Matrix nanotopography as a regulator of cell function

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The architecture of the extracellular matrix (ECM) directs cell behavior by providing spatial and mechanical cues to which cells respond. In addition to soluble chemical factors, physical interactions between the cell and ECM regulate primary cell processes, including differentiation, migration, and proliferation. Advances in microtechnology and, more recently, nanotechnology provide a powerful means to study the influence of the ECM on cell behavior. By recapitulating local architectures that cells encounter in vivo, we can elucidate and dissect the fundamental signal transduction pathways that control cell behavior in critical developmental, physiological, and pathological processes.

Introduction

Living tissues are intricate ensembles of multiple cell types embedded in a complex, well-defined ECM. The ECM possesses topographical and adhesive features ranging from nanometers to micrometers. Many ECM proteins form large-scale structures up to several hundred micrometers in size that interact with multiple individual cells to coordinate complex multicellular behavior. For instance, collagen fibrils, with diameters ranging from 20–200 nm, can form hierarchically structured microscale collagen fibers (Birk et al., 1989, 1995; Canty et al., 2004). Interestingly, the nano- and microscale architecture of these fibrils/fibers influence cell polarity and promote migration along collagen fibrils by providing contact guidance cues (Dickinson et al., 1994; Wang et al., 2002; Meshel et al., 2005; Provenzano et al., 2008; Perentes et al., 2009). Basement membrane complexes are another class of ECM superstructures. Recent ultrastructural analysis using EM reveals that the basement membrane of epithelia and endothelia exhibits a complex 3D texture in the nanometer range (Fig. 1 A; Abrams et al., 2002, 2003). Moreover, during several pathological conditions, such as cancer cell invasion, the ECM is commonly remodeled (Fig. 1 B), whereas ECM architecture influences numerous pathophysiologic and physiological events ranging from glioma progression (Fig. 1 C) to development and remodeling of cardiovascular tissue (Fig. 1 D). As cells contain nanoscale features whose sizes are compatible with these ECM structures, such as focal contacts/adhesions, and fine processes (e.g., cilia and filopodia), one can reasonably conclude that nanoscale features of ECM influence cell function in vivo.

Despite the complex topographical features of 3D cell microenvironments in various tissues in vivo (Fig. 1), most in vitro studies examine cells cultured on flat 2D rigid substrates. Although these studies have been instrumental in elucidating fundamental principles in cell biology, they do not recapitulate the complexity found in 3D microenvironments. This difference is important, as extensive experimental evidence suggests that cell behavior is often profoundly different in deformable 3D matrices versus flat, rigid 2D culture substrates made of glass or plastic (Cukierman et al., 2001; Grinnell, 2003; Yamada and Cukierman, 2007). Arguably, the primary reason for consistent use of standard 2D culture is its ease and simplicity. Indeed, it is considerably more difficult to measure and control the details of 3D microenvironments. Thus, a useful compromise allows one to mimic aspects of the natural 3D cell environment while retaining the convenience of working in 2D. Recent technological advances have made this possibility a reality.

The utilization of microtechnology has heralded attractive innovations to classical cell biology experimentation (Bhadriraju and Chen, 2002; Paguirigan and Beebe, 2008), including the use of sophisticated microfluidic devices that allow detailed analysis and control of live cells (Li et al., 2003; Gupta et al., 2010). Microengineered or micropatterned cell adhesion substrates have enabled selective cell attachment to study cell phenotype and signaling (Chen et al., 1997; Tan et al., 2003; Xia et al., 2008). As a result, the impact of microscale ECM features has been well documented and is viewed as being central to many cellular functions (Lim and Donahue, 2007; Ruiz and Chen, 2008). More recent advances in nanoscale fabrication techniques have allowed rapid accumulation of evidence supporting the significance of ECM nanotopography on cellular

