP. Axonal growth could result from the selective orientation of the centrosome towards regions coated with laminin (Gupta et al., 2010). Interestingly, in addition to its composition, the geometry of dendrite adhesion was also shown to affect axon maturation. A continuous, rather than an interrupted (dashed) line of the non-specific adhesion primer amino-silane, promotes axon maturation (Stenger et al., 1998). However the effect of a dashed line of adhesion primer has been contested (Vogt et al., 2004). Moreover, the length of L1-coated lines was shown to also affect axon guidance and determination (Shi et al., 2007). These results have revealed how neurons integrate geometrical and biochemical cues to orient their polarity and to specify axon maturation.

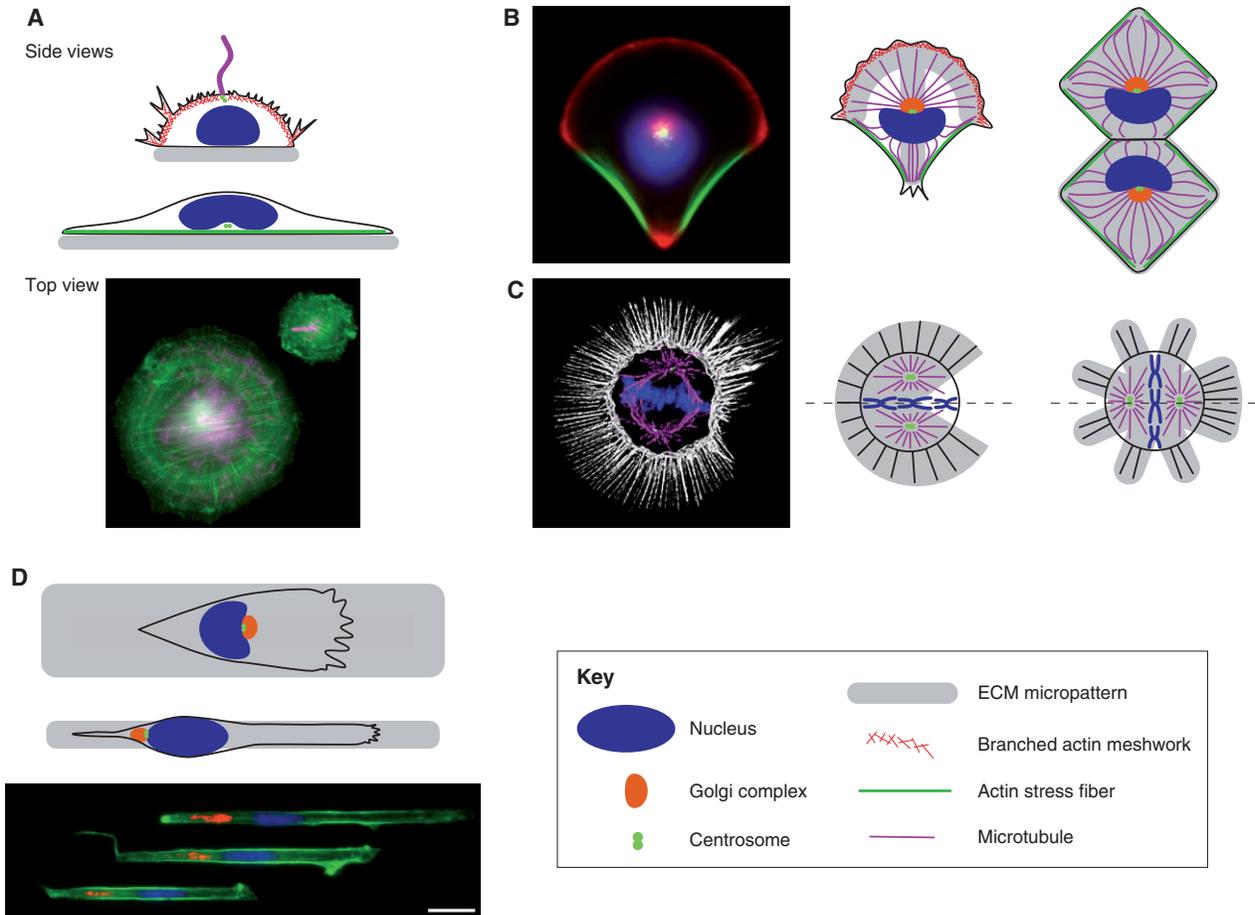


Fig. 3. Effect of the cell adhesion pattern on cell polarity and migration. In a cell, the intra-cellular compartmentalisation is organised with respect to external adhesive cues. (A) When confined to a small micropattern, a cell grows a primary cilium on its dorsal surface (top illustration). By contrast, in a cell grown on an enlarged micropattern, the centrosome is close to the ventral surface without primary cilium. The micrograph shows a top view of cells grown under these conditions with actin (green) and acetylated tubulin (magenta) as a marker for the primary cilium [adapted and published with permission from Pitaval et al. (Pitaval et al., 2010)]. (B) On a crossbow-shaped fibronectin micropattern, microtubules grow along stress fibres and stop growing when they reach branched actin meshwork. Here, the nucleus is off-centred towards the contractile non-adhesive edge, whereas the centrosome and the Golgi complex are located at the cell centre as illustrated in the image that shows the averaged location of F-actin (green) along cell contour, of cortactin (red), along cell contour, nucleus (blue), centrosome (green) and Golgi complex (red) [adapted with permission from Théry et al. (Théry et al., 2006b)]. On bowtie-shaped micropatterns (shown on the right), which constrain a cell doublet, nuclei are off-centred toward cell-cell junction. (C) Cell division is also oriented relative to the microenvironment geometry. When a cell rounds up in mitosis, it forms retraction fibres that connect cell adhesion pattern to the round cell body (shown as black lines). Spindle poles are pulled toward these fibres and orient the mitotic spindle. A localised reduction in cell adhesion, as to the right of a ‘pacman’-shaped micropattern (left), prevents spindle pole positioning towards this region, as seen in the image on the left. Here, retraction fibers are shown in white, spindle poles in green, microtubules in magenta and DNA in blue [adapted from Théry et al. (Théry et al., 2007)]. By contrast, a localised increase in cell adhesion, as to the right of the star-shaped micropattern (right), attracts spindle poles. Here, if a particular region of the cortex attracts spindle poles more than other areas do – such as the right sector of the star-shaped micropattern – spindles will adopt an asymmetric orientation with the two poles along the cell symmetry plane (dashed line), rather than on both sides like in a symmetric division. (D) Micropatterning also allows to mimic cell migration in situ. On wide micropatterned tracks (top), a cell migrates with its centrosome in the front, whereas on thin micropatterned tracks (below), it migrates with its centrosome in the back. This is illustrated in the image shown underneath, in which F-actin staining reveals membrane protrusions at the cell front (pointing to the right). Micrograph adapted and published with permission from Pouthas et al. (Pouthas et al., 2008)].

