

# Presentation Counts: Microenvironmental Regulation of Stem Cells by Biophysical and Material Cues

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microenvironment, niche, biomaterial, biophysics,  
mechanotransduction

## Abstract

Stem cells reside in adult and embryonic tissues in a broad spectrum of developmental stages and lineages, and they are thus naturally exposed to diverse microenvironments or niches that modulate their hallmark behaviors of self-renewal and differentiation into one or more mature lineages. Within each such microenvironment, stem cells sense and process multiple biochemical and biophysical cues, which can exert redundant, competing, or orthogonal influences to collectively regulate cell fate and function. The proper presentation of these myriad regulatory signals is required for tissue development and homeostasis, and their improper appearance can potentially lead to disease. Whereas these complex regulatory cues can be challenging to dissect using traditional cell culture paradigms, recently developed engineered material systems offer advantages for investigating biochemical and biophysical cues, both static and dynamic, in a controlled, modular, and quantitative fashion. Advances in the development and use of such systems have helped elucidate novel regulatory mechanisms controlling stem cell behavior, particularly the importance of solid-phase mechanical and immobilized biochemical microenvironmental signals, with implications for basic stem cell biology, disease, and therapeutics.

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## INTRODUCTION

In the early twentieth century, scientists observed that some but not all cells could give rise to multiple specialized cell types in blood (Danchakoff 1916) and that cell proliferation and lineage specification were required for embryonic development. These observations were among the first to support the concept that stemness—the capacity for extended self-renewal and multilineage differentiation—is attributed to individual cellular entities. The idea of the stem cell was further supported by the first bone marrow transplant in 1956 (Thomas et al. 1957), in which the proliferation and differentiation of cells from the grafted marrow repopulated the hematopoietic system of a cancer patient following radiation and chemotherapy. In the 1960s, McCulloch, Till, and colleagues (Becker et al. 1963, Siminovitch et al. 1963) provided the first definitive and quantitative evidence for the existence of stem cells by demonstrating that bone marrow cells injected into irradiated mice formed colonies in the spleen

that were clonal in nature but gave rise to cells from three different hematopoietic lineages.

Although some initially thought stem cell behavior to be determined in a purely stochastic fashion (Bjerknes 1985, Nakahata et al. 1982, Till et al. 1964, Vogel et al. 1968), a wealth of research has established that numerous exogenous factors—including growth factors, morphogens, cytokines, small molecules, extracellular matrix (ECM) proteins, and ligands presented by adjacent cells—can strongly affect stem cell self-renewal and differentiation. This regulatory influence of the extracellular microenvironment was formally conceptualized by Schofield (1978) as the stem cell niche. Taken to an extreme, because the cell's behavior cannot be fully realized without exogenous cues, stemness can be regarded as a collective function of the stem cell and its microenvironment, a view supported by several lines of evidence. The loss of key regulatory proteins or supporting cells in the niche can lead to the depletion of stem cells in multiple tissues (Adams & Scadden 2006, Hayashi et al. 2009, Tanentzapf et al. 2007). In addition, the natural niche can actively convert nonstem cells into stem cells (Cheng et al. 2008), and remarkably, this dedifferentiation may be the primary source of germline stem cells in the *Drosophila* testes (Sheng et al. 2009). Furthermore, exogenous soluble factors can help induce multipotency in specialized progenitors (Kondo & Raff 2000) and pluripotency in nonstem cells (Marson et al. 2008). Finally, misregulation of niche properties may lead to tumorigenesis (Paszek et al. 2005). These examples illustrate the importance of efforts to learn more about the stem cell microenvironment.

Toward this goal, there has been major progress in elucidating the roles of small, often soluble protein factors in stem cell systems, such as Wnt proteins (Kalani et al. 2008, Lie et al. 2005, Reya et al. 2003), insulin and fibroblast growth factors (FGFs) (Bendall et al. 2007), and cytokines (Zandstra et al. 1997, Zhang & Lodish 2008). This important work has been extensively reviewed elsewhere (Boonen & Post 2008, Martinez-Agosto et al.

**Extracellular matrix (ECM):** a meshwork of proteins, polysaccharides, and glycoproteins that provides structural and adhesive support to cells and tissues

**Microenvironment:** the soluble and solid-state surroundings of a cell, including the biochemical and mechanical properties of the ECM, adjacent cells, and interstitial fluid

2007, Shenghui et al. 2009, Suh et al. 2009). In addition to soluble signals, however, it is becoming increasingly clear that biology encodes and conveys regulatory information in other ways. Specifically, there are numerous aspects of the solid-state microenvironment—in particular ECM factors, proteins immobilized to the ECM, and neighboring cells—that may play a role in regulating stem cell behavior; however, these components are comparably difficult to study because of experimental challenges in recapitulating complex cell-matrix and cell-cell interactions *in vitro*. To address this challenge, engineered material systems in combination with analytical methods developed over the past half century have provided platforms to perform reductionist biology on solid-state biochemical and biophysical aspects of the niche. This work initially has been phenomenological, conceptually akin to cloning a new growth factor without yet knowing its receptor or downstream signaling pathways, but it has benefited from parallel progress in the fields of signal transduction and mechanobiology. As a result of these efforts, it is becoming increasingly apparent that numerous solid-state biochemical aspects of the stem cell microenvironment are important regulators of cell behavior, including the conformational, spatial, and temporal presentation of immobilized signaling factors and adhesive ligands. Also key is the biophysical context in which these factors are presented, such as the stiffness, topography, stresses, strains, and dimensionality of the system. This review will therefore discuss the manners and in some cases the mechanisms by which biophysical and solid-state biochemical signals can regulate stem cell function and fate.