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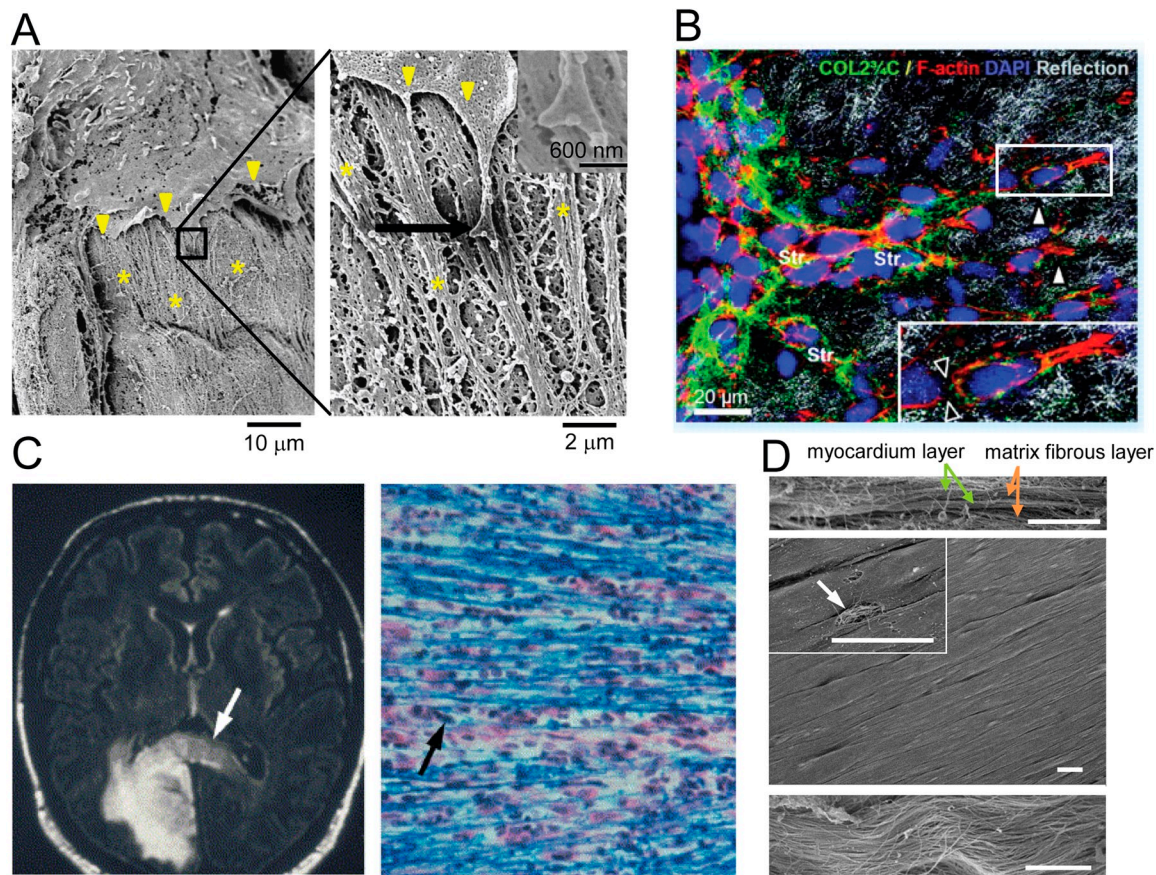Abbreviation used in this paper: hMSC, human mesenchymal stem cell.

Figure 1. **Anatomical features of highly oriented ECM in various tissues.** (A) SEM images showing interaction of an aortic endothelial cell with the basement membranes in an intact vessel. The left image shows the edges of the cell membrane (arrowheads) interacting with the rough ECM structures forming the basement membrane (asterisks). The boxed area marks the area that is magnified in the right image and displays an end process of the cell membrane adhering to the basement membrane. The higher magnification allows better visualization of the bumps, ridges, pits, and grooves forming the complex topography of the basement membrane. The arrow marks the area magnified in the inset (top right), which highlights the specific interaction between an end foot of the cell membrane and the nanotopography of the ECM. The images are adapted from Liliensiek et al. (2009), reprinted with permission from Mary Ann Liebert, Inc. (B) Remodeling of ECM structures by motile HT1080 fibrosarcoma cells. Transition from individual to collective invasion is displayed in 3D spheroids cultured within a 3D collagen lattice. Single cells (white arrowheads) generate small proteolytic tracks (black arrowheads in inset; detected by cleavage site–specific COL2 3/4C antibody) that become further remodeled and widened by solid strands of multiple cells (Str). The box marks the area magnified in the inset (bottom right). Images are from Friedl and Wolf (2008), reprinted with permission from American Association for Cancer Research. (C, left) Invading brain tumor cells move along the corpus callosum of the human brain. An MRI of brain tumor (bright mass) and cells migrating along the corpus callosum (arrow) is shown. (right) Individual brain cancer cells (arrow) can be seen migrating along myelinated fibers (blue) of white matter tracts. The images are adapted from Bellail et al. (2004), reprinted with permission from Elsevier. (D) SEM images of ex vivo myocardium of adult rat heart. The side view (top) and top view (middle) show well-aligned myocardium. The inset in the middle image and the magnified view (bottom) demonstrate that the structural organization of the myocardium correlates with matrix fibers (arrows) aligned in parallel. Bars, 10 µm. The images are adapted from Kim et al. (2010b), reprinted with permission from *Proceedings of the National Academy of Sciences USA*.

behavior (Curtis, 2004; Bettinger et al., 2009; Kim et al., 2010a). A variety of nanoscale topographic features, <1 µm in size, can now be incorporated into in vitro experimental platforms to model the structural and mechanical intricacies of 3D in vivo ECM environments. Experiments using engineered substrates with nanoscale features (Fig. 2) demonstrate that nanotopography strongly influences cellular form and function. However, detailed understanding of the mechanisms by which nanotopographic cues are integrated to regulate cell behaviors remains largely unknown, but, with growing advances in nanotechnology tools, we are poised to answer fundamental questions at the single-cell level. Here, we survey current efforts using nanoscale technologies to investigate the influences of ECM nanotopography on cell behaviors, such as polarity, migration, proliferation, and differentiation.

Nanotopographic cues as regulators of cell behavior

Nanotopography-induced changes in cell shape and polarity. Characteristic functions of complex tissues depend heavily on cell shape and polarity (Chen et al., 1997; McBeath et al., 2004; Kim et al., 2010b). For instance, establishment and maintenance of epithelial cell polarity is critical for organ development (Bryant and Mostov, 2008; Miller and McCrea, 2010), and loss of cell polarity is involved in many human disease processes, including cancer metastasis (Bryant and Mostov, 2008; Petrie et al., 2009). Cell–cell- and cell–matrix-associated signaling complexes, particularly the Par (partitioning defective) complex (composed of Par3, Par6, and aPKC) and associated Rho GTPase signaling, have emerged as regulators of polarity during numerous cellular processes, including basal–apical polarity, asymmetric cell

division, and directional migration (Etienne-Manneville and Hall, 2003; Aranda et al., 2008; Petrie et al., 2009). However, the role of local ECM architecture on cell polarity is not well understood.