Cell migration

The orientation of the actin cytoskeleton and its polarity in response to geometrical cues governs cell migration. On teardrop-shaped micropatterns, cells form a more-rigid actin network close to the curved end compared with that at the pointed end (Su et al., 2007). Cell polarity orients from pointed towards curved edges, and cells that are released from the patterned shapes escape from the curved edge, indicating that the orientation of cell migration is not intrinsically defined but, rather, depends on external geometrical constraints (Jiang et al., 2005). When triangular shapes are used as

a micropattern, cells form actin-based membrane protrusions at cell apices and an alignment of triangles will guide sequential cell movement from one triangle to the next (Mahmud et al., 2009). How specific geometrical constraints impose a defined architecture and dynamics to the actin network remains, however, unclear. Interestingly, not only the geometry but also the mechanical properties of cell microenvironment can orient cell migration. For example, microfabricated substrates, in which substrate rigidity is higher in one direction compared with another direction that is perpendicular to it, are able to orient focal adhesion, stress

fibers and cell migration along the stiffest direction (Saez et al., 2007).

Interestingly, even symmetric environments have a dramatic effect on cell migration. Cells plated on wide tracks of fibronectin migrate in a similar manner to cells grown on Petri dishes, with their centrosome oriented in front of the nucleus and towards the lamellipodia. By contrast, in cells that are confined to thin tracks, the centrosome is located in the back of their nucleus (Fig. 3D) (Pouthas et al., 2008). Cells grown on a thinly lined substrate migrate faster than those grown on a homogeneous substrate surface and display most of the characteristics of cells that migrate in collagen gels; i.e. a coordinated displacement of cell front and cell body, and dependency on acto-myosin contractility and microtubules (Doyle et al., 2009; Pouthas et al., 2008). These results suggest that cell migration on thin micropatterned tracks recapitulates better the in situ situation than cell migration on homogeneous surfaces, such as Petri dishes. Interestingly, during oriented cell migration on tracks, the Src signalling pathway – one of the main pathways that conveys signals from cell adhesions – is activated homogeneously throughout in the cell, whereas Rac activation, which promotes lamellipodia formation, occurs only at the leading edge (Ouyang et al., 2008). Equally noteworthy, regular oscillatory behaviours of nuclear motion have been observed in non-migrating cells that had been plated on narrow linear tracks, and have been attributed to displacement of the microtubule networks within these cells (Szabo et al., 2004).