## ENGINEERED STEM CELL CULTURE SYSTEMS

The microenvironments surrounding stem cells are structurally complex, which renders experiments to explore the effects of this structure on cell function difficult. For example, the biophysical characteristics of a tissue are the aggregate properties of numerous ECM

macromolecules and resident cells. Thus, it is not trivial to independently control and vary the biochemical and biophysical properties of this amalgam, which makes it challenging to study the specific effects of, for example, various microenvironmental mechanical properties on cell function. Likewise, many regulatory proteins are presented in a complex manner that is difficult to control and emulate *in vitro*, for instance because of complex posttranslational modifications (Mann & Beachy 2004, Zeng et al. 2001), presentation as transmembrane proteins from adjacent cells (Bray 2006), or spatially structured 3D presentation.

To conduct reductionist biology on such complex environments, engineered material systems have recently been developed with the capacity to quantitatively tune one or more regulatory properties in a modular manner, which has enabled detailed mechanistic studies. These systems have several characteristics that enable them to emulate natural microenvironments. For example, biological tissues are hydrogels, networks of insoluble, natural biopolymers that absorb sufficiently large quantities of water that the majority of the resulting material is aqueous. Accordingly, many natural (e.g., collagen, fibrin, and hyaluronan) and synthetic (e.g., polyacrylamide, alginate, polyethylene glycol, and self-assembling synthetic peptides) gels have been utilized as ECM scaffolds. Many of these hydrogels can be used to study stem cells in both 2D and 3D. Furthermore, synthetic materials provide several advantages over natural ones, including the ability to generate a wide range of possible stiffnesses (in 2D:  $10\text{--}10^6$  pascals, Pa, where Pa is a unit that denotes the stress, or force per area, required to induce a measured material deformation), the potential inclusion of degradable cross-links (e.g., peptide substrates for matrix metalloproteinases or photolabile linkages), the capacity to form complex geometrical structures such as ridges and microposts by polymer-casting techniques, and the inclusion of protein adsorption-resistant surfaces (e.g., polyacrylamide, polyethylene glycol) to avoid fouling by soluble or secreted proteins in culture over time.

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**Topography:** a surface's shape and physical geometrical features

**Stress:** amount of force exerted per unit area

**Strain:** spatial deformation of a material

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**RGD:** arginine-glycine-aspartic acid

**Shear stress:** stress applied parallel or tangential to a surface of a material or cell

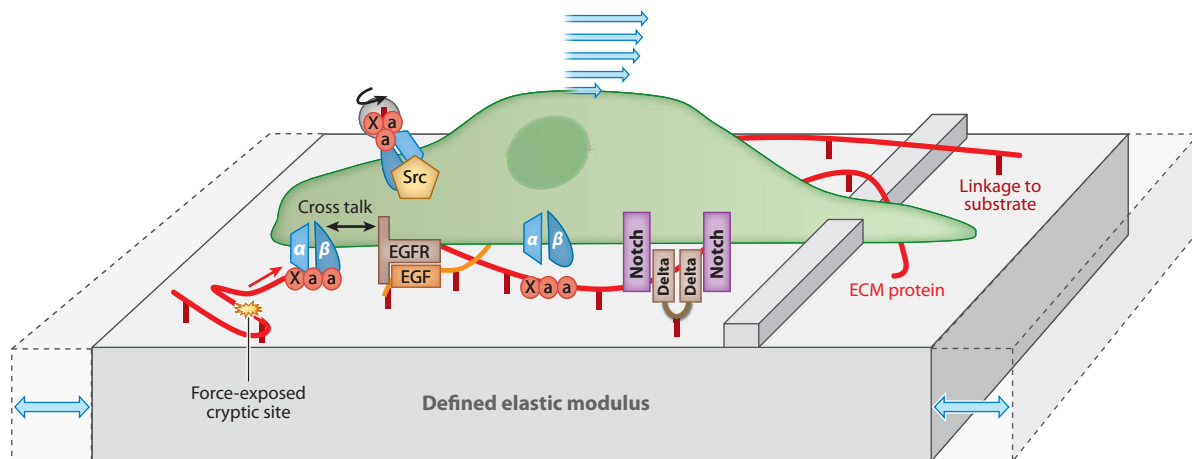
Synthetic systems can also be engineered to independently modulate biochemical properties. Adhesive ligands and/or regulatory proteins can be grafted onto hydrogels at controlled densities, while the material's mechanical properties can be adjusted independently by tuning the cross-linking density of the hydrogel's inert polymer skeleton. The bioactive ligands typically used to functionalize synthetic hydrogels include natural proteins such as laminin, fibronectin, collagen, and fibrinogen (Peyton et al. 2008). More specific interactions can be studied by conjugating small biomimetic peptides containing sequences such as arginine-glycine-aspartic acid (RGD), an integrin-engaging motif in ECM proteins such as fibronectin and collagen (Pierschbacher & Ruoslahti 1984), onto hard surfaces or synthetic hydrogels (Massia & Hubbell 1990a,b). RGD and other ligands can also be spatially patterned onto synthetic surfaces using micro-contact (Kane et al. 1999) or inkjet (Phillippi

et al. 2008) printing to study the effects of ligand patterning on stem cell function.