Substrate topography can strongly influence the polarity of many different cell types through a process known as contact guidance (Dunn and Ebendal, 1978; Clark et al., 1991; Dickinson et al., 1994). Arrays of parallel nanogrooves, or nanoridges (Fig. 2), have been used to provide an in vitro experimental model of the nanotopography of the in vivo ECM. Interestingly, contact guidance cues from these substrates compel multiple cell types to preferentially elongate and align parallel to these grooved nanopatterns (Fig. 3 A, left; Rajnicek et al., 1997; Rajnicek and McCaig, 1997; Karuri et al., 2004; Teixeira et al., 2004; Diehl et al., 2005; Yim et al., 2005; Biela et al., 2009; Kim et al., 2009a, 2010b). Neurons in the peripheral nervous system polarize along nanogrooves fabricated specifically to imitate polarization along neurite bundles (Nagata and Nakatsuji, 1991; Nagata et al., 1993), suggesting that nanotopographic features of neurite bundles may regulate polarity of neuronal cells. Furthermore, quantitative analysis with corneal epithelial cells showed that the extent of alignment varied with the specific dimensions (e.g., depth and pitch) of the nanogrooves and culture media conditions, suggesting a synergy between substrate architecture and soluble chemical factors (Teixeira et al., 2003, 2006). The geometry of the underlying nanotopography also affected cell shape and polarity (Fig. 3, B and C). Filopodia and lamellipodia generally extended parallel to the underlying nanoridge features with aligned actin stress fibers and vinculin-positive focal adhesions that scaled with the width of the underlying substrate (Teixeira et al., 2003, 2006). The nanoscale width of the grooves can also limit cell penetration into them, thus limiting the cell–substratum adhesion (Fig. 3 D). Yet, the role of these features in regulating cell polarity in response to nanotopography remains poorly understood. However, these studies do demonstrate that even extremely small (nanoscale) differences in architecture can profoundly influence cell polarity and provide a unique forum to dissect the molecular mechanisms driving cell polarity.

The influence of nanoarchitecture on cell morphology is further exemplified by the cells' ability to acutely sense variability in topographic cues. For instance, nanoscale differences in ligand spacing significantly influenced polarity of osteoblasts and fibroblasts (Arnold et al., 2008; Kim et al., 2009a). Interestingly, growing NIH 3T3 cells on a gradient of nanoridges with varying groove widths led to increased cell polarity on denser patterns, i.e., those with narrower groove widths (Fig. 3 A, right; Kim et al., 2009a). Organization of the actin cytoskeleton was also sensitive to the local topographic pattern density. Polarized fibroblasts aligned with dense groove patterns showed aligned actin stress fibers and robust focal adhesions at the cell's leading edge. In contrast, on locally sparser patterns (i.e., those with greater groove widths), vinculin-positive focal adhesions were more randomly distributed, demonstrating that varying topography alters adhesion organization, which may play a role in dictating cell polarity.

Nanotopographic guidance during cell migration. Cell migration is essential for numerous physiological and pathological processes, including embryogenesis, wound repair,
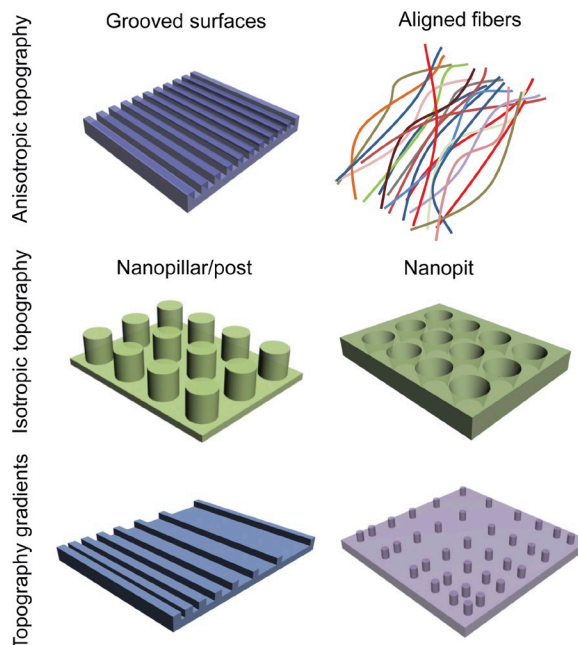

Figure 2. **Nanotopographically defined in vitro cell culture tools.** Schematics of three representative nanotopography geometries commonly used as cell culture substrates, including nanogroove/ridge arrays, nanopost arrays, and nanopit arrays. Anisotropic topographies are directionally dependent, in this case, providing cues along a single axis. Isotropic topographies are uniform in all directions, providing cues along multiple axes. Topography gradients provide cues through gradual changes in physical features (e.g., groove spacing) along a particular direction. Schematics are not drawn to scale. The ranges of relevant feature sizes could vary between 100 nm and 1 μm, depending on the design dimensions of the substrates' nanofeatures.