Cell division

The polarity of the actin cytoskeleton, i.e. the relative position of protrusive and contractile regions of the network, which is established in interphase in response to adhesive cues, is maintained during mitosis and orients the cell division axis (Théry and Bornens, 2006). When cells enter mitosis, they round up and, when the cell membrane retracts, form retraction fibers that originate from their adhesive contacts with ECM. Actin-associated proteins that are present in the protrusive part of the actin network accumulate at the proximal part of the retraction fibers that are in contact with the round cell body and, thereby, constitute cortical cues (Théry et al., 2005). By changing the shape of a micropattern to manipulate cell adhesion and the associated location of cortical cues, it was shown that these cues are instrumental in guiding the orientation of the mitotic spindle (Théry et al., 2005). It has been hypothesised that the cues induce tension on microtubules, thereby pulling on the spindle poles; the amount of force produced being proportional to the local density of retraction fibers. Modifications of the cell microenvironment geometry, in order to induce localised deprivation or accumulation of cell adhesion and change the local density of cortical cues, were shown to affect the force balance on the spindle pole and switch the spindle from a symmetric to an asymmetric orientation (Fig. 3C) (Théry et al., 2007). However, whether these conditions actually induce a genuine asymmetric division with unequal segregation of cell fate determinants remains to be investigated.

Interestingly, cortical cues also affect the division of cancer cells. A characteristic feature of cancer cells is centrosome supernumeracy, which can lead to the formation of multipolar spindles during mitosis. Multipolar divisions generate highly aneuploid cells that eventually die. To avoid these detrimental effects of multipolar divisions, cancer cells can form bipolar spindles with multiple centrosomes per pole, which will allow their survival. The geometry of the microenvironment affects the

location of cortical cues that orient the spatial distribution of forces acting on the additional centrosomes. This, in turn, induces either centrosome coalescence (when the microenvironment is bipolar, such as on H-shaped micropatterns), or centrosome separation (when microenvironment is multipolar, e.g. on Y-shaped micropatterns) and, thus, eventually dictates the proportion of multipolar and bipolar spindles in cancer cells (Kwon et al., 2008). These results demonstrate that the cell microenvironment can either promote or hinder cancer progression, depending on the geometry of the microenvironment.

Cell growth and differentiation

The effects of geometrical and mechanical constraints are not limited to structural cell changes and have been shown to also interfere with the regulation of fundamental cell fates, notably cell growth and cell differentiation. In situ, these regulations depend on many different parameters, such as the presence of neighbouring cells, the molecules they can secrete, or the mechanical stimulation through cell-cell contacts; and it is difficult to delineate their respective contributions. Micropatterning techniques have proven useful to clearly distinguish and characterise these different parameters in individual isolated cells and, by controlling the geometry of multi-cellular groups, conclusions could be further extended to investigate tissue-like structures.

Cell growth

The first seminal study that used micropatterning techniques to control cell shape by using adhesive islands of various size demonstrated that geometrical confinement reduces cell growth and promotes the differentiation of human epidermal keratinocytes (Watt et al., 1988). Later on, it was shown that, in endothelial cells, highly restricted cell spreading (by using square shapes with a width of less than 10 μm) even induces cell apoptosis. Here, it appears that the crucial factor that regulates the switch between apoptosis, survival and growth is not the degree of cell adhesion per se, but the area in which the cell can spread (Chen et al., 1997). It appears that the level of cell contraction, which increases with cell spreading, is responsible for activation of cell growth in highly spread cells (Huang et al., 1998; Mammoto et al., 2004). Similarly, cell elongation reduces proliferation rates and F-actin content in vascular smooth muscle cells (Thakar et al., 2009; Thakar et al., 2003). The role of anisotropic stimulation was further confirmed by the observation that the orientation of mechanical strain on mesenchymal stem cell and endothelial cells is important; proliferation is stimulated with mechanical strain along the axis of cell elongation, but is not affected when the strain is perpendicular to it (Kurpinski et al., 2006; Wu et al., 2007). However, in vascular endothelial cells, cell elongation reduces contractility without reducing proliferation rates (Roca-Cusachs et al., 2008). Therefore, cell contractility is either not a direct regulator of cell cycle progression, or such a role is cell type specific. Interestingly, two different studies of cell elongation and cell cycle progression, which observed opposing roles for cell contractility, came to the same conclusion that reduction of cell growth is systematically correlated with small nuclear volume (Roca-Cusachs et al., 2008; Thakar et al., 2009), suggesting that nuclear distortion in response to changes in cell shape (Khataou et al., 2009) might be the crucial underlying parameter.