In addition to presenting constitutive cues, materials systems can be engineered for dynamic variation in properties or application of external mechanical forces. For example, hydrogels or flexible membranes can be compressed or stretched to assess stress and strain effects on cells, which can, for example, simulate the effects of pulsatile blood flow. Fluid can also be flowed over cells at defined velocities and shear stresses. In sum, such engineered systems have been applied to present a variety of static or dynamic biochemical and biophysical cues in a modular and quantitative fashion to explore new mechanisms through which the niche can instruct stem cell biology (Figure 1).

### INFLUENCE OF SOLID-PHASE BIOCHEMICAL PROPERTIES

Specificity of interactions in biological systems is crucial for developing and maintaining the



**Figure 1**

Numerous solid-state biochemical and biophysical microenvironmental cues regulate stem cell behavior. These include immobilized adhesive (i.e., Xaa amino acid/peptide sequence), growth [e.g., epidermal growth factor (EGF)], and morphogenic (e.g., Delta) biochemical factors interacting with cell surface receptors, for example integrins ( $\alpha$ ,  $\beta$ ), EGF receptors (EGFRs), and Notch receptors. In addition, steric availability of receptor-ligand binding (e.g., Xaa on the free end of a protein versus in the middle of a protein), cryptic sites exposed by cell-exerted contractile forces (red arrow), and ligand clustering (e.g., Delta) may be necessary for or enhance biochemical signaling. Biophysical regulators include extracellular matrix (ECM) elastic modulus, topography such as ridges, and strains and stresses imposed by stretching the ECM, flowing fluid over cells, and locally twisting magnetic microbeads on cell surfaces [gray sphere] functionalized with arginine-glycine-aspartic acid (RGD) peptide. Blue arrows signify external applications of force; Src is a mechanotransductive tyrosine kinase associated with focal adhesions.

structure and function of organisms, tissues, and cells. For stem cells, this specificity is largely determined by the biochemical nature of the surrounding microenvironment, i.e., the molecular identities of soluble factors, ECM components, or factors on the surfaces of other cells. Much work has focused on the specific identities of these factors and their important effects on different stem cell types; however, the contextual manner in which these moieties are presented is also highly important, including potential immobilization on scaffolds or particles, molecular conformation and clustering, and temporal presentation. Here we acknowledge the importance of biochemical specificity in stem cell-microenvironment interactions but emphasize the effects of the contextual presentation of solid-state biochemical factors on stemness.

### Adhesive Ligands

Specific ECM-cell and cell-cell interactions are important in providing spatial anchors as well as signals that regulate stem cell maintenance, survival, and differentiation. Cell adhesion is also required for a cell to sense other contextual information, such as the mechanical properties of the microenvironment. Therefore, we begin by reviewing the importance of the specific identities of biochemical ligands in the solid phase of natural systems as well as ways in which engineered systems have been utilized both to identify functional adhesive peptide sequences and to investigate their interactions with stem cells.

Anchoring or localization to proper niches is important for stem cell viability and function because without proper localization, stem cells may not be exposed to the appropriate survival and differentiation signals. The earliest known example of adhesive ligands regulating stem cell location is in the reconstitution of the hematopoietic system of cancer patients, in which transplanted hematopoietic stem cells (HSCs) were found to relocate to bone marrow niches following chemotherapy or radiation (Krause et al. 2001, Thomas et al. 1957). This

clinical observation has motivated subsequent mechanistic research. In nonhuman primates, injection of antibodies against integrin  $\alpha_4\beta_1$ —which is expressed on HSCs and binds to fibronectin (Williams et al. 1991) and to the cell surface sialoglycoprotein vascular cell adhesion molecule 4 (VCAM-4) (Frenette et al. 1998)—mobilizes CD34<sup>+</sup> hematopoietic progenitors and granulocyte/macrophage colony-forming cells to the bloodstream (Papayannopoulou & Nakamoto 1993). Furthermore, conditional ablation of  $\beta_1$  integrins yields HSCs that are unable to engraft in irradiated recipient mice (Potocnik et al. 2000). The concept that key adhesive interactions are necessary for niche localization has been extended to other systems. In mice, ablation of  $\beta_1$  integrins but not the cell-cell adhesion protein E-cadherin impairs the ability of mouse spermatogonial stem cells to repopulate recipient testes, likely through a decreased ability to associate with the adhesive protein laminin (Kanatsu-Shinohara et al. 2008). Interestingly, in *Drosophila* testes the anchoring interactions of germline stem cells appear not to be integrin-based but instead to rely on *Drosophila* E-cadherins presented by adjacent “hub” cells; however, integrins, specifically those containing the  $\beta$ PS subunit, do regulate the localization of the hub cells to the niche (Yamashita et al. 2003, Tanentzapf et al. 2007).

In addition to anchoring and maintaining stem cells within their niche, adhesive ECM and cell surface proteins also activate signals well known to regulate maintenance and differentiation. For example, the RGD sequence known to bind  $\beta_1$  integrins increases expression of integrin-linked kinase, whose subsequent activation of protein kinase B, or Akt, supports human mesenchymal stem cell (hMSC) survival (Benoit et al. 2007). Similarly, survival of erythroid progenitors is enhanced by their binding to fibronectin via integrin  $\alpha_4\beta_1$ , which upregulates the antiapoptotic transmembrane mitochondrial protein Bcl-xL (Eshghi et al. 2007). Stem cell differentiation can also be regulated by adhesion to ECM proteins. hMSCs can be induced toward an osteogenic lineage

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**HSC:** hematopoietic stem cell

**MSC:** mesenchymal stem cell

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**NSC:** neural stem cell

**IKVAV:** isoleucine-lysine-valine-alanine-valine

**ESC:** embryonic stem cell

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by culturing them on laminin-5, which ligates integrin  $\alpha_3\beta_1$ , activates extracellular signal-regulated kinase (ERK), and leads to phosphorylation of the osteogenic transcription factor Runx2/CBFA-1 (Klees et al. 2005). These studies demonstrate the integral role of the adhesive microenvironment in activating canonical cell signaling pathways.