and metastasis (Sahai, 2007; Petrie et al., 2009; Friedl and Wolf, 2010). Although a great deal is known about the fundamental mechanisms of cell migration, the molecular mechanisms by which cells integrate signals resulting from local 3D matrix architectures to promote directional migration are not well understood (Petrie et al., 2009). In conjunction with its effects on cell polarity, nanotopography influences cell migration. Numerous human cell types exhibit enhanced motility when grown on nanostructured surfaces (Ranucci and Moghe, 2001; Mello et al., 2003; Brammer et al., 2008). In contrast to flat substrates where the trajectory of migration is essentially random, corneal epithelial cells migrate parallel to nanoridges (Diehl et al., 2005), consistent with in vivo findings with carcinoma cells migrating along aligned ECM (Provenzano et al., 2006; Provenzano et al., 2008). Furthermore, neutrophils migrate more rapidly on grooved surfaces than on flat surfaces (Tan and Saltzman, 2002), which may have important implications for immune surveillance as well as wound repair in which ECM architecture is disrupted and reestablished. Hence, these studies bring to light the influence of nanoscale architectures as regulators of directional migration, a behavior that is important during both normal and pathological conditions such as proper tissue organization during development, wound repair, and focal invasion leading to metastasis.

Cell migration is also sensitive to variation in the density of nanotopographic cues. For example, the migration speed of
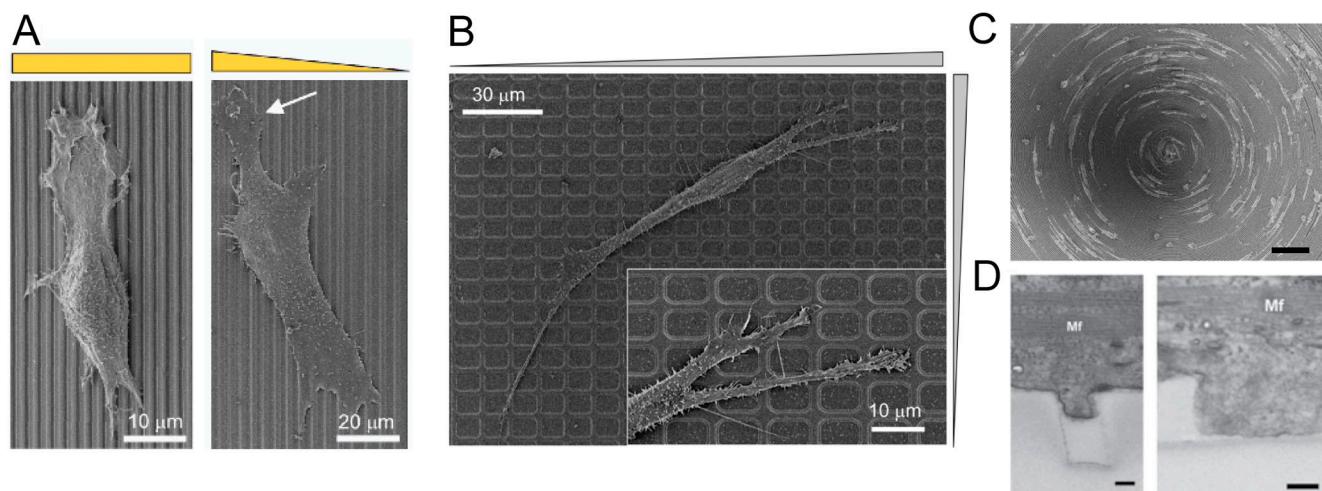
Figure 3. Mechanosensitivity of cells to changes in substrate nanotopography. (A) SEM images of NIH 3T3 fibroblasts cultured on nanoridge arrays with regular spacing (left) and graded spacing (right). The yellow rectangle represents regularity in spacing between nanoridges, whereas the triangle represents a gradient of spacing between the nanoridges. The white arrow indicates membrane protrusion extending toward more closely spaced ridges. The images are adapted from Kim et al. (2009a), reprinted with permission from Elsevier. (B) Representative SEM image of the morphology of cells on square lattice pattern arrays of different local densities. The gray triangles describe the changing dimensions of the rectangular gaps in the lattice. A magnified image at the motile edge is shown in the inset. The images are adapted from Kim et al. (2009b), reprinted with permission from Wiley. (C) Directed cell migration along grooves of different curvature. Bar, 50 µm. (D) Differential degree of primary cardiac cell protrusion into a 400-nm-wide (left) and an 800-nm-wide (right) groove. Mf, myofilament. Bars, 200 nm. The images are adapted from Kim et al. (2010b), reprinted with permission from *Proceedings of the National Academy of Sciences USA*.

NIH 3T3 fibroblasts is dependent on the width of nanopatterned ridges. With this biphasic dependence, nanoridges that are either too close or too far apart do not promote directed migration to the degree of nanoridges 200–300 µm apart (Kim et al., 2009a), suggesting a complex interplay between cell sensing of microscale and nanoscale features. In these cases, cell movement could be guided by gradients in patterned substrates, which can be termed topotaxis (i.e., directional migration toward particular topography density gradients), as seen by the trajectories of migrating fibroblasts toward denser regions of patterns (Fig. 3 A; Kim et al., 2009a). Interestingly, this behavior was also observed for both individual and collective cell migration, suggesting a role for matrix nanotopography in coordinated cellular responses such as wound repair. Aggregation and orientation of fibroblasts in areas of optimal ECM content and organization might facilitate very important functions of these cells in the reconstruction of damaged ECM. As ECM is repaired and the density of organized matrix is increased, fibroblasts can naturally migrate to areas of less-organized matrix, thus ensuring self-organized repair propagation (Fig. 4). By analyzing fibroblasts grown on a rectangular lattice of variable local density and anisotropy (Fig. 3 B), which mimics the complexity of randomly organized ECM components, preferential migration and planar congregation toward areas of optimal topographic density were observed (Kim et al., 2009b). The direction of cell movement was also guided by grooves of different curvature (Fig. 3 C). These results support the conclusion that nanotopography may influence cell localization and migration during tissue repair. Thus, cell motility can be enhanced and directed by both the local density and anisotropy of the nanoscale features of their immediate matrix environment.

