

Material Cues as Potent Regulators of Epigenetics and Stem Cell Function

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Biophysical signals act as potent regulators of stem cell function, lineage commitment, and epigenetic status. In recent years, synthetic biomaterials have been used to study a wide range of outside-in signaling events, and it is now well appreciated that material cues modulate the epigenome. Here, we review the role of extracellular signals in guiding stem cell behavior via epigenetic regulation, and we stress the role of physicochemical material properties as an often-overlooked modulator of intracellular signaling. We also highlight promising new research tools for ongoing interrogation of the stem cell-material interface.

Introduction

The stem cell niche is much more than a set of soluble factors and cell-cell interactions; it is also a physical space with definable mechanical and chemical properties that influence a variety of intracellular events. Both in vivo developmental studies and in vitro experiments using substrates with defined mechanical properties have made it increasingly clear that the mechanosensitivity of cells strongly influences their decision-making and that the substrate upon which a stem cell is grown is therefore itself a potent stimulus. While tissue culture-treated plastic is invaluable in research as a reproducible, standardized culture substrate, it possesses physical properties—high stiffness and surface homogeneity—that are non-physiological and known to affect cell fate decisions (Dalby et al., 2007; Engler et al., 2006).

Studies using materials specifically designed to recapitulate individual aspects of a cell's complex physical and mechanical environment have repeatedly shown that a number of physicochemical stimuli affect cell behavior (Stevens and George, 2005). These include factors such as material stiffness (Engler et al., 2006), microstructure and nanostructure (Dalby et al., 2007; McMurray et al., 2011), and three-dimensionality (Levenberg et al., 2003; Mabry et al., 2016). There has already been a highly productive focus on developing and defining stem cell culture conditions in terms of biomolecular cues; a decade-long refinement has allowed the field to move away from the usage of xenogeneic feeders and undefined serum toward fully defined culture media such as 2i + LIF. These innovations have led to greatly improved experimental reproducibility, which is critical for basic biological understanding and eventual clinical translation. In parallel, defined material systems with tunable parameters have provided a framework for studying how (stem) cell fate can be influenced through changes in the extracellular space. These factors are more influential than might be generally appreciated, and the physicochemical properties of culture substrates used for stem cells and their progeny therefore merit additional attention. The application of materials in the biological realm will continue to supplement the role of conventional cues in specifying desirable stem cell behavior.

In this Review, we discuss the biophysical relationship between a cell and its surroundings, particularly focusing on how epigenetic status is influenced by extracellular stimuli. We first describe some of the key mechanisms by which cells sense physical signals from their microenvironment, and we examine the current model for physical linkage of the nuclear envelope to the extracellular space. We then categorize the external inputs that experimentalists have introduced to cells, review the application of materials systems to studying (stem) cell biology and epigenetics, and discuss the intracellular machinery implicated in signal transduction in each case. Finally, we highlight key research tools that we believe hold great promise for ongoing investigations at the interface of stem cell biology and materials science.

Extracellular Mechanosensing

From a cell's perspective, biophysical cues ultimately result in a change in protein conformation in response to tension or compression. Conversion of mechanical inputs to biological responses occurs at several levels, each with varying layers of complexity and often happening simultaneously. At the level of the plasma membrane, cell-matrix and cell-cell adhesions are formed mostly by integrins and cadherins, respectively; these transmembrane adhesive structures are tethered between the cytoskeleton and an external anchor, physically linking the extracellular and intracellular compartments. In response to tension, integrins and cadherins undergo a conformational change, which initiates a variety of cytosolic signaling cascades including activation of the kinases Src and PI3K (Tzima et al., 2005). For a comprehensive review of cell-ECM (extracellular matrix) homeostasis and integrin signaling, the reader is referred to Humphrey et al. (2014). Mechanosensitive ion channels may be similarly activated by tension between the ECM and the cytoskeleton (Ko et al., 2001). Heterotrimeric G-proteins (Gudi et al., 1998) and ion channels (Maroto et al., 2005) can also respond directly to changes in membrane tension or fluidity caused by fluid shear stress or changes in cell shape. Alternatively, although its components constantly turn over, the cytoskeleton forms a rigid network that transmits physical forces to the cell as a whole.

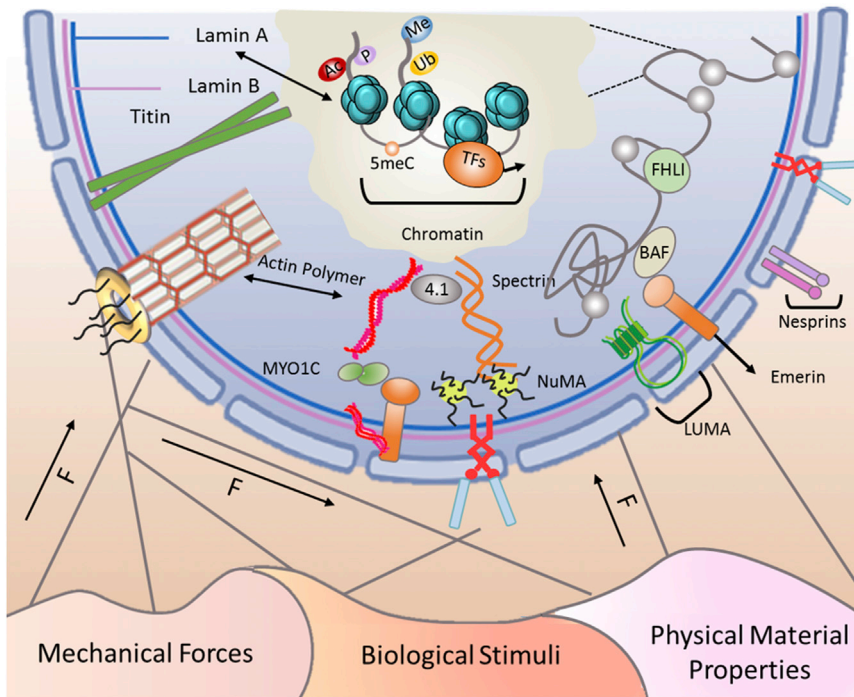


Figure 1. Extracellular Forces Influence the Cell's Epigenetic Status through Interactions with the Nuclear Membrane

The cell is an interconnected entity with cytoskeletal components linking the membrane to the nucleus. Cells sense biophysical signals and propagate forces along the cytoskeleton to the nucleus, altering the epigenetic status and gene expression profile. Here, we classify these cues as mechanical forces, biological stimuli, and physical material properties. The nuclear envelope contains components of the LINC (Linker of Nucleoskeleton and Cytoskeleton) complex including nesprin and SUN proteins, emerlin, and LUMA. These LINC complex components act as receivers and transmitters of mechanical forces to the chromatin and the nucleoskeleton, which includes polymerized actin, nuclear mitotic apparatus protein (NuMA), intermediate filaments, spectrins, protein 4.1, titin, A- and B-type lamins, and nuclear pore complex (NPC)-linked filaments. External forces induce mechanosensitive changes in the nucleoskeletal complexes that, in turn, alter the epigenome and chromatin accessibility. Therefore, these extracellular signals are perceived at the nuclear level as the cell adapts its transcriptome in response to the signals it perceives.

From the cell-extracellular interface, forces can be transduced through these stiff linkages directly to other sites such as the mitochondria (Wang et al., 2001) or the nucleus (Maniotis et al., 1997). Although there are various mechanisms through which extracellular signals generate gene-, protein-, and whole cell-level changes, we focus primarily on mechanotransduction and the downstream behaviors generated in response to external cues for the scope of this Review.