To date, many *in vitro* studies examining the role of the ECM in stem cell systems have involved adsorption of natural ECM proteins such as laminin and fibronectin to traditional cell culture surfaces; however, the use of intact proteins presents several challenges. These large macromolecules contain numerous receptor-binding motifs, which renders it difficult to determine which one or ones are functionally important in regulating a key cell function. In addition, recombinant production of ECM proteins is difficult, and their isolation from tissues often results in biochemically heterogeneous mixtures. Therefore, engineered systems often instead have utilized synthetic, ECM-based motifs or peptides, singly or in combination, thereby in principle enabling a dissection of the relative importance of specific receptors in transducing an ECM signal.

For example, RGD-containing peptides, which engage a subset of integrins, have been increasingly used to functionalize synthetic matrices for stem cell culture (Saha et al. 2007) and were recently adapted to form self-assembling peptide hydrogels capable of encapsulating neural stem cells (NSCs) without the need for synthetic polymer matrices (Gelain et al. 2006). Another peptide sequence utilized in synthetic matrices, the isoleucine-lysine-valine-alanine-valine (IKVAV) motif found naturally in laminin, enhances the differentiation of neuronal progenitor cells when incorporated into self-assembling peptide hydrogels (Silva et al. 2004). In addition, some stem cell cultures, such as human pluripotent stem cells, require culture on complex blends of proteins or feeder cells with multiple unknown binding motifs to maintain growth and pluripotency. For example, Matrigel, a complex mixture of hundreds of ECM and other proteins

derived from Engelbreth-Holm-Swarm mouse sarcoma (Hansen et al. 2009), has emerged as a prevalent substrate for human embryonic stem cell (hESC) and human induced-pluripotent stem cell culture. To investigate adhesive interactions involved in Matrigel maintenance of hESC pluripotency, Meng and colleagues (2010) used blocking antibodies to identify  $\alpha_v\beta_3$ ,  $\alpha_6$ ,  $\beta_1$ , and  $\alpha_2\beta_1$  integrins as functionally contributing to hESC attachment to Matrigel. Adhesive peptide sequences adopted from laminin-111 were then chosen based on their ability to bind those integrins, and the authors found that whereas three peptides individually are able to support hESC growth and pluripotency for short periods of time (4 days), their combination enhances both the quality of cultures (i.e., the number of colonies) and the duration over which pluripotency was maintained (>7 days). This strategy emphasizes the ability of engineered systems to parse out the synergistic contribution of individual motifs within full-length natural proteins and may inspire future mechanistic studies.

However, one challenge for the field is that beyond RGD and several others, there are simply limited numbers of known ECM-based motifs that engage specific adhesion receptors. The existence of numerous families of ECM proteins and cell surface receptors [e.g., 24 known integrin heterodimers in mammals (Hynes 2002)] suggests that developing other peptidomimetic ligands will enable the investigation of a broader range of ECM-cell interactions. Rational identification of short adhesive motifs from ECM has yielded the peptides widely utilized to date; however, library approaches may lead to the identification of additional natural sequences, and it is not even necessarily clear that an optimal adhesive peptide must exactly correspond in sequence to an ECM protein. One recent study employed phage display of a library of random 12-mer peptides to “pan” for peptides that bind hESCs. Two novel sequences that did not align to any known extracellular protein were found to support extended hESC proliferation and maintenance of pluripotency



on a self-assembled monolayer (SAM) surface. Interestingly, these peptides apparently did not bind via integrins or proteoglycans (Derda et al. 2010), which suggests that adhesive interactions used for ex vivo culture of stem cells need not be limited to those found in vivo, although it remains to be determined whether other proteins adsorb to the SAM surface over time. In all, the combined use of rational and library-based screening methods will provide an increasing number of ligands for functionalization of synthetic systems and may aid mechanistic investigation of specific receptors and signaling events involved in regulating stem cell responses to their microenvironments.

### Immobilization of Growth Factors and Morphogens

The ECM offers sites for cell adhesion, but it can also serve as a platform for the presentation of other biochemical factors and orchestrate cell-cell interactions. Whereas the stem cell field has often investigated growth factors, morphogens, and cytokines as soluble factors, many of these proteins have matrix-binding domains such that they may be presented within the niche as “solid-phase” ligands. For example, Sonic hedgehog (Shh) binds vitronectin (Pons & Marti 2000) whereas Hedgehogs in general, FGFs, platelet-derived growth factors (PDGFs), vascular endothelial growth factors (VEGFs), transforming growth factor  $\beta$ s (TGF $\beta$ s), and several cytokines have heparin-binding domains (Hasan et al. 1999, Khachigian & Chesterman 1992, Krilleke et al. 2009, McLellan et al. 2006, Ye et al. 2001). Furthermore, numerous important ligands are integral membrane proteins presented from the membranes of adjacent cells, such as the Notch ligand families Delta and Serrate/Jagged (Fortini 2009). Immobilization of factors may have several consequences including increasing their local concentration and establishing concentration gradients emanating from the source (Saha & Schaffer 2006), promoting sustained signaling by inhibiting receptor-mediated endocytosis (Kuhl & Griffith-Cima 1996, Tayalia

& Mooney 2009), and modulating the spatial organization or molecular conformation of factors to enhance signaling. Several engineered systems have been utilized to study these effects.