Studies in which surface architecture, stiffness, ligand type and density, and chemical stimuli can be simultaneously modulated have an increased potential to provide additional insights into the physical mechanisms of cell motility. With nanoscale tools, quantitative models can be tested with additional rigor, and more complex models can be formulated to gain valuable insights into the mechanisms of cell migration. For example, seminal work by Lauffenburger and Horwitz (1996) provided an important insight into the physical mechanisms of cell migration on planar surfaces. Later work has confirmed early findings and produced additional mechanistic data to describe mechanical aspects of cell migration (Gardel et al., 2010). As many of the fundamental processes that regulate cell migration identified to date operate on the microscale and, particularly, the nanoscale (Arnold et al., 2008; Gardel et al., 2010; Kanchanawong et al., 2010), tools specific to these scales should be instrumental in obtaining new findings that can shape more complete quantitative models to accurately describe cell migration in complex microenvironments.

Nanotopographic control of cell proliferation. Cell proliferation is heavily influenced by the structure of the surrounding microenvironment. Seminal work by Chen et al. (1997) defined a role for cell shape as a determinant of cell proliferation and apoptosis using microscale-patterned substrates. More recent work demonstrated that proliferation in human cell lines is sensitive to nanoarchitecture by culturing cells on substrates consisting of randomized nanoscale bumps or nanoislands of varied heights <100 nm (Lim et al., 2005; Milner and Siedlecki, 2007). Cells maintained significantly lower densities and lower rates of proliferation on patterns with larger nanoscale features. Continued reduction of feature sizes eliminated the discrepancy between nanotextured and flat substrates with respect to cell proliferation (Milner and Siedlecki, 2007). Indeed, cell proliferation in multiple cell types is reduced on nanopatterned

surfaces when compared with flat substrates (Yim et al., 2005; Liliensiek et al., 2006; Bettinger et al., 2008; Dulgar-Tulloch et al., 2009), which may explain, in part, why cells proliferate at much greater rates in vitro than in native in vivo microenvironment. In fact, corneal epithelial cells showed reduced proliferation when cultured on nanogrooves, mimicking the effective sizes of collagen fibrils in the corneal stroma (Liliensiek et al., 2006). Furthermore, culturing cells on surfaces with nanoscale roughness showed that even altering roughness by a few nanometers influenced cell proliferation (Washburn et al., 2004; Brunetti et al., 2010), highlighting the exquisite sensitivity to even minute nanoscale cues.

Further support for the extreme sensitivity of cell proliferation to the specific size of nanoscale features was observed through experiments with rat neural stem cells cultured on meshes composed of nanofibers (Christopherson et al., 2009). The number of proliferative cells in a population depended heavily on the diameters of the nanofibers, wherein proliferation increased with decreasing fiber size. In contrast, Oh et al. (2006) reported enhanced proliferation of mouse osteoblasts on hollow nanotubes of diameters near 100 nm versus the flat control. Hence, nanotopography may regulate cell proliferation in a cell-specific manner, consistent with differences in cell proliferation found for cells residing in diverse ECM microenvironment in vivo.

Nanotopographic control of cell differentiation. During development and tissue homeostasis, pluripotent cells integrate numerous signals that determine their ultimate fates. Among the numerous cues affecting differentiation (soluble factors, ECM composition, etc.), the nanotopography of the cells' surroundings also plays an important role (Kshitiz et al., 2011). For instance, either nanopits or nanotubes stimulated osteogenic differentiation of human mesenchymal stem cells (hMSCs) in the absence of osteogenic induction media (Dalby et al., 2007; Oh et al., 2009), suggesting that nanotopography may be sufficient to guide differentiation but not optimal, as osteogenesis of hMSCs was synergistically enhanced by culture on nanostructured surfaces with osteogenic induction media (You et al., 2010). Likewise, Sjöström et al. (2009) reported skeletal differentiation by exposing hMSCs to nanopillar structures of different heights (0–100 nm), finding maximal differentiation on pillars of 15 nm, again highlighting the influence of even subtle differences in nanoarchitecture on cell behavior.