The Nucleus Is Physically Linked to the Extracellular Space

The nucleus is physically linked to the ECM and other cells, acting as a part of a continuous, transcellular tensile network composed of extracellular and intracellular fibers. External biophysical cues, such as the classifications listed in Figure 1, can mechanically alter the nuclear matrix via cytoskeletal filaments, impacting gene expression through recruitment of epigenetic modifiers or reorganization of chromatin and the nuclear lamina (reviewed in Wang et al., 2009a). The nucleoskeleton is a multi-component, intra-nuclear scaffold composed of several classes of proteins—including A- and B-type lamins, spectrin, titin, and nuclear actin—that couple the nuclear envelope to chromatin and whose combined effects impart viscoelastic properties to the nucleus (Swift et al., 2013). The nucleoskeleton is physically connected to the cytoskeleton by LINC (Linker of Nucleoskeleton and Cytoskeleton) complex proteins (Lu et al., 2012), which include emerlin and various isoforms of SUN and nesprin. The LINC complex helps to transduce biophysical forces into functional, intra-nuclear changes, including the expression of mechanosensitive genes (Lammerding et al., 2005). Nesprin proteins connect the nucleus to cytoskeletal components, with the four isoforms exhibiting different binding partners. Nesprin-1 and -2 are the largest (giant) isoforms, connecting to nuclear envelope proteins via C-terminal spectrin repeats and to poly-

merized actin via N-terminal calponin homology (CH) domains (Padmakumar et al., 2004; Zhen et al., 2002). In contrast, the smaller nesprin-3 and -4 isoforms lack the N-terminal CH domain and link the nuclear envelope to either intermediate filaments via plectin (Wilhelmsen et al., 2005) or the motor protein kinesin-1 (Roux et al., 2009), respectively.

Material-Derived Cues Regulate Epigenetic Status

Direct linkage of the nuclear envelope to chromatin implies that physical forces exerted on the nucleus should have direct consequences on chromatin organization and accessibility of DNA for transcription. Epigenetics define the various changes in gene expression that may occur without alteration in the DNA sequence; these include chromatin modifications, DNA methylation, and non-coding RNAs (reviewed in Flynn and Chang, 2014; Laugesen and Helin, 2014; and Lee et al., 2014, respectively). To date, few studies have investigated how synthetic-material-derived cues can regulate epigenetic status, although available data suggest exciting possibilities. For example, when mesenchymal stem cells (MSCs) were cultured on parallel microgrooves, the level of histone H3 acetylation was significantly enhanced relative to that of the flat material control. In addition, when either compressive or tensile force was exerted perpendicular to the alignment of cells within the microgrooves, MSCs demonstrated a decrease of histone deacetylase (HDAC) activity (Li et al., 2011b). In a follow-up study, Downing et al. demonstrated that forced elongation improves the reprogramming efficiency of embryonic fibroblasts by decreasing HDAC activity, which modulates the acetylation pattern of histone H3 and the subsequent susceptibility to reprogramming factors (Downing et al., 2013). This discovery directly demonstrated the ability of a seemingly-subtle material cue to influence the epigenetic signature, as well as the functional consequence in priming cells for reprogramming. Recently, our group has shown

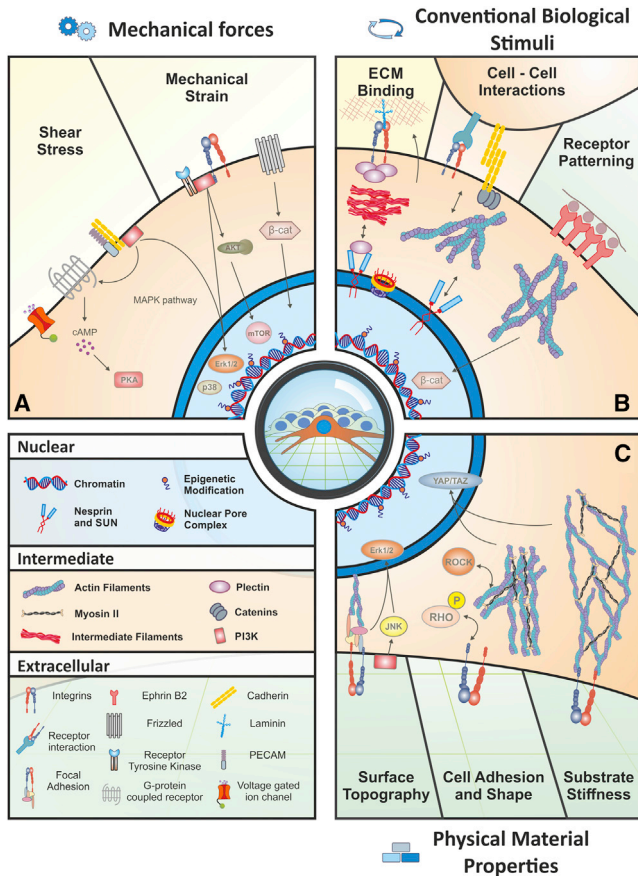


Figure 2. External Biophysical Regulation of Cell Fate and Function Extrinsic cues can be divided into three general categories.

(A) Direct applications of mechanical forces, such as fluid shear stress and mechanical strain, are relatively well-characterized examples of how biophysical signals from the microenvironment activate specific signaling pathways and alter the epigenetic status. Fluid shear stress is transduced through multiple mechanisms, and a few key examples listed here include voltage-gated ion channels, G-protein-coupled receptors (GPCRs), and cell adhesion molecules such as cadherins and platelet-endothelial cell adhesion molecule (PECAM). These sensors activate classical elements such as the MAPK pathway, which leads to the nuclear translocation of ERK1/2 and p38 to act on downstream target genes, and activation of PI3K and PKA. Mechanical strain involves a myriad of receptor tyrosine kinases as well as the Wnt receptor, Frizzled, and an array of integrins. These extracellular mediators can activate the PI3K/AKT pathway, which stimulates mTOR activity.

(B) Conventional biological stimuli include cell-ECM and cell-cell interactions and receptor patterning/clustering. These events exhibit a feedback response: the cell receives specific input from one of these effectors, and in turn it changes the signal by altering the composition or organization of the ECM. A cell can then respond with an additional signal to a neighboring cell, propagating the message into something dynamic. Cell-ECM interactions are dominated by integrins at the cell surface that exert their effect directly on the nucleus via cytoskeletal filaments. Cell-cell interactions act in a similar manner but also include cell adhesion molecules such as cadherins. Receptor patterning occurs naturally in the sense that the spatial distribution of signaling or adhesion molecules affects cell behavior. In a synthetic system, receptors such as Ephrin-B2 can be encouraged to cluster by presenting the ligand at varying densities along a physically linked backbone, which stimulates a stronger response than a single ligand alone; in this case, Ephrin-B2 stimulates the stabilization and nuclear translocation of β -catenin.

(C) Physical material properties at the macro-, micro-, and nano-scales include surface topography, cell adhesion and shape, and substrate stiffness. Topography of the material surface, such as the distribution and regularity of nanopit spacing, activates signaling pathways such as JNK and Erk1/2 and affects cytoskeletal tension. Cell adhesion and shape can be controlled by alteration of the adhesive sites on a synthetic substrate. This effect regulates

that parallel microgrooves also enhance the trans-differentiation of cardiac progenitors into cardiomyocyte-like cells. We showed that the grooved substrates increase histone H3 acetylation and promote sumoylation of myocardin, a modification that strongly activates cardiogenic gene activity (Morez et al., 2015). Therefore, the ability to regulate the epigenetic landscape and subsequent stem cell function with relatively simple material cues suggests that a wealth of unexplored intracellular processes might be controlled by individual or combined extracellular cues.