Micropatterning experiments have also been performed with multi-cellular groups to investigate the effects of confinement within tissues. The geometry of the used micropattern defines the overall size of the cell group, as well as the length and shape of its

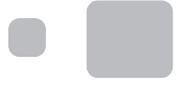
boundaries. In multicellular groups, cells with specific attributes are segregated into defined spatial domains and the attribute is regulated by a number of factors. For example, cell proliferation is favoured at the periphery of large multicellular colonies (Nelson et al., 2005) and results from higher contraction levels in peripheral regions compared with those in central regions (Li et al., 2009; Nelson et al., 2005). Cell proliferation also depends on cell-cell contacts, because downregulation of E-cadherin abrogates differences in cell growth rates within a cell group (Kim et al., 2009). Micropatterns have been used to control the exact number and location of cell-cell junctions. It was shown that the presence of only a few cell-cell junctions between endothelial cells promotes cell growth (Nelson and Chen, 2002), whereas that of many junctions tends to inhibit it (Gray et al., 2008).

Cell differentiation

Importantly, the level of cell contraction also dictates stem cell fate. For example, individual human mesenchymal stem cells

(hMSCs) cultured in differentiating medium and plated on 1000 μm^2 micropatterns have low contraction levels and differentiate into adipocytes, whereas those plated on 10,000 μm^2 micropatterns are highly contracted and differentiate into osteoblasts (McBeath et al., 2004). These differences could be abrogated with the addition of myosin II inhibitors. Several other examples of shape- and contractility-dependent differentiation patterns have been reported and are summarised in Fig. 4A. For example, reduced levels of cell spreading (Watt et al., 1988) and the presence of cell-cell contacts promote the differentiation of epidermal keratinocytes (Charest et al., 2009). Individual elongated hMSCs cultured in non-differentiating medium on micropatterned lines, reduced their spreading area, had smaller nuclei and adopted myocytes-like phenotypes (Tay et al., 2010). hMSCs treated with transforming growth factor β (TGF- β) differentiate into chondrocytes when plated on small micropatterns, but into myocytes when plated on large micropatterns (Gao et al., 2010). Furthermore, matrix metalloproteinase-3-induced epithelial-to-mesenchymal transition

A

Cell type	Conditions		Cell fate	References
	ECM micropattern LOW contractility HIGH contractility	Biochemical cues		
Mesenchymal stem cells		Mixed medium inducing adipogenic and osteogenic differentiation	 Adipocytes	(McBeath et al., 2004) (Kilian et al., 2010)
			 Osteoblasts	
		TGF- β	 Chondrocytes	(Gao et al., 2010)
Epidermal stem cells		Growth factors	 Differentiated epidermal cells	(Connelly et al., 2010)
			 Epidermal stem cells	
Epithelial cells		Matrix metalloproteinase-3 Low concentration of TGF- β	 Epithelial cells	(Nelson et al., 2008) (Gomez et al., 2010)
			 Mesenchymal cells	

B

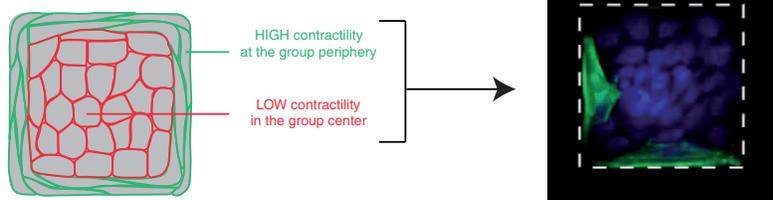


Fig. 4. Effect of cell adhesion pattern on contractility and cell differentiation. (A) Overview of the effect of cell contractility on different cell types. Cells were subjected to geometrical constraints by ECM micropattern and biochemical cues with soluble signals. ECM micropattern geometries either reduce or increase cell contractility. The level of contractility orients the biochemically induced cell differentiation. (B) The location of a cell within a multicellular group also affects cell shape and contractility and, therefore, differentiation. Cells at the periphery are more contractile than cells at the group centre. This further impacts on the spatial distribution of the mesenchymal marker smooth-muscle actin (image on right, green), which shows the epithelial cells that have undergone the epithelial to mesenchymal transition upon TGF- β stimulation (image on left, all nuclei are shown in blue). Published with permission from Gomez et al. (Gomez et al., 2010).