In addition to hMSC osteogenic differentiation, the role of nanotopography as a factor promoting differentiation toward other lineages has been investigated. Dang and Leong (2007) found that aligned nanofibers promote cytoskeletal reorganization as well as cellular and nuclear elongation of hMSCs, which biases hMSCs toward myogenic differentiation. Nanostructures have also been used to bias MSCs and mouse embryonic stem cells toward neuronal lineages (Christopherson et al., 2009; Tsuji et al., 2009; Xie et al., 2009; Migliorini et al., 2011). Furthermore, the diameter of nanofibers influenced the differentiation of rat neural progenitor cells, showing a 40% increase in oligodendrocyte differentiation on 283-nm fibers and a 20% increase in neuronal differentiation on 749-nm fibers, relative to standard tissue culture surfaces (Christopherson et al., 2009). In a separate study using rat pheochromocytoma cells, which terminally
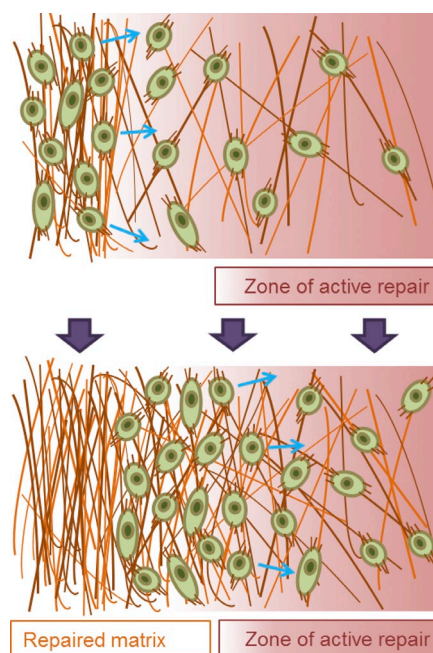

Figure 4. **Model of the role of fibroblasts in ECM remodeling during wound healing.** During the wound healing process, fibroblasts are functionally essential cells that migrate (arrows) toward and into the open wound, deposit collagen, and restructure the ECM. Fibroblasts can aggregate in zones of intermediate ECM density that may become the zones of active ECM repair, which can change as cells progressively create denser matrix and clear these now dense zones for the less-dense adjacent zone. This, along with apoptosis, can be an efficient mechanism of cell clearance from the restructured wound zones before reepithelialization. The schematics are adapted from Kim et al. (2009a), reprinted with permission from Elsevier.

differentiate in the presence of NGF, the authors found that the NGF threshold for induction of neuritogenesis was lowered when combined with specific surface nanotopography (Foley et al., 2005). Hence, these studies suggest that nanotopographic cues of precise dimensions can be used to bias precursor, pluripotent, and adult stem cells toward particular fates; yet, the molecular mechanisms driving these processes are not currently known.

Synergistic effects of nanotopography and mechanical stimuli on cell motility

In the complex microenvironment, cells encounter a multitude of distinct, simultaneously active stimuli that are biochemical, structural, and mechanical in nature. For example, directed migration can be controlled by many different mechanisms, which may include chemotaxis, galvanotaxis (i.e., directional movement of cells in response to an electric field), haptotaxis (i.e., the directional motility guided by gradients in adhesion), and durotaxis (i.e., cell migration guided by gradients in substrate rigidity) as well as contact guidance. Cells exposed to combinatorial stimuli decipher their crosstalk to determine cell behavior in any given situation. Although a limited body of work has been presented on synergistic effects between nanoarchitecture and growth factor stimulation (Teixeira et al., 2003, 2006; Foley et al., 2005; Patel et al., 2007; You et al., 2010) as well as nanoarchitecture and electric field stimuli (Au et al., 2007; Rajnicek et al., 2007; Heidi Au et al., 2009), here, we will discuss recent exciting data demonstrating crosstalk between nanoarchitecture and mechanical signals.

The rigidity of the surrounding ECM plays a role in regulating cell behavior (Pelham and Wang, 1997; Lo et al., 2000; Paszek et al., 2005; Provenzano et al., 2009; Provenzano and Keely, 2011) and distinctly influences cell migration (Pelham and Wang, 1997; Lo et al., 2000). Interestingly, the intricate nanotopographic features of the cell environment comprise a critical element of mechanical stimulation (Tzvetkova-Chevolleau et al., 2008; Park et al., 2012). When using nanostructured polymeric materials to simulate the cell environment, it is possible to vary the stiffness of the substrate by altering its chemical composition. For example, CHO cells are more polarized along stiffer nanogrooved substrates (Park et al., 2012), whereas NIH 3T3 and cancerous SaI/N mouse fibroblasts cultured on nanoposts and nanogrooves (Fig. 2) display changes in morphology and motility in response to modulated nanosubstrate rigidity (Tzvetkova-Chevolleau et al., 2008). NIH 3T3 fibroblasts obtained more polarized morphologies on softer patterned nanosubstrates, whereas SaI/N fibroblasts demonstrate enhanced migration speed on patterned surfaces, which was further amplified on more rigid nanosubstrates. Hence, changing the stiffness of nanostructures has a profound influence on cell motility. As cells sense the stiffness of their local environment, which in turn influences cellular traction force, and many of the fundamental processes that regulate cell migration are in the nanoscale (Arnold et al., 2008; Gardel et al., 2010; Kanchanawong et al., 2010), nanotechnology tools should be instrumental in obtaining new findings regarding the physical and molecular mechanisms of cell migration.