Categorization of Cues

Each cell is presented with enormous spatiotemporal complexity that results from the summation of soluble and insoluble inputs from the ECM and neighboring cells. Biophysical signals are a particularly interesting category of cue that encompasses both extracellular-derived inputs (fluid shear stress, cyclic strain, and cell confinement) and cell-generated responses (intracellular tension and focal adhesion formation). At the cellular level, however, discrimination between types or sources of biophysical cues might not be as clear as the experimentalist intended. The cell may not *independently* sense complex physical cues such as cell shape, microtopography, or force application, but rather, these cues may differentially impact overlapping tension-sensitive signaling pathways. Although tremendous work has been done in these fields, the mechanisms by and extent to which a cell distinguishes these signals as *distinct* information are yet to be fully understood.

Here, we categorize the “cues” presented to a cell from a materials-based standpoint and classify them simply, but acknowledge through our discussion that the resultant cellular response or responses can overlap. Broadly, we identify three main categories (Figure 2): (1) direct application of mechanical force, such as fluid shear stress or cyclic stretching; (2) conventional biological stimuli, covering the incorporation of soluble cues and tethered adhesion molecules; and (3) physical material properties at varying length scales (macro-, micro-, and nano-scales), covering stiffness, topography, and control of cell shape. By organizing our discussions into these three categories, we can explore the intended efforts of researchers to study specific biological responses while emphasizing how seemingly-different cues can generate similar intracellular responses.

Category 1: Direct Application of Mechanical Forces

The effects of mechanical strain and fluid shear stress on cell behavior are well-characterized examples of “outside-in” signaling that affect epigenetic status. Although these forces are not material derived, they have provided useful information for monitoring how physical extracellular cues can potentially regulate intracellular signaling and the epigenome. Mechanical strain and fluid shear stress are sensed by cells cooperatively—the organization of the cytoskeleton physically links adjacent cells and allows a concerted response to mechanical forces, often through mechanically gated ion channels or transmembrane

cell fate decisions in hMSCs through the Rho/ROCK pathway, which is necessary for the activity of the YAP and TAZ transcription factors. Finally, the physical stiffness of the extracellular substrate heavily influences cell behaviors, such as fate decisions, and this signal is also transduced through YAP/TAZ and the actin cytoskeleton.

proteins (Figure 2A). These interactions converge by regulating the activity of the small GTPase Rho at the RhoGAP and RhoGEF domains, and they are then further transduced via the downstream effector Rho-associated kinase (ROCK) to increase myosin II contractility and intracellular tension. Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding domain (TAZ), both members of the Hippo signaling pathway, are subsequently translocated to the nucleus where they co-activate multiple transcription factors and regulate a host of target genes and cell fate decisions (Dupont et al., 2011). Cells respond to various extracellular stimuli via YAP/TAZ, as will be discussed in more detail in later sections.

The endothelium is a particularly pertinent model for exploring how fluid shear stress affects epigenetic status. Because alterations to the vascular flow profile are associated with the onset and progression of atherosclerosis, changing the type of fluid shear stress experienced by endothelial cells leads to alterations of their epigenetic profile. Laminar fluid shear stress, or unidirectional flow that occurs in healthy vasculature, induces phosphorylation of H3S10, H3K14 acetylation, and nuclear export of HDAC5, which together result in a more accessible genome because of increased acetylation (Illi et al., 2003). Conversely, oscillatory fluid shear stress, which occurs in diseased vasculature, induces the expression and nuclear accumulation of class I and class II HDACs and increases DNA methyltransferase 1 (Dnmt1) expression, which subsequently leads to DNA hypermethylation (Lee et al., 2012; Zhou et al., 2014). Laminar fluid shear stress has been shown to guide mouse embryonic stem cells (ESCs) to differentiate toward the cardiovascular lineage, as evidenced by enhanced transcriptional activity of lineage-specific markers, via altered histone methylation profile, including enhanced acetylation of H3K14 (Illi et al., 2005). Shear stress sensing is an intricate process that requires various mechanisms, notable examples of which include protein kinase A (PKA)-dependent phosphorylation of cAMP-responsive elements (Boo, 2006), extracellular signal-related kinase (ERK)1/2 activation (Huddleson et al., 2005), and PI3K-dependent chromatin remodeling (Takahashi and Berk, 1996).

A wide variety of cells experience mechanical strain and compression in their native physiological location. Cardiac rhythm produces cyclic strain not only locally on cardiac tissue but also on the entire vasculature, in particular nearby arteries, following systole. Vascular smooth muscle cells respond to cyclic, isotropic strain by upregulating HDAC7 and downregulating the level of HDAC3/4, which leads to H3 hyperacetylation and a block in cell migration (Yan et al., 2009). As shown in Figure 2, mechanical strain can be modulated by the Wnt/ β -catenin pathway (Case et al., 2008), PI3K-protein kinase B (Akt) (Danciu et al., 2003), and mitogen-activated protein kinase (MAPK)-mediated p38 signaling (Peng et al., 2010).

Furthermore, LINC complex components have been shown to be critical for transmitting mechanical forces. Knockdown of nesprin-1 reveals its importance in the propagation of mechanical strain from the actomyosin components to the nucleus, as nesprin-1-depleted endothelial cells are unable to reorient in the presence of cyclic strain (Chancellor et al., 2010). Disruption of SUN or nesprin proteins also prevents the physical propagation of ultra-low-magnitude mechanical perturbation in MSCs, indicating that LINC-mediated mechanocoupling between peri-

nuclear actin and the nucleus is required (Uzer et al., 2015). These data illustrate the sensitive epigenetic changes that occur in response to external forces, as well as the intricate signaling pathways involved, and are a driving force for conceptualizing and designing materials for regulating epigenetic status.

Category 2: Conventional Biological Stimuli

The balance between cell-cell and cell-matrix interactions is a critical theme of developmental biology, tissue homeostasis, and disease progression, and materials can be leveraged to bias cells toward one interaction over the other. Attempts to simplify these relationships, as well as to study their fine balance, have used an array of (pseudo)synthetic techniques mimicking the in vivo relationship between cells and their environment (Figure 2B). In a physiological setting, integrin-mediated interactions with the ECM are indispensable for normal development and cell function (De Arcangelis and Georges-Labouesse, 2000). In vitro, arrays of self-assembled peptides have been used as cell culture substrates, demonstrating that heparin-binding peptides that interact with glycosaminoglycans (GAGs) on the cell surface most effectively promote human pluripotent stem cell (hPSC) self-renewal (Klim et al., 2010). Further investigation revealed that combinatorial surfaces that engage both GAGs and integrins promote differentiation of hPSCs toward ectoderm, whereas surfaces that interact exclusively with GAGs enhanced differentiation to mesoderm and endoderm (Wrighton et al., 2014). Materials can also be designed to respond specifically to a remodeling enzyme. A common strategy is to crosslink a hydrogel with an enzymatically degradable peptide so the cellular production of proteolytic remodeling enzymes, in particular matrix metalloproteinases (MMPs), specifically induces localized degradation (Lutolf et al., 2003). This design allows sequestered biomolecules to be released only when a certain enzyme is present to cleave the crosslink and free the molecule.