One biomimetic strategy to immobilize factors harnesses the affinity of some for heparin. In one study, heparin-binding peptides were cross-linked to a fibrin gel to enable noncovalent attachment to heparin, and the material was then loaded with the heparin-binding factors neurotrophic factor 3 (NT-3) and PDGF. The resulting material was shown to induce neuronal and oligodendrocytic differentiation of mouse NSCs while inhibiting astrocytic differentiation (Willerth et al. 2008). The protein factors were released over 1–14 day ranges, a capability that could be utilized for studying kinetic effects of signaling, controlled delivery of factors in transplanted engineered tissues, or potential extensions in the active life span of factors in vitro. In addition to natural, noncovalent matrix binding, covalent linkage of factors is an effective means to biofunctionalize materials. For example, Shh covalently grafted to a polymer hydrogel surface was shown to promote the osteogenic differentiation of MSCs (Ho et al. 2007), whereas linkage of leukemia inhibitory factor (LIF) to thin film polymer coatings supported mouse ESC pluripotency for 2 weeks without the addition of soluble LIF (Alberti et al. 2008). In addition, covalent tethering of epidermal growth factor (EGF) was shown to sustain mitogen-activated protein kinase kinase (MAPKK)/ERK signaling in hMSCs and to achieve greater cell spreading and survival over unfunctionalized substrates in the presence of saturating levels of soluble EGF (Fan et al. 2007). Finally, in work that extended this concept beyond proteins, the small chemical phosphate, *tert*-butyl, and carboxyl groups were tethered to synthetic scaffolds to mimic the functional moieties exposed in mineralized bone, the hydrophobic lipids in adipose tissue, and the glycosaminoglycans prevalent in native cartilage, respectively. Interestingly, these chemical groups were shown to induce hMSC differentiation into osteogenic, adipogenic, and chondrogenic lineages, respectively, in the

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**MAPKK/ERK:**  
mitogen-activated  
protein kinase  
kinase/extracellular  
signal-regulated kinase

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absence of traditional soluble or immobilized morphogenic factors (Benoit et al. 2008).

There is also evidence that immobilized growth factors, morphogens, and integral membrane protein ligands may act synergistically with one another or with ECM adhesive ligands. For example, culturing NSCs on immobilized NT-3 with fibronectin, but not laminin, enhances both neuronal and astrocytic differentiation (Nakajima et al. 2007). Known cross-talk between growth factor receptor and integrin signaling through their intracellular domains may be responsible for this synergy (Schwartz & Ginsberg 2002, Yamada & Even-Ram 2002), and immobilization of ligands for both receptor classes may enhance this synergy by clustering their intracellular signaling domains. The above studies demonstrate that immobilization has important and sometimes necessary functional roles in stem cell systems, and the ability to immobilize factors in well-controlled and defined engineered cell culture systems may allow deeper mechanistic questions to be addressed in the future.

### Ligand Conformation

In addition to their manner of presentation, the molecular structure or conformation of these factors as well as the accessibility or presentation of binding motifs within these factors are important for their function. Altering the molecular conformation of ligands may form novel active sites or expose cryptic binding sites. For example, cell-generated forces have been found to unfold fibronectin, thereby exposing cryptic sites (Antia et al. 2008, Klotzsch et al. 2009) that have various biological activities, including self-assembly into fibronectin fibrils, binding of tenascin, and cleavage of collagen (Ingham et al. 2004, Schnepel & Tschesche 2000, Sechler et al. 2001).

The molecular conformation of immobilized ligands is also dependent on the chemical nature of the surfaces to which they are adsorbed. For example, fibronectin adsorbed to hydroxyl- and amine-terminated surfaces promoted osteogenic differentiation more so than

adsorption to carboxyl- and methyl-terminated surfaces. These observations correlated with differences in the binding of antibodies to epitopes within fibronectin adsorbed to these different surfaces, a result attributed to different conformations of the fibronectin (Keselowsky et al. 2003). In addition to passive adsorption, covalent attachment chemistry and the steric availability of ligands for binding can also regulate the activity of grafted synthetic peptides (Salinas & Anseth 2008).

### Spatial Presentation of Regulatory Factors

In addition to the properties of individual ligands, collections of multiple ligands can exhibit higher degrees of spatial organization at the nanoscale as well as microscale. Nanoscale spatial clustering of ligands and receptors, such as that at focal adhesions (Turner 2000), can bring them into closer relative proximity, increase the local intracellular concentrations of signaling effectors (e.g., focal adhesion kinase, paxillin, and Src), and thereby enhance activation of downstream pathways such as the MAPKK/ERK cascade (Giancotti & Ruoslahti 1999, Igishi et al. 1999). For example, clustered RGD ligands attached to the termini of star-shaped polymers promote motility in nonstem cells (Maheshwari et al. 2000), likely via their clustering of integrins and subsequent enhancement of downstream signaling events such as focal adhesion kinase activation (Kornberg et al. 1992). In addition, clustering of the Notch ligand Delta is necessary for Notch activation in numerous systems (Hicks et al. 2002). For example, in neural crest stem cell cultures, addition of antibody-clustered Delta inhibited neuronal and promoted glial differentiation (Morrison et al. 2000). Interestingly, in other stem cell systems, immobilization of Delta on a cell culture substrate or beads is necessary for downstream Notch signaling (Varnum-Finney et al. 2000), T cell differentiation from HSCs (Taqvi et al. 2006), and the activation of hematopoietic cord blood progenitor cells for subsequent engraftment in bone marrow



(Delaney et al. 2005). Some evidence suggests that mechanical forces exerted by ligation to clustered or immobilized Delta may be necessary for exposure of the Notch cleavage site (Gordon et al. 2007).