(EMT) occurs only in spread cells and not confined cells (Nelson et al., 2008). It is worth noting that the level of cell contraction not only depends on the extent of cell spreading, but also on the convexity of cell edges (James et al., 2008; Théry et al., 2006a). Consequently, hMSCs grown on convex geometries, such as a pentagon-shaped micropattern, differentiate preferentially into adipocytes, whereas those grown on concave geometries, such as a star-shaped micropattern, tend to differentiate into osteoblasts (Fig. 4A) (Kilian et al., 2010). Changes in cell shape as well as actin remodelling are also required for the differentiation of mammary epithelial cell and lactation. Confinement of mammary epithelial cells on small micropattern induces cell rounding, prevents formation of stress fibers, and induces nuclear compaction and deacetylation of histones H3 and H4, prerequisites for cell differentiation (Le Beyec et al., 2007; Lelievre et al., 1998). These effects of contractility on the fate of individual cells are also observed in geometrically controlled cell groups, in which cell contraction is higher at the periphery (Fig. 4B) (Gomez et al., 2010; Li et al., 2009; Ruiz and Chen, 2008).

The molecular mechanism of cell-shape-dependent regulation of differentiation have only recently started to be elucidated (Connelly et al., 2010; Gomez et al., 2010) and include the activation of serum response factor (SRF) in response to changes in actin polymerisation. Cytoplasmic G-actin binds myocardin-related transcription factor (MRTF), which is a SRF co-factor. Upon G-actin polymerisation, MRTF is released and accumulates in the nucleus, where it binds to SRF and promotes the transcription of SRF target genes that are directly responsible for cell differentiation (Vartiainen et al., 2007). In epidermal keratinocytes, sequestering of actin monomer by latrunculin A was shown to increase the levels of G-actin, preventing nuclear localisation of MRTF and, thus, cell differentiation. By contrast, filament blocking with cytochalasin D decreases the levels of G-actin, promoting MRTF nuclear localisation and cell differentiation (Connelly et al., 2010). In epithelial cells, the formation of numerous contractile F-actin filaments reduces the cytoplasmic concentration of G-actin and, thereby, promotes nuclear localisation of MRTF; consequently, it promotes the expression of smooth muscle actin and of other proteins that are involved in their differentiation into mesenchymal cells upon TGF- β stimulation (Gomez et al., 2010).

How geometrical constraints affect the differentiation patterns in early embryos is an exciting question that has begun to be addressed. The degree of confinement in colonies of human embryonic stem cells (hESC) plated on micropatterns appears to influence their differentiation (Bauwens et al., 2008; Peerani et al., 2007). For example, hESCs tend to express endogenic markers rather than neurogenic markers as the colony size increases (Bauwens et al., 2008). Since cells proliferate within the colony, the size of the colony affects local cell density. Cell differentiation depends on the concentration of secreted morphogen and, therefore, on local cell densities. A high cell density, i.e. in the centre of the colony, promotes expression of *Oct4*, a hallmark of stem cell pluripotency (Peerani et al., 2007).

Coordination in multicellular arrangements

Micropatterning methods have also been successfully used to control the shape of multicellular groups and to follow their behaviour. This makes it possible to analyse the different intercellular communication mechanisms that are based on mechanic or chemical signalling as discussed below.

Cells interact with each other mechanically. Two endothelial cells plated on a micropattern tend to turn around each other in a remarkably regular fashion (Huang et al., 2005) by coordinating the orientations of their leading edges to move together in a confined space. Although such rotation movements probably do not occur in vivo, they highlight that cells are capable of coordinated movements, which is likely to be relevant for the establishment and maintenance of the mechanical equilibrium in a tissue. Other coordinated movements were analysed in migrating epithelial cell sheets (Poujade et al., 2007). Here, microfabrication methods were used to define a linear sheet border within multicellular assemblies, which can be removed to allow the cells to move freely. In this setup, cells do not migrate forward all at once; instead, they form finger-like multicellular outgrowths that arise from the movement of a single cell that progresses faster than the rest. Cells directly adjacent to this leader cell, and also all the cells in the outgrowth, align their migration almost perfectly with that of the leader (Petitjean et al., 2010). The leader appears to act here as a guide rather than a locomotive engine. Mechanical interactions between cells do not pull the cell along but, instead, bias individual cell migration. Interestingly, this bias influences not only adjacent cells but also cells that are several cell diameters away from the leader (Treat et al., 2009).