The stem cell niche involves interactions with supporting cell types and this has been well defined in experimentally tractable model organisms like *Drosophila* and *C. elegans* (Li and Xie, 2005), but it is also known to be true in mammalian systems. For example, maintenance of the hematopoietic stem cell (HSC) niche in the bone marrow requires interactions with multiple supporting cell types, and HSC attachment to osteoblasts is required, in part, to maintain the hematopoietic pool (Calvi et al., 2003). Engineering materials that promote cell-cell interaction through aggregation can be useful for enhancing the soluble and insoluble factors that drive the therapeutic phenotype. A straightforward example is the use of microwells for MSC aggregation; MSCs cultured in hanging drops form spheroids but exhibit practical issues with scalability. By contrast, 100- μ m-deep, microwell-patterned surfaces can be seeded with a single MSC in each well, which then proliferates to produce a large quantity of uniformly sized aggregates (Wang et al., 2009b). Encapsulation of hMSC spheroids is another approach for producing homogeneous populations, a strategy that is particularly beneficial for the delivery of therapeutically active aggregates (Chan et al., 2013). The synthetic recapitulation of cell-cell interactions using bioactive peptides can also induce beneficial regenerative effects. Poly(ethylene glycol) (PEG) hydrogels are a useful tool for these experiments due to their biocompatibility, ease of chemical modification, aqueous solubility, and active

sites for tethering peptides and growth factors. In particular, the experimentalist can incorporate an adhesive RGDS (Arginine-Glycine-Aspartic Acid-Serine) sequence into a 3D PEG gel to encourage cell spreading and vary other mimetic peptides as well. For example, incorporating a peptide that mimics N-cadherin into 3D hydrogels enhances the chondrogenic potential of hMSCs (Bian et al., 2013) and exemplifies how physicochemical and biological cues can be productively combined.

Clustering multiple versions of the same cell surface receptor, including integrins or growth factor receptors, can strengthen or activate the input from a physical signal or soluble ligand, and materials can be engineered that present ligands to enhance or permit this clustering (Figure 2B). For example, by controlling the density of the ligand ephrin-B2 along a soluble biopolymer backbone, the differentiation of neural progenitors was enhanced both *in vitro* and *in vivo*, and similar conjugates were able to promote the differentiation of hPSCs toward a dopaminergic phenotype (Conway et al., 2013). Materials that are engineered to present bioactive molecules in a defined spatial manner are also able to enhance signaling potency (Figure 2B). The spatial control of stem cell fate was achieved by co-presentation of two artificial peptides that bound subunits of the TGF- β receptor II without blocking their ligand-binding domain, causing pre-assembly of the mature complex and sensitizing the cell (Li et al., 2011a).

Category 3: Physical Material Properties with Varying Length Scales

Surface topography. Altered topographical cues within the natural extracellular environment are linked to changes in cell function. For example, the architecture of the *in vivo* microenvironment of cardiomyocytes consists of highly aligned, uniform fibers upon which the healthy cells can contract and transduce signals; in contrast, disease states, such as myocardial infarction, disrupt this architecture via formation of fibrous scar tissues, interrupting normal function. These microstructural cues influence function at the multicellular level by defining organ architecture but may also affect mechanotransduction pathways in individual cells. Similarly, manipulation of nano- and micro-scale topography of a 2D culture substrate surface has a profound effect on the cellular response (Figure 2C). Cues at the cell-material interface can be as simple as the organization of nanosized pits; surfaces with a disordered pattern of 120-nm-diameter pits bias hMSCs toward osteogenic differentiation (Dalby et al., 2007), while highly ordered 120 nm pits maintain long-term, undifferentiated cultures (McMurray et al., 2011). Exactly how artificial stimuli such as these relate to *in vivo* stimuli is not clear, but the authors postulated that differences in physical interactions at the interface might reflect the types of signals that hMSCs receive in their native niche. The effects of the surface topography order extended to changes in metabolism, with undifferentiated cells exhibiting a reduced metabolic load relative to their differentiating counterparts that correlated with regulation by ERK1/2, c-Jun N-terminal kinase (JNK), and low-density lipoprotein (LDL) effectors (Tsimbouri et al., 2012). On the sub-nanometer scale, the cell's recognition of the surface chemistry and shape of ECM proteins may be partially altered by the structure of a substrate polymer itself (Celiz et al., 2015), which enables it to act as a growth or adhesion factor substitute.

Cell shape. Similarly, materials can be used to force a cell to adopt a particular shape. Culturing a cell in 3D by encapsulation in a gel allows retention of a rounded morphology rather than the spreading that occurs on a 2D surface. This is critical for retention of a chondrocyte phenotype (Takahashi et al., 2007) and also encourages MSCs to adopt a chondrogenic fate (Mauck et al., 2006). Finer control of cell shape on a 2D surface can be accomplished by microcontact printing of cell adhesion molecules in particular patterns so that a single cell adhering to the material surface spreads into the desired shape. In an early study, McBeath and coworkers demonstrated that MSCs could be directed toward an adipogenic or osteogenic fate when a small or large surface area for spreading, respectively, was provided; this spreading behavior was shown to be regulated by Rho/ROCK signaling (McBeath et al., 2004). Further evidence suggests that cell-shape-dependent contractility of hMSCs stimulates the expression of JNK, ERK1/2, and Wnt effectors (Kilian et al., 2010). The control of cell shape by the ECM also has profound effects on chromatin structure and gene transcription. 3D culture of human mammary epithelial cells leads to deacetylation of histones H3 and H4, chromatin condensation, and reduced gene expression, and this effect can be recapitulated by forced cell rounding or by inhibiting actin polymerization (Le Beyec et al., 2007). In parallel, fibroblast spreading and actin fiber formation were shown to *inversely* correlate with the nuclear localization of HDAC3, serum response element (SRE), and myocardin-related transcription factor A (MRTF-A), as well as increased H3K9 acetylation and transcription of cell homeostasis-related genes (Jain et al., 2013). These data indicate that spreading leads to less acetylated histones in two different cell types, demonstrating that a complex combination of intracellular contraction/tension-related signaling activity can regulate acetylation profile depending on a cell's shape/adhesion status.

Matrix stiffness. Tissues throughout the body display a wide range of stiffness values, from relatively soft neuronal tissue to far stiffer cortical bone. The composition and organization of the tissue dictate the resulting mechanical properties and influence the behavior of resident cells. Monitoring tissue stiffness changes *in vivo* during development has proven difficult, but evidence from *Drosophila* indicates that developmental ECM dynamics result in changing elasticity, which drives neuronal migration and fate decisions (Kim et al., 2014). Similarly, the developing chick heart becomes stiffer over time as type I collagen is deposited, enabling the tissue to withstand the enhanced contractile forces generated by maturing cardiomyocytes (Majkut et al., 2013). However, the stiffness of fully formed, healthy tissues (i.e., those not undergoing wound healing or fibrosis) remains relatively constant until aging or disease onset. Recapitulation of matrix elasticity has been a focus in the biomaterials community owing mainly to the availability of substrates with tunable mechanical properties. In a seminal 2006 paper, Engler et al. demonstrated the ability of matrix stiffness alone to drive fundamental changes in stem cell behavior. hMSCs cultured on polyacrylamide hydrogels of varying stiffnesses differentiated into specific cell types that corresponded to the compliance of their underlying matrices (Engler et al., 2006). Soft gels ranging from 0.1–1 kPa have been reported to enable adipogenic differentiation while very stiff gels resembling bone tissue drove them toward the osteogenic lineage (Dupont

et al., 2011). When the mechanosensitive signal transduction mechanism was examined, YAP/TAZ were found to be responsible for translating extracellular mechanical cues into functional responses (Dupont et al., 2011), similar to their role in mediating shear stress and extrinsic mechanical forces. Chromatin organization is also regulated by extracellular elasticity: stiff matrix substrates (>50 kPa) that allow the generation of cellular tension lead to increased nuclear area, decondensed chromatin, and H3 acetylation relative to the soft matrix counterparts (Kocgozlu et al., 2010). How these specific extracellular signals stimulate changes in the nucleus has been an open topic of debate, with one promising explanation that physical forces on the nucleus (dis)allow the transport of key transcription factors across the nuclear envelope (Wang et al., 2009a).