In addition to nanoscale features, micrometer-scale patterning of adhesive or regulatory factors may regulate subcellular localization of signaling proteins, thus affecting cytoskeletal organization and organelle localization. In stem cells, asymmetric spatial presentation, in which only one side of the stem cell is exposed to specific adhesive ligands, has been shown to regulate cell behavior in natural niches, including asymmetric divisions of stem cells in hematopoietic (Adams & Scadden 2006), keratinocyte (Lechler & Fuchs 2005), hair follicle (Jaks et al. 2008), esophageal epithelial (Seery & Watt 2000), and germinal (Li & Xie 2005) stem cells. This effect can occur through orientation of the centrosome and mitotic spindle perpendicular to the adhesive ligands (Yamashita et al. 2003).

The degree of asymmetric signal presentation can be finely controlled in culture through microcontact printing, which can be utilized to control ligand density and even cell shape. By patterning small and large islands of adhesive protein on a 2D surface, Chen and colleagues (McBeath et al. 2004) demonstrated that small and round MSCs preferentially differentiate into adipocytes, whereas spread cells differentiate into osteoblasts. These shape-based effects are regulated by RhoA signaling and downstream actomyosin contractility, which connects cell shape changes induced by biochemical patterning of ligands to changes in cellular mechanics, properties that will be discussed in detail below. Another mechanism through which adhesive patterns, and therefore cell shape, may affect stem cell function is by directly altering nuclear shape, which has been suggested to modulate gene expression in osteogenic cells (Thomas et al. 2002).

Micrometer-scale presentation of ligands can also regulate the multicellular organization of stem cells, as shown *in vivo* and in engineered systems. Early in development, the multicel-

lular organization of stem cells is partially regulated by the spatial patterns of cell-cell and cell-ECM contacts during important processes such as germ layer segregation and neural tube formation (Hammerschmidt & Wedlich 2008). ECM proteins have also been shown to differentially pattern epidermal stem cells and their progeny, transit-amplifying cells, on the basis of the higher expression levels of integrins  $\alpha_2\beta_1$  and  $\alpha_3\beta_1$  on the stem cells (Jones et al. 1995). The higher integrin expression levels anchor epidermal stem cells to collagen IV and the tips of the dermal papillae while allowing for the migratory behavior of transit-amplifying cells away from the stem cells toward the tips of the rete ridges nearer the dermis (Jensen et al. 1999). Finally, micrometer-scale patterning can also affect multicellular MSC shape and mechanics. Patterned multicellular structures of hMSCs exhibit distinct differentiation patterns, as cells on the concave edges of structures experience high tension and differentiate into osteoblasts, whereas those on the convex or low-tension edges generate adipocytes (Ruiz & Chen 2008). This study strongly emphasizes the intimate connection between the spatial organization of a material's biochemical properties and its control over the mechanical properties of stem cells. In addition, this example motivates the need to investigate how biophysical and biochemical properties of an environment can collaborate to regulate cell function.

## INFLUENCE OF BIOPHYSICAL PROPERTIES

Just as the mammalian body exhibits incredible diversity in biochemical interactions and specificities, it also exhibits a wide range of biophysical properties defined not by the specific identities of interacting molecules but by their collective structural and mechanical characteristics. Examples of this diversity include the palpable differences in the stiffnesses of fat versus bone tissue and the different topographies of layered 2D-like epithelial and intestinal sheets and of bulk 3D liver and pancreatic parenchyma. In addition to

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**Actomyosin contractility:** intracellular forces generated by the dynamic interaction of myosin motors and actin fibers

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**Complex/dynamic modulus:** the ratio of stress to strain under oscillatory deformation conditions

differences in static biophysical properties, organisms are inherently dynamic, as is evident in bulk motions such as joint bending, muscle contraction, compressive impact and strains on tissues, and pulsatile flow of the circulatory system. There is even evidence for the generation of strong forces owing to cell adhesion and migration during embryonic development (Keller et al. 2003). These large internal variations in the structure and mechanics of various tissues, and consequently in their resident stem cell niches, suggests that in addition to solid-state biochemical signals, stem cells may respond to biophysical properties of the microenvironment.

### Elastic Modulus

Of all the many mechanical properties of biological systems, stiffness or rigidity is perhaps the most apparent and widely studied. In general, the mechanical stiffness of a material can be determined by measuring its complex modulus, the ratio of stress (force per unit area) to strain (fractional deformation) applied to a material. This value reflects the material's ability to store and frictionally dissipate the applied mechanical energy, as reflected by a storage (elastic) modulus and loss (viscous) modulus, respectively. Tissues and cells are often viscoelastic in that they exhibit both fluid- and solid-like properties, but the viscous component has proven challenging to systematically measure and vary, and its investigation awaits the development of future material systems. However, the elastic modulus, the measure of the stress required to achieve a specific strain in a material without any permanent deformation, has emerged as an important regulator of stem cell function. The elastic moduli of various tissues range over four orders of magnitude from <1 kPa for fat (Wellman 1999), brain (Gefen et al. 2003), and mammary tissue (Paszek et al. 2005) to ~10 kPa for skeletal muscle (Engler et al. 2004) and 10 MPa for bone (Goldstein et al. 1983). Individual tissues can also contain significant internal heterogeneities in stiffness, such as the nearly threefold variations in stiffness reported within

the hippocampus of the brain (Elkin et al. 2007). In stark contrast, the typical surfaces used to culture cells (e.g., plastic and glass) have supra-physiological stiffnesses (>1 GPa) (Miyake et al. 2006) as much as 10 million-fold stiffer than a natural stem cell microenvironment. This raises the question of whether stiffness can contribute to regulating stemness.