In addition to force transmitted to and from the ECM, many cells types (e.g., vascular endothelial cells) encounter mechanical stimulation from fluid flow in vivo. Accordingly, many in vitro experimental platforms intentionally and unintentionally expose cells to this phenomenon. Interestingly, bovine aortic endothelial cells respond to flow-induced shear stress differentially when cultured on parallel grooves versus flat control conditions (Uttayarat et al., 2008). Under static conditions, these cells align their focal adhesions and migrate parallel to the grooves. High levels of shear stress alter this response. The number of aligned, parallel migrating cells are increased by parallel flow and decreased by orthogonal flow (Uttayarat et al., 2008). Hence, these studies suggest complex mechanisms for integrating multiple mechanical signals arising at different scales and modalities as well as a combinatorial influence from ECM structure that is yet to be well described.

Mechanisms of cellular responses to nanotopography

Significant research has now been conducted to observe the behaviors of cells in the presence of nanotopographic cues. However, the underlying molecular mechanisms governing these processes remain elusive. One of the most challenging questions is: how do cells sense matrix nanotopography? In efforts to describe these complex systems, scientists have recently used nanofabricated substrates that mimic the organized matrix in vivo. Although, to date, few distinct answers have arisen, initial clues come from defined micro- and nanoscale substrates and the integrin family of transmembrane adhesion molecules.

Integrins transduce extracellular forces into biochemical signals through focal adhesions, a cluster of integrin-associated proteins localized at the interface between integrin receptors and the actin cytoskeleton. Integrins play a major role in adhesion- and force-dependent signal transduction, serving as key regulators of cell motility, growth, and differentiation (Giancotti and Ruoslahti, 1999; Katsumi et al., 2004). Interestingly, work using substrates with defined microscale features has provided novel insight into focal adhesion signaling (Balaban et al., 2001; Tan et al., 2003; Goffin et al., 2006). Gallant et al. (2005) used micropatterned substrates to measure focal adhesion strength and reported a novel role for FAK, a primary regulator of focal adhesion signaling, in integrin activation and adhesion strengthening (Michael et al., 2009). Extending these studies using nanopatterning techniques presents cell-adhesive signals on scales comparable with individual focal adhesion complexes (Arnold et al., 2004; Zhu et al., 2005) and subsequently provides tools to facilitate novel insight into focal adhesion signaling. For example, CHO cell polarization along nanogrooves is $\beta 1$-integrin dependent (Park et al., 2012), whereas culturing fibroblasts on nanocolumn substrates increases filopodia (Dalby et al., 2004). This increased surface contact guided cell migration by regulating the strength of focal adhesions through FAK and myosin II (Frey et al., 2006), whereas aligned fibers or grooves promote actin polymerization and protrusion in the direction parallel to the fibers/grooves (Li et al., 2005). This process can result in aligned focal adhesions and traction force through the actin cytoskeleton in the same direction, which likely plays a role in cell polarity and directed migration. Furthermore, the addition of actin-disrupting agents attenuates alignment of human embryonic stem cells in response to nanotopography (Gerecht et al., 2007). These findings illustrate the importance of interplay between adhesion signaling, the actin cytoskeleton, and substrate interactions in mediating contact guidance.

Other contact guidance studies have focused more heavily on intracellular signaling cascades. RACK1 (receptor of activated protein kinase C) inhibits the response to contact guidance by nanogrooves while positively promoting adhesion (Dalby et al., 2008). In hippocampal neuritis, calcium influx and protein kinase C activity regulate alignment on nanoarchitectured substrates (Rajnicek and McCaig, 1997), whereas the PI3 kinase pathway was similarly necessary for contact guidance in fibroblasts and cardiomyocytes (Au et al., 2007). Likewise, STRO-1^{+} skeletal stem cells cultured on nanotopographies displayed significant FAK-dependent down-regulation of extracellular signal–regulated kinase (ERK)/MAPK signaling molecules (Biggs et al., 2009). In addition, ERK/MAPK pathways exhibited marked changes in transcription factor expression as a result of variation in nanoarchitecture, whereas nanotopography-induced changes in many MAPK pathway component proteins (e.g., heat shock protein 70 [Hsp70] and galectin-8 [Gal-8]) have also been reported (Kantawong et al., 2009).

Examination of the temporal sequence of dynamic events involved in contact guidance has provided interesting insight into the mechanisms of the guidance response. Immunocytochemical analysis of microtubules, focal contacts, and actin filaments in fibroblasts aligned along grooved substrates
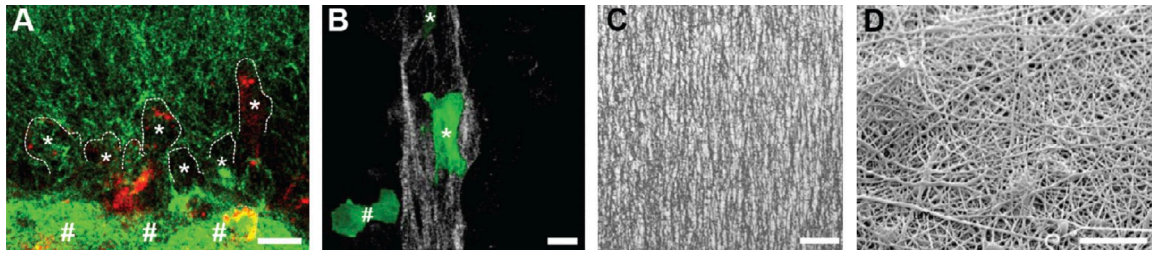
Figure 5. **Engineering nanoarchitectures to study cell behavior in 3D environments.** (A) Combined multiphoton excitation (red; endogenous cellular fluorescence) and second harmonic generation (green; collagen) microscopy capturing 3D cell migration into magnetically aligned collagen matrices. MDA-MB-231 breast carcinoma cells (asterisks; dashed lines highlight the leading edge of each cell) preferentially migrate through aligned collagen (top) versus randomly oriented collagen (bottom; highlighted by #). The image was adapted from Provenzano et al. (2008), reprinted with permission from Elsevier. (B) Bovine aortic endothelial cell expressing GFP-tubulin (green) on collagen aligned in microchannels (white) shows cell alignment (asterisks) along the collagen matrix versus random alignment on a flat surface (#; bottom left of the micrograph). The image was adapted from Lee et al. (2006), reprinted with permission from Springer. (C) Consistent generation of highly aligned collagen fibrils using flow-through microchannels. The image was adapted from Lanfer et al. (2008), reprinted with permission from Elsevier. (D) SEM micrograph of polyamide nanofibers coating a glass slide. The image was adapted from Schindler et al. (2005), reprinted with permission from Elsevier. If technologies such as these can be extended to provide highly defined 3D microenvironments with nanoscale and microscale features for embedded cells, they may be of great value to unlocking fundamental questions in cell biology. Bars: (A, B, and C) 10 µm; (D) 5 µm.