In a landmark finding, nuclear stiffness, as determined by the ratio of the intermediate filaments lamin A and B, was shown to correlate with bulk stiffness of tissues throughout the body, and altering this ratio by knocking down or overexpressing lamin A enhanced the elasticity-induced differentiation of hMSCs (Swift et al., 2013). Matrix-permitted cytoskeletal tension stabilizes lamin A at the inner nuclear envelope and stimulates expression of YAP, TAZ, and retinoic acid receptor gamma (RARG) target genes, promoting osteogenic differentiation in hMSCs. Recently, the accessibility of specific epitopes of lamin A/C was shown to be mechanoresponsive; the Ig-domain of lamin A/C is less accessible in the basal nuclear envelope, relative to apical, when the cell is undergoing osteogenic differentiation, during cell spreading, or in the presence of compressive force (Ihalainen et al., 2015). Of note, this epitope was equally available in both apical and basal locations during adipogenic differentiation, or for cells cultured on soft hydrogels, and it overlaps with important binding sites for maintaining efficient mechanotransduction.

Matrix elasticity and composition, as well as the resulting role of the actomyosin contractile machinery, also influence the maintenance and fate decisions of hematopoietic stem and progenitor cells (HSPCs). Soft culture substrates fabricated from full-length tropoelastin (a naturally occurring elastic material) promote the expansion of murine HSPCs (Lin⁻Sca-1⁺c-kit⁺, LSK cells) and human progenitors (Holst et al., 2010). However, this effect was reduced when HSPCs were cultured on stiffer gels of truncated or crosslinked tropoelastin, or in the presence of inhibitors of myosin heavy or light chains, indicating a role of the contractile machinery. Furthermore, the specific myosin II heavy chain isoforms A and B exhibit divergent roles in HPSC regulation in vitro and in vivo. Myosin IIB regulates asymmetric cell division and is downregulated during differentiation of human HSPCs; partial knockdown of myosin IIB promotes symmetric division and increases the number of human CD34⁺ progenitors expanded in culture (Shin et al., 2014). In contrast, dephosphorylation of myosin IIA (pS1943), which renders it more active, increases during cytokine-induced differentiation or following culture on stiff substrates (34 kPa). In vivo, myosin IIA was shown to be required for sustained hematopoiesis in an allogeneic transplantation model, as LSK cells were ten times less abundant in the marrow after 8 weeks when myosin IIA was deleted from donor cells; myosin IIB, however, contributes to differentiation, but not survival, of xenogeneic transplanted cells (Shin et al., 2014). These data demonstrate the fundamental

role of mechanosensing and mechanoregulation in HSPC fate decisions.

Promising Research Tools for Studying the Cell-Material Interface

Increasingly sophisticated cell-influencing materials are under constant development, and concurrent advances in techniques that aim to analyze the interactions between a cell and its environment have provided methods to monitor these behaviors. Below, we have selected examples of some of the most promising aspects of ongoing research that will contribute to the evolution of our field (Figure 4).

Atomic Force Microscopy

The ability to reproducibly image nano-scale features with high spatial resolution in living cells, as well as measure the associated physical properties, has been developed to a high standard using atomic force microscopy (AFM). These microscopes are now accessible to many biologists and interdisciplinary researchers, and the development of combinatorial AFMs with other types of microscopes (i.e., confocal fluorescent) has provided a range of tools that can explore cellular processes in real time. For example, the stiffness of patient-derived metastatic cancer cells was measured by AFM and shown to be 70% softer than local benign cells, ranging from ~0.50 kPa to ~2.0 kPa for cancer and healthy cells, respectively (Cross et al., 2007). AFM has also been used extensively to measure the surface modulus (stiffness) of synthetic culture scaffolds as well as the force required to detach an antibody from its ligand (Wen et al., 2014), allowing investigation of how the cell interacts with the microenvironment at relevant length and force scales.

Scanning Ion Conductance Microscopy

An emerging technique that offers similar advantages to AFM in terms of high spatial resolution and versatility is scanning ion conductance microscopy (SICM). Although SICM is not nearly as available to biology researchers as AFM, the customizable features are promising for a range of applications. Non-invasive imaging by SICM is achieved using a nanopipette in aqueous, conducting media through which changes in the ion current are measured and then used as feedback to prevent the tip from physically interacting with the surface. This information is then converted into measurements of the proximity and physical properties of the cell surface, providing a high-resolution image of the sample of interest without direct interaction (Korchev et al., 1997). This approach has been leveraged to image the precise location of individual ATP-regulated K⁺ channels in living cardiac myocytes with ~50 nm resolution, volumes of whole cells and their subcellular protrusions with 10⁻²⁰ liter resolution, and the organization of single proteins or complexes at the cell surface with a smaller nanopipette that allowed 3–6 nm lateral resolution (Shevchuk et al., 2006).

Super-resolution Microscopy

Advances in light microscopy have resulted in unprecedented resolution and image quality that has enhanced our understanding of how cellular processes and cells interact with their microenvironment. The diffraction limit of light has long been considered an inherent roadblock to enhancing spatial resolution. Various forms of super-resolution microscopy exist, including lattice light-sheet microscopy, structured illumination microscopy (SIM), photoactivation localization microscopy (PALM),