**Mesenchymal stem cells.** Because MSC-derived lineages are typically associated with load-bearing connective tissues that possess diverse mechanical properties (e.g., bone, muscle, and fat), MSCs are a particularly appropriate system for investigating mechanoregulation. In landmark work, Engler and colleagues (2006) found that hMSCs cultured on polyacrylamide gels (functionalized with type I collagen) that mimicked the stiffnesses of bone, muscle, and neural tissue preferentially differentiate into these corresponding specialized cell types. This effect requires inclusion of a cocktail of soluble differentiation factors; however, culturing MSCs on substrates of different stiffnesses in the absence of these soluble factors restricts their potency to the corresponding cell type upon later addition of soluble factors. This suggests that ECM stiffness alone may have the capability to restrict potency, with subsequent differentiation requiring soluble factors.

In addition to its role in modulating lineage commitment, there is also evidence that substrate stiffness can regulate MSC self-renewal. Similar to many specialized cell types that proliferate faster on stiffer substrates, hMSCs remain quiescent on soft substrates but proliferate on stiffer substrates functionalized with a mixture of type I collagen and fibronectin (Winer et al. 2009). Likewise, partially committed osteoblastic cells proliferate at a higher rate on stiffer substrates (Hsiong et al. 2008); however, multipotent mouse MSCs proliferate at similar rates on RGD-functionalized substrates of varying stiffnesses. Thus, whereas ECM stiffness is an important regulatory cue for MSC behavior, specific phenotypes may depend on details such as the adhesive ligand(s) and the species or tissue origin.

**Neural stem cells.** The brain is not exposed to exogenous mechanical forces in the same manner as bone and cartilage; however, brain function is exquisitely sensitive to altered intracranial pressure, and NSCs normally exist in mechanically heterogeneous niches. For example, the hippocampus varies in elastic modulus from 100 to 300 Pa in the CA1 and CA3 subregions, respectively (Elkin et al. 2007). In addition, brain tumors can be delineated by ultrasound based on the density differences in tumor versus normal tissue (Unsgaard et al. 2006), and glial scars may in part prevent nerve regeneration by forming mechanical barriers, an effect interestingly attenuated by implantation of a soft hydrogel material (Horner & Gauge 2000, Woerly et al. 2004). In this context, K. Saha and colleagues (2008) cultured adult hippocampal NSCs on RGD-functionalized, variable modulus hydrogels in the presence of soluble factors that promote either cell proliferation or differentiation. They found that NSCs optimally proliferate on an intermediate stiffness (~500 Pa) characteristic of brain tissue, and under conditions that strongly promote neuronal differentiation, they optimally mature into neurons at the same intermediate stiffness. Furthermore, under conditions that promote mixed neuronal and astrocytic differentiation, NSCs differentiate predominantly into neurons on soft substrates (>90% neurons on 10 Pa gels) and into astrocytes on hard surfaces (>50% astrocytes on 10 kPa gels) (K. Saha et al. 2008). A subsequent study in which NSCs were embedded in 3D alginate gels of variable stiffness reported analogous findings (Banerjee et al. 2009), and collectively these results indicate that NSCs respond strongly to a combination of biochemical and mechanical cues.

For NSCs derived from the subventricular zone (SVZ) of the adult forebrain, a similar increase in neuronal differentiation is observed on soft, laminin-coated, methacrylamide chitosan substrates (Leipzig & Shoichet 2009). However, astrocytic differentiation is low on all substrates (<2%) for these NSCs, and oligodendrocytic differentiation is favored on stiffer substrates (>7 kPa). These differences in

glial differentiation could be due to different anatomical origins of the adult NSCs. Likewise, NSCs derived from rat embryos and cultured on fibronectin rather than laminin exhibited increased astrocytic differentiation on softer substrates and low neuronal differentiation (<10%) on all substrates (Teixeira et al. 2009), which indicates that both NSC origin and ECM can influence mechanoregulation of fate choice.

### **Potential mechanisms of modulus response.**

A rich mechanobiology literature suggests many possible mechanisms that may regulate ECM modulus effects on stem cell behavior. The roles of several mechanotransductive proteins, including G-protein coupled receptors (Chachisvilis et al. 2006) and focal adhesion kinase (Hanks & Polte 1997) as well as integrins and Rho GTPases (Ridley 2000), in regulating cellular processes have been studied. In addition, biophysical cellular responses such as changes in cell shape, contractility, stiffness, or cytoskeletal architecture may regulate stem cell responses by modulating nuclear architecture and/or transcription and transcription factors (Mammoto et al. 2009, Thomas et al. 2002), intracellular and cytosol-nucleus transport (Kamal & Goldstein 2000), or localization of signaling factors through cytoskeleton-mediated sequestration (Mammoto et al. 2007, Wang et al. 1997).