Multicellular movements are also directed by secreted diffusive morphogens. For example, homogeneous secretion of inhibitory morphogens in a tubule-like structure made of mammary epithelial cells promotes tubule elongation rather than broadening, and a curvature in the tubule favours outgrowth on the external side (Nelson et al., 2006). By controlling tubule shape in micromolded wells together with computational modeling, it could be shown that tubule geometry organises morphogen gradients and thereby dictates the position of future branches and accounts for the branching morphogenesis in the ductal tree of the mammary gland (Nelson et al., 2006).

Tissue-like morphogenesis

Multicellular interactions and coordinations result into large morphogenetic changes resembling those observed in situ, and which can also be studied with dedicated micropatterning methods. 2D guiding cues, such as micropatterned lines on a flat substrate, were shown to be able to guide 3D morphogenesis. For instance, when plated at confluency on thin 10 μm wide micropatterned lines, endothelial cells spontaneously stop dividing and form hollow tubes, mimicking actual blood vessels (Dike et al., 1999). On lines wider than 30 μm , endothelial cells form hollow vessels only when they are overlaid with ECM gel (Dike et al., 1999; Okochi et al., 2009). The concentration of adhesion proteins on the micropatterned lines also affects the self-assembly of endothelial cells into tubular structures. Low concentration of adhesion peptide RGD (<10 $\mu\text{g}/\text{cm}^2$) is not sufficient to induce cell attachment, whereas high concentration (>100 $\mu\text{g}/\text{cm}^2$) prevents formation of the tubular structure that only occurs at an intermediate RGD concentration of ~20 $\mu\text{g}/\text{cm}^2$ (Moon et al., 2009). Similar vessels form when endothelial cells are cultured in microfabricated 3D channels filled with ECM gel (Raghavan et al., 2010b). Noteworthy, endothelial cell elongation on micropatterned lines was shown to be sufficient to promote the expression of immunogenic genes that are required to reduce inflammatory capability of blood vessels, indicating that engineering of cell shape contributes to the full recapitulation of cell function in vitro (Vartanian et al., 2010).

In addition to morphogen secretion and contact with homotypic cells, morphogenesis and full cell differentiation in situ also depends on interactions with other cell types (Steinberg, 2007). The co-culture of two micropatterned cell types allowed to identify the parameters in homo- and heterotypic interactions that determine the acquisition of defined multicellular functions, such as liver morphogenesis (Bhatia et al., 1998; Khetani and Bhatia, 2008). Innovative dynamic micropatterns, in which contact between patterned cell population can be promoted and prevented at will, were used to show that, although the contact between hepatocytes and fibroblasts is required to ensure liver functionality, only transient initial contact is necessary (Hui and Bhatia, 2007).

Future perspectives

Taking together, the micropatterning methods discussed above have revealed important insights into how the geometry of the microenvironment impacts on cellular physiology, from intracellular organisation to multicellular morphogenesis. The microenvironment is, therefore, a parameter that should be addressed and ideally controlled in studies aimed at investigating these cellular functions.

As demonstrated above, the culture of cells on engineered substrates that precisely mimic the structure, composition and mechanical properties of tissues is already feasible and, thus, provides a possible means to overcome the limitations of classic cell culture. The micropatterning approach is particularly useful in this respect, because it not only places cells in physiologically relevant conditions but, in addition, also makes it possible to manipulate and fine-tune these conditions and, thus, interfere with cell behaviour. This level of control is a tremendous advantage over in situ studies on animals.

Future progress in micropatterning approaches are anticipated to allow us to investigate how cells sense changes in their microenvironment and, thus, will help us to understand the core mechanism of morphogenesis during normal development and tissue renewal, as well as during pathological transformation. Directly guiding cell architecture, polarity, migration and division during tissue formation in vitro are other possibilities of micropatterning and these tools would revolutionise the future of regenerative medicine.

This review covers technological progress in manipulating cells with micropatterning but has not taken into account the fact that the cellular microenvironment itself, which also consists of cells, is able to sense and respond to cells. Thus, the far future of micropatterning is not simply a switchable microenvironment – but an active microenvironment, in which sensors are used to measure the behaviour of the surrounding cells and adapt to it.

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