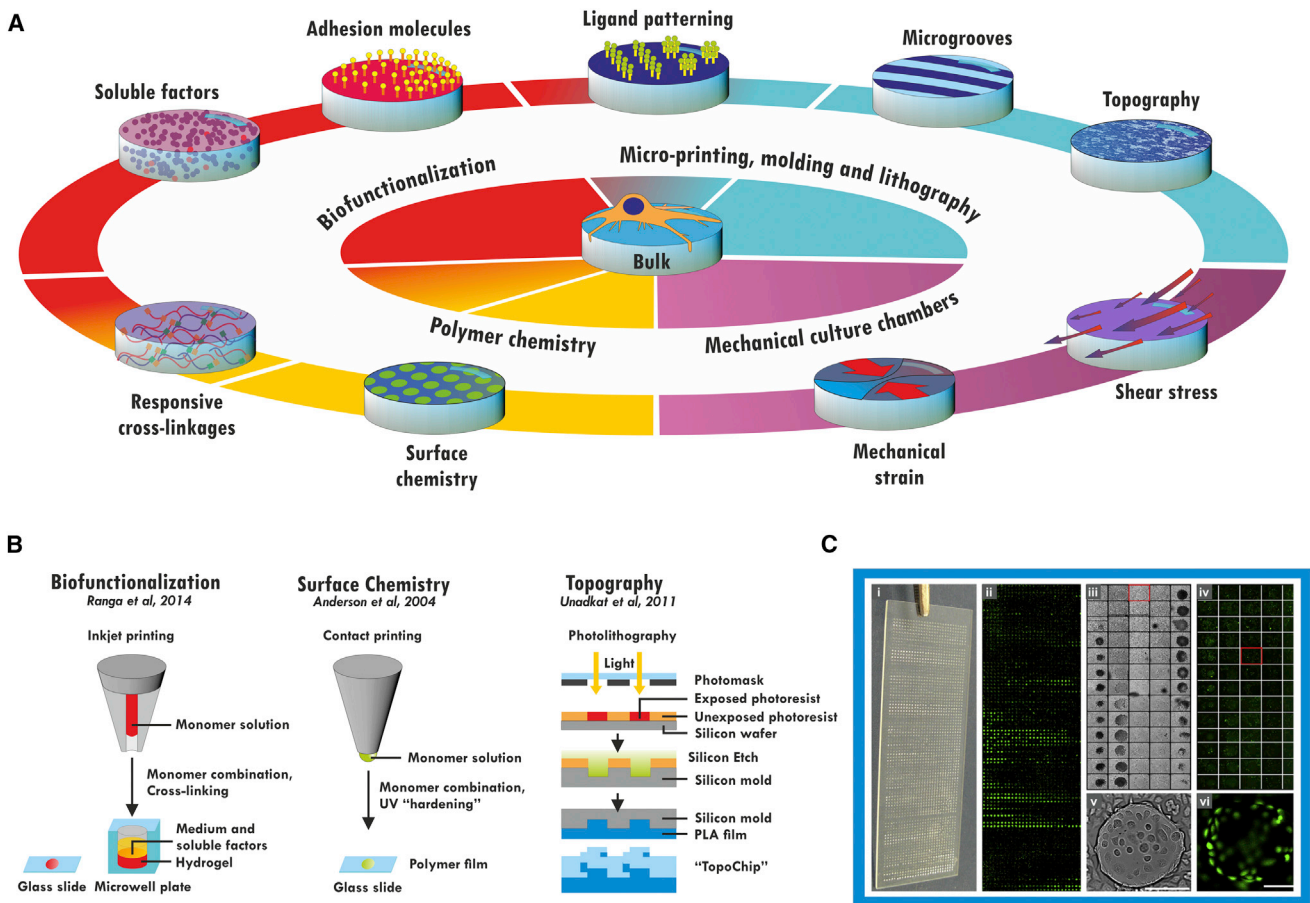


Figure 3. Accessing Complexity

(A) A bulk material can be further functionalized by a number of routes. Soluble biomolecules can be incorporated into the bulk of the material or presented on the surface, and hydrogels may be made biologically responsive by use of protease-degradable crosslinkages. Micropatterning techniques allow these cues to be patterned within the material, and they also allow the material itself to be patterned into grooves or more complex topographical cues on the micro- or nano-scales. The chemistry of the material itself can be altered (e.g., by being blended with other polymers) to confer responsiveness or alter the cell-material surface interface. With the appropriate culture apparatus, cells may also be exposed to mechanical strain or fluid shear stress in combination with material-derived cues. (B) High-throughput screening (HTS) has been applied from a discovery-driven standpoint to identify material properties that elicit specific effects on cell behavior. Biofunctionalizations have been screened using micro-inkjet systems to generate hydrogels within a 1,536-well plate or on a glass slide. Surface chemistry can be investigated by micro-contact printing and UV crosslinking of monomer combinations on a glass slide. Topographical cues have been investigated using photolithography to etch micro-scale geometric cues onto a poly-lactic acid (PLA) chip. The response of cells is screened by imaging (C) the entire surface, generating values for cell attachment or proliferation (iii, v) and fluorescent reporter expression (ii, iv, vi) for each individual material spot. (C) is adapted with permission from Zhang et al. (2009). Scale bar, 200 μm .

and stochastic optical reconstruction microscopy (STORM) (Chen et al., 2014) (reviewed in Sydor et al., 2015). Here, we discuss stimulated emission depletion (STED) microscopy, which has overcome the diffraction limit by quenching the excited molecules at the periphery of a focal spot, providing a smaller excitation point, which results in higher resolution. This approach confers a 4-fold improvement in x-y resolution and a 16-fold reduction in focal-spot cross-sectional area compared to confocal microscopy, which means it allows unprecedented observation of cellular events at the nano-scale. Illustrating the particular significance of enhanced spatial resolution, the arrangement of organizational proteins in the mitochondria was measured for the first time using STED, and defining features with no known function such as a highly periodic spatial distribution of the proteins were observed (Jans et al., 2013).

Raman Spectroscopy

Raman spectroscopy (RS) provides information about the types and relative abundance of chemical bonds present in a sample by calculating shifts in photon frequency due to inelastic scattering. This provides a global snapshot of the distribution of biomolecules, such as proteins, lipids, and DNA, in a cell or tissue and can be used to characterize spatiotemporal changes in cell composition, deposited ECM, and heterogeneous cell populations. Some of the earliest applications of RS to biology focused on monitoring stem cell phenotype during differentiation as a means to identify discernible differences such as mRNA levels in mouse ESCs (Nottingham et al., 2004). In 2009, our group used RS to capture “fingerprints” of mineralized bone nodules derived from osteoblasts, MSCs, and ESCs (Gentleman et al., 2009). Following thorough characterization, we concluded that hMSCs and osteoblasts could generate nodules similar to native

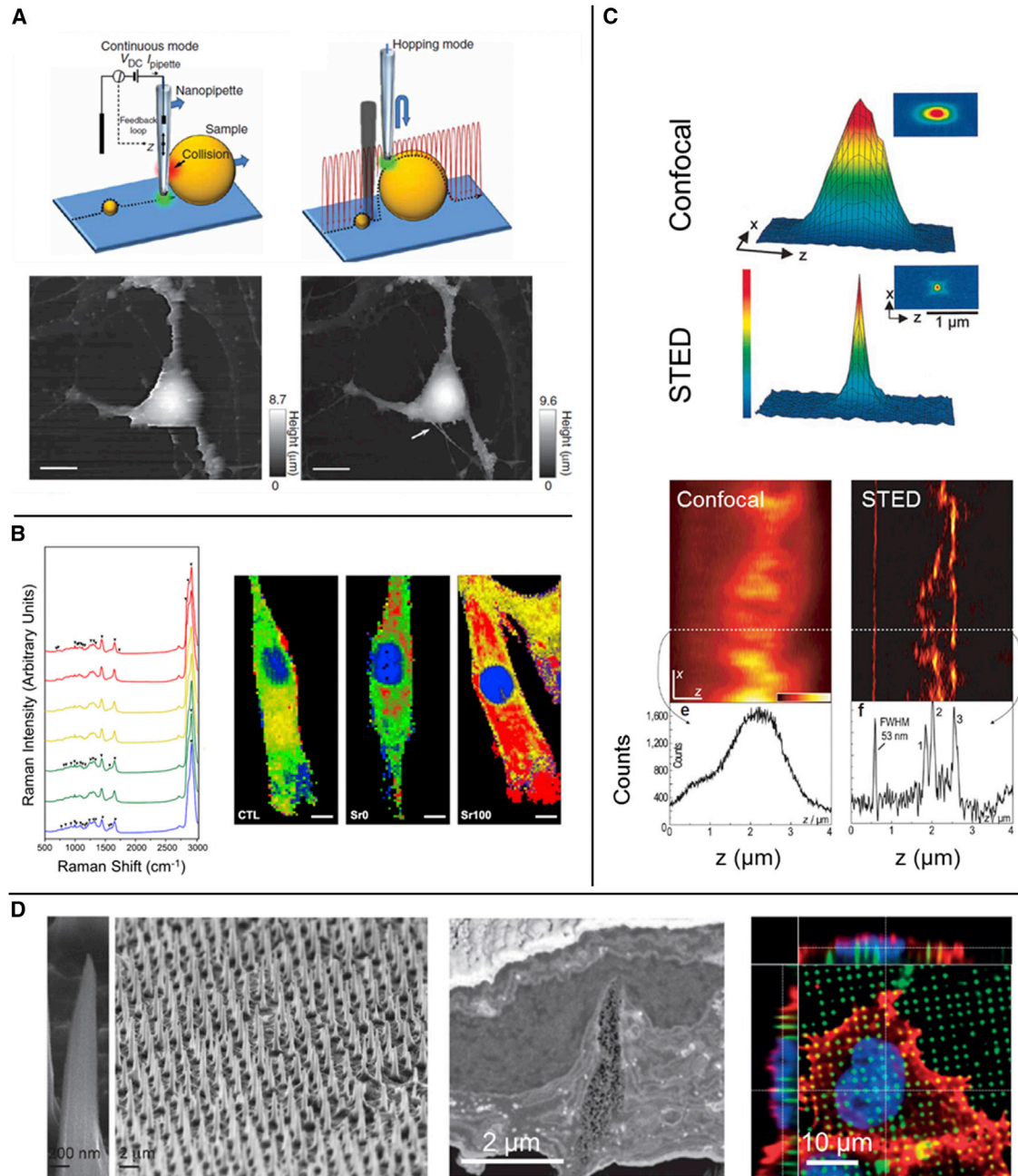


Figure 4. Promising Research Tools: Characterization Techniques, Imaging Modalities, and Novel Materials

(A) Scanning ion conductance microscopy (SICM) uses a nanopipette to produce an image of a living cell without physical interaction. Two different modes of SICM are shown here: continuous mode, in which the probe does not significantly change its z position, and hopping mode, in which the probe is constantly approaching the sample and then retreating. In hopping mode, small features that are not observed with continuous mode can become spatially resolved. Scale bars, 10 μm .