(Oakley and Brunette, 1993) showed that microtubules are the first element to orient parallel to the grooves followed by actin microfilaments and then focal contacts. Based on the assumption that the initially aligned cytoskeletal component is the major determinant of cell orientation, these findings implicate microtubules as a key regulator of contact guidance. Combining live-cell time-lapse imaging with fluorescent proteins could further enhance our knowledge of the molecular mechanisms underlying topographic guidance.

Applications and outlook

It is clear from the numerous investigations described herein and elsewhere that the nanotopography of the ECM plays a critical role in regulating cell behavior. These interactions, which occur at an extremely small-length scale, are often overlooked when experiments are conducted in the context of larger-length scales. However, specific tools to study the influence of intricate nanoscale features of the ECM are available, and scientists should now be able to take full advantage to gain additional insight into fundamental mechanisms driving cell behavior. Although no in vitro system can perfectly emulate the characteristics of the in vivo environment, these systems hold advantages in their reduced, but relevant, complexity. Nanotopographically defined cell culture models described in this review provide a unique middle ground, maintaining the simplicity of traditional in vitro systems while mimicking small-scale 3D features of the ECM down to the molecular level.

The numerous studies with micro- and nanoengineered substrates described in this review demonstrate influences on cell morphology, migration, proliferation, and differentiation. In 3D, ECM composition, density, and architecture have a profound influence on cell behavior (Amatangelo et al., 2005; Alcaraz et al., 2008; Provenzano et al., 2008). For instance, 3D collagen matrices provide contact guidance, wherein cells preferentially orient parallel to aligned collagen fibrils/fibers and migrate in the direction of collagen alignment (Fig. 5 A; Guido and Tranquillo, 1993; Dickinson et al., 1994; Dallon et al., 1999; Provenzano et al., 2008). This behavior is often

recapitulated on 1D microtracks of fibrillar fibronectin (Doyle et al., 2009). Interestingly, 1D guidance more closely mimics motility in 3D matrices than planar 2D surfaces (Doyle et al., 2009), consistent with observations of carcinoma cells moving along collagen fibers in vivo (Wang et al., 2002), suggesting that fundamental aspects of 3D cell migration in vivo may be successfully studied in more reductionist 1D and 2D culture systems that provide relevant architecture. To date, efforts to engineer 3D microenvironments with defined architecture have focused on microscale technologies, particularly microfluidics (Lee et al., 2006; Lanfer et al., 2008; Sung et al., 2009). Using microscale channels, collagen alignment is achieved in a flow-mediated fashion (Fig. 5, B and C; Lee et al., 2006; Lanfer et al., 2008) and can be prepared to contain embedded cells (Fig. 5 B; Sung et al., 2009). Whereas the matrices are formed in microfluidic channels, nanoscale features arise from the dimensions of the comprised collagen fibrils (Fig. 5 C). As many synthetic matrices can be integrated with natural ECM components and/or reconstituted ECMs (Fig. 5 D; Schindler et al., 2005) and have tunable mechanical properties, such nanoscale constructs may provide valuable resources to study fundamental cell biology in 3D microenvironments.

Precise nanotopographic control of cell behavior will likely allow better understanding of signaling and cellular functions and inspire novel strategies to manipulate cell motility, proliferation, and differentiation. By predefining nanoarchitectures found in vivo with the capability to manipulate surface architectures as well as stiffness, ligand type and density, and chemical stimuli to essentially provide single cells with choices, a wealth of new information becomes available. Although such levels of control are not yet available, they may play a fundamental role in the future of nanotechnology in cell biology. For instance, it will be of interest to investigate whether the presentation of nanotopographic cues that mimic the anisotropic, filament-like properties of ECM would lead to results distinct from cell exposure to disorganized, rough nanoscale surfaces, whose features lack particular geometric definition (Washburn et al., 2004; Brunetti et al., 2010). By virtue of being able to vary the cues regulating

cell behavior in a very precise manner down to nanometer scale, quantitative models can be devised and refined by comparing differing experimental and theoretical outcomes in response to even minute variations in cellular environment. As such, one can now revisit many of the dogmas about regulation of fundamental cellular processes with a more quantitative rigor.

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