(B) Raman spectroscopy produces a chemical “fingerprint” of the cell, cells, or tissue that is studied, providing information about the specific chemical bonds present in the sample. This information can then be used to produce a 2D or 3D map that shows the localization of certain classes of molecules. For example, bioactive glass materials with a significant amount of strontium ion (Sr100) lead to a shift in the lipid distribution in MSCs (lipid, red). Scale bars, 10 μm .

(C) Stimulated emission depletion (STED) microscopy overcomes the diffraction limit typically associated with light-based imaging techniques. The focal point of a STED microscope is much smaller (bottom graph) than that of a standard confocal (top graph). This allows enhanced spatial resolution, such as that able to image the microtubule network in monolayer cell culture. Typical confocal (left image) produces an image in which the network cannot be observed, but sub-diffraction STED imaging allows crisp visualization of the subcellular processes.

(D) High-aspect ratio nanoneedles can be produced into high density, 2D arrays to act as cell culture substrates. HeLa cells cultured on nanoneedles interact directly with the underlying material, and the needles physically deform both the cell membrane and the nucleus.

Figures are adapted with permission from the following. (A) Nature Publishing Group (NPG) Ref: [Novak et al. \(2009\)](#); (B) Ref: [Autefage et al. \(2015\)](#); and (C) Ref: [Klar et al. \(2000\)](#) and NPG Ref: [Dyba et al. \(2003\)](#). (D) was reprinted with permission from Ref: [Chiappini et al. \(2015c\)](#). Copyright 2015 American Chemical Society; NPG Ref: [Chiappini et al. \(2015b\)](#) and Ref: [Chiappini et al. \(2015a\)](#).

bone in terms of their composition and nano-scale architecture, but ESCs deposited bone that was reminiscent of mechanically compromised, aging tissue. RS has also shown promise for non-destructive, label-free identification of cell phenotypes such as cell lines that most closely resemble the *in vivo* counterpart (Swain et al., 2010). An emerging application of RS will be the non-destructive observation and characterization of stem cell dynamics during culture on advanced material platforms such as those listed above; however, only very recent studies have demonstrated the feasibility of these processes (El-Said et al., 2015; von Erlach et al., 2015) and interdisciplinary collaboration to achieve these goals will undoubtedly be necessary.

Next-Generation Chromatin Imaging

It is well accepted that genome organization regulates the transcriptional profile by enabling direct physical interactions between gene promoters, distal regulatory elements, and transcriptional proteins. Electron microscopy and fluorescence *in situ* hybridization (FISH) assays have been employed to study the spatial organization of chromatin; however, these methods are still currently limited in throughput, analyzing only a few loci at a time. Molecular assays based upon chromosome conformation capture (3C) have allowed advanced understanding of how physical interactions between distant genomic loci affect the biophysical properties of chromatin (Dekker et al., 2002) as well as the functional relevance of chromatin interactions with the proteome (Alabert et al., 2014). For example, a modified version of 3C (Hi-C) applied to monitor higher-order chromatin dynamics in hESCs during differentiation revealed that extensive chromatin reorganization occurs during lineage specification and provided new insights for allelic expression biases that have been previously observed (Dixon et al., 2015). The application of high-resolution, time-resolved chromatin rearrangement, as well as interactome information, for (stem) cells in contact with materials would shed more light on how external biophysical forces result in whole-cell-level changes.

Developing New Materials Systems: Discover or Design?

The development of new materials can either follow design- or discovery-driven approaches. Discovery-driven approaches are most appropriate when the underlying mechanisms of an effect are unclear, as is the case for how polymer chemistry influences cell behavior (Celiz et al., 2014; Simon and Lin-Gibson, 2011). The approaches used and discoveries made have been extensively reviewed elsewhere; briefly, combinations of monomers are typically printed on a glass slide and polymerized *in situ* to form microarrays of films (Anderson et al., 2004; Celiz et al., 2015) (Figure 3B). Combinations of biological functionalizations of hydrogels have also been screened in microwell plates (Ranga et al., 2014), and topologies have been screened by algorithmic design of a poly-lactic acid chip (Unadkat et al., 2011).

As a problem involving the synthesis of complex polymers whose properties and biological effects cannot be fully predicted *a priori* from the properties of their monomers, high-throughput screening (HTS) of materials is comparable to the directed evolution of proteins. Well-established key criteria for successful directed evolution experiments that may be relevant to materials HTS studies include (1) appropriate selection criteria, (2) a sufficiently large and functionally diverse library, and (3) appropriate

intergenerational candidate mutation and recombination (Romero and Arnold, 2009). As cell response in materials HTS studies is typically evaluated by one or two parameters—cell number (attachment and survival) and expression of a single fluorescent marker—other effects and responses may change if unchecked. Following their initial screening, Ranga and colleagues found that differently biofunctionalized hydrogels that were apparently similar in their ability to support ESC proliferation and Oct4 expression were varied in their induction of other pluripotency markers such as SSEA-1 and Rex1. The physical properties of materials are also susceptible to this effect—the final polymer identified by Celiz and coworkers cracked after coating on tissue culture flasks, though fortuitously, they had also identified blends with similar properties that did not. So far, the largest materials libraries have been many orders of magnitude smaller than most protein libraries (Celiz et al., 2015; Romero and Arnold, 2009). This may limit movement away from local fitness optima; several screens have identified homopolymers as the best-performing, final candidates, and moving away from these materials in favor of co-polymers that might potentially perform better is difficult when poorly performing candidates lie in between (Celiz et al., 2015; Hook et al., 2013; Mei et al., 2010). Unlike proteins, a material's properties are not necessarily acutely dependent on an exact sequence of monomers. Altering higher-order structure and organization may be a productive, if challenging, route to library enlargement and diversification, in addition to screening multiple co-polymers. Multigenerational biomaterials screens have so far manually curated candidates for each stage, while directed evolution (DE) experiments diversify and recombine the fittest candidates in a stochastic fashion. Adoption of this strategy, coupled with less stringent selection criteria over more generations, may permit tolerance of intermediate, low-fitness stages as the screen moves away from local optima and toward a more effective exploration of the potential polymer space.

By contrast, design-based approaches are most useful when an underlying structure-function relationship is already known or hypothesized. New classes of materials that exhibit complex physical behaviors are emerging and can be used to further probe how cells respond to their biophysical microenvironment. For example, structural components of cells and ECM are viscoelastic in nature, yet most synthetic materials used in cell culture display only the elastic component. Materials that exhibit stress *relaxation* (reduced stress under constant strain) promote cell spreading on soft substrates comparable to that of cells on stiff, elastic substrates (Chaudhuri et al., 2015a), and hMSCs cultured in 3D gels of increasing stress relaxation rates exhibit enhanced osteogenic differentiation (Chaudhuri et al., 2015b). Synthetic materials that exhibit stress *stiffening* (increased stiffness when a particular stress is applied) mimic the temporal response of gels formed from cytoskeletal and ECM components, and the onset of stiffening can be tuned by changing the polymer length (Kouwer et al., 2013). When used as 3D culture substrates, stress-stiffening gels of the same bulk stiffness can determine commitment of hMSCs toward either adipogenic or osteogenic lineages, depending upon the point at which stress-stiffening is experienced (Das et al., 2015). Furthermore, scaffolds fabricated from methacrylated dextran (DexMA) present fibers at the cell-material interface, instead of a flat surface like a classical

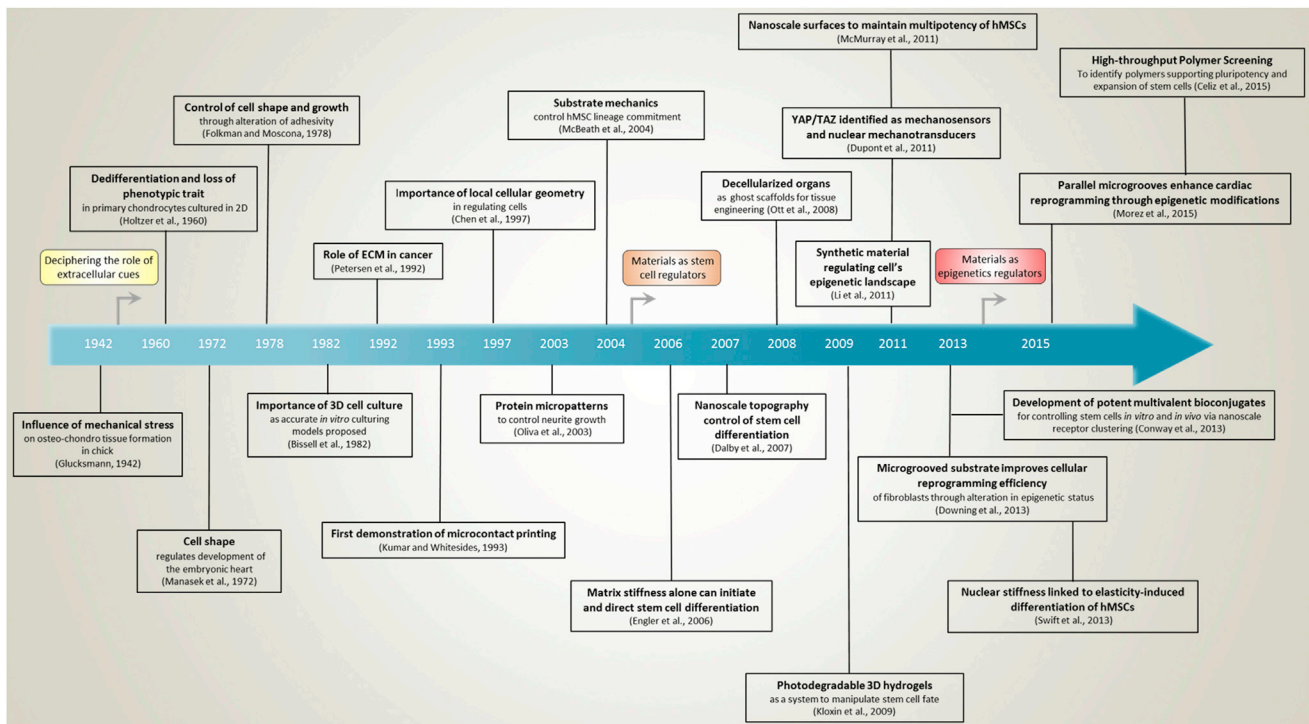


Figure 5. Key Events in the Field of Biomaterials as Stem Cell Regulators

hydrogel, but still offer user-defined control over cell adhesiveness and mechanical properties (Baker et al., 2015). When cultured on DexMA gels of low fiber stiffness, hMSCs exhibited enhanced spreading and focal adhesion formation due to their ability to physically reorganize the matrix by bringing fibers and the associated adhesive sites into closer proximity to the cell; these effects were strikingly similar to behaviors observed for cells on fibrous collagen substrates, but not homogenous, non-fibrillar hydrogels (Baker et al., 2015). It is difficult to pinpoint a specific relationship between the mechanical properties of these gels and the cellular response, but elucidation of exactly how this outside-in signaling occurs will continue to progress the status of the field.

Dynamic systems that allow real-time manipulation of substrate properties have demonstrated that cells respond to immediate alterations in the extracellular microenvironment. Hydrogel chemistry can be exploited to increase stiffness by exposure to specific wavelengths of light; when gels underwent an increase in stiffness from 3 to 30 kPa, the spreading of hMSCs was increased along with bias toward osteogenic differentiation (Guvendiren and Burdick, 2012). Taken one step further, the ability to soften hydrogels by exploiting photodegradable crosslinkers has revealed that hMSCs possess a “mechanical memory” by which the duration of culture on a stiff substrate influences the subsequent ability of the cells to adapt and respond to culturing on a soft substrate (Yang et al., 2014). YAP/TAZ responsiveness in this hydrogel system could be attenuated after 7 days pre-culture on a stiff substrate and was lost completely if the cells were cultured on a stiff substrate for 10 days. When translated into 3D scaffolds, additional responsive cues can be added to hydrogel

systems to allow the study and exploitation of cell behavior. The ability to degrade the surrounding matrix directly affects hMSC fate decisions by (dis)allowing force generation; non-degradable gels inhibit cell spreading and traction force generation, biasing the cells toward adipogenic differentiation, while MMP-degradable gels allow cell spreading and force generation in a microenvironment that favors osteogenesis (Khetan et al., 2013). Patterning the 3D environment using elegant chemistry and two-photon microscopy has provided a means for specific tailoring of material properties and protein presentation with high spatial resolution. This technique has proven useful for controlling the dynamic addition and removal of the Notch ligand, Delta, and vitronectin to guide stem cell fate (DeForest and Tirrell, 2015). Next-generation material systems that are tailorable in real-time are promising platforms for elucidating the response of cells cultured in/on them and will allow more complex biological questions to be addressed.

Advances in biomaterial fabrication techniques have made it possible to create nanostructures with precise geometry, textures, and rigidity. An emerging class of nanomaterials for cell-interfacing are nanoneedles and nanopillars. These can be produced in different formats, from nanostraws with a hollow core (VanDersarl et al., 2012) to high-aspect ratio porous needles (Chiappini et al., 2015b). These materials have shown tremendous potential for biological applications such as delivery of biomolecules, electrophysiological measurements of neuronal cells, and pH sensing (Chiappini et al., 2015a; VanDersarl et al., 2012). We found that our newly developed class of porous silicon nanoneedles was not only effective at cellular interfacing for delivery to the cells and biosensing applications, but its application in vivo

did not elicit any notable inflammatory response (Chiappini et al., 2015b, 2015c). Since nanoneedles can modify the shape of both the cell and the nuclear membrane, they will be of future interest in studying associated effects on epigenetics.

Conclusion

Within solid tissues, there is no such thing as an isolated cell; all cells exist interconnected in a tensile network that they both secrete and comprise. Unified movements of cell sheets and deposition of ECM are critical events in development. For tissue stem cells such as MSCs, and even for cells such as ESCs and iPSCs that lack true stable physiological counterparts, the finding that mechanosensing and other physical cues exert a control on fate is, in retrospect, hardly surprising. As the field progresses, details of the signal transduction pathways and epigenetic changes underlying these events are starting to emerge, and we have outlined some key events in the development of this field from basic insights into cell mechanosensing to recent applications of materials biology to cell fate control (Figure 5). Cross-disciplinary collaboration and increased adoption of tractable materials systems by biologically focused research groups are both likely to accelerate development of an increasingly complete understanding of these events. In parallel, new emerging materials systems will provide increasingly sophisticated investigative and therapeutic tools. We suggest that together, these strands of research have great potential to both illuminate basic biological questions on stem cell decision-making and inform progress toward increasingly relevant models of disease and effective regenerative therapies.

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