Several studies indicate that a combination of such mechanisms may be important in stem cells, in particular changes in cellular contractility regulated through RhoA signaling and actomyosin-based forces. In hMSCs, inhibition of myosin II abrogates the effect of ECM stiffness on hMSC differentiation into all lineages (Engler et al. 2006). Furthermore, in hMSCs decreasing ECM stiffness decreases RhoA activity and subsequently  $\text{Ca}^{2+}$  signaling (Kim et al. 2009), pathways known to regulate actomyosin contractility. Interestingly, RhoA signaling may also regulate NSC differentiation, as suppression of Rho GDI $\gamma$  decreases RhoA expression and increases the neuronal but not glial differentiation of immortalized murine neuronal precursors (Lu et al. 2008). Although

### Compressive and tensile strains:

fractional deformation of a material with a decrease or increase in displacement, respectively

it is unclear if this is a mechanical effect, recent work indicates that Rho GTPase signaling transduces ECM modulus cues into biases in adult hippocampal NSC lineage commitment (A.J. Keung, E.M. de Juan-Pardo, D.V. Schaffer, & S. Kumar, unpublished data). Interestingly, changes in cellular stiffness may also be intimately linked to cellular shape, as RhoA was also implicated in regulating the hMSC differentiation response to cell shape (McBeath et al. 2004). Future work may reveal additional mechanistic links between solid-state biochemical and biophysical cues.

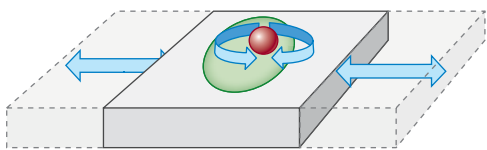
### Stress and Strain

In addition to intrinsic mechanical properties of the microenvironment such as modulus, extrinsic mechanical perturbations, specifically the application of forces or stresses that induce deformation or strain, are important characteristics of microenvironments surrounding stem cells. Tissue-scale examples of such dynamic, mechanical perturbations include stretching and contraction of tendons, ligaments, and

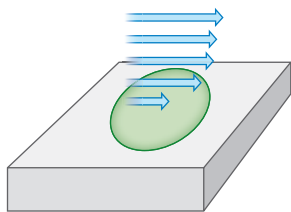
musculature, as well as cyclic loading of vasculature. The mechanically dynamic nature of tissues suggests the potential importance of stress and strain in regulating stem cell behavior in native settings (Albinsson et al. 2004, Saitoh et al. 2000). In addition, the different modes of stress application (**Figure 2**) including tensile, compressive, torsional, and shear forces may influence stem cell behaviors in diverse ways.

**Tensile and compressive strains.** Tensile (stretching or elongating) and compressive strains have been observed at the cellular level in embryonic systems. In *Drosophila* embryos, artificial compression of cells induces expression of Twist, an important factor regulating germ layer specification and patterning (Farge 2003). Natural tissue dynamics during development, such as germ layer extension, may utilize this compressive mechanism to induce expression of patterning genes. Similarly, tensile strains may also be important in development and were recently shown to regulate zebrafish

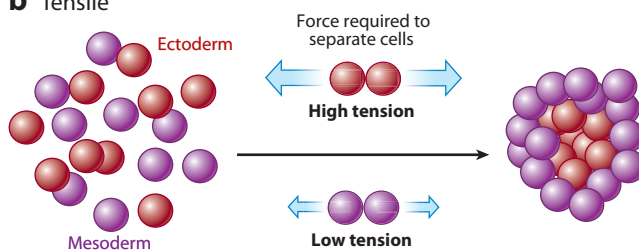
**a** Cyclic tensile and torsional



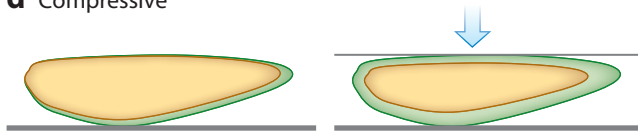
**c** Shear



**b** Tensile



**d** Compressive



**Figure 2**

Mechanical forces have been applied to stem cell microenvironments and stem cells themselves in several distinct modes. (a) Cyclic tensile (*linear arrows*) and torsional (*rotational arrows*): Cyclic stretching of cell culture substrates regulates mesenchymal stem cell and embryonic stem cell differentiation. (b) Tensile: Distinct tensile forces between cells govern zebrafish germ layer organization (Krieg et al. 2008). Greater forces (*blue arrows*) are required to separate two ectodermal (*red*) compared with mesodermal (*purple*) cells. (c) Shear: Shear stress/strain regulate vascular and endothelial stem cell differentiation. (d) Compressive: Compression upregulates *twist* expression (*green region*) in the *Drosophila* blastoderm embryo (Farge 2003). In all panels, blue arrows signify applications of force.

gastrulation, the first stage in vertebrate development in which progenitors undergo sorting and assembly into the distinct germ layers (Krieg et al. 2008). Contractile tension in the actin-myosin mesh composing the cell cortex, measured by atomic force microscopy (AFM) indentation, was found to vary almost twofold within the embryo, with ectodermal progenitors exhibiting the highest tension and endodermal progenitors the lowest. When individual progenitors from different germ layers are mixed *in vitro*, ectodermal progenitors sort to the inside of heterotypic mixtures, as anticipated owing to their high cell cortex tension. Interestingly, this germ layer sorting does not correlate with cell-cell adhesion strengths as determined by AFM, whereas genetic and pharmacological reduction of cellular contractility ablates the cell-sorting behavior, which supports the hypothesis that cell cortex tension is important in regulating germ layer patterning. Given the wealth of literature on the role of cell-cell adhesions in development and the requirement for cell adhesions to transmit tensional forces, it is likely that a combination of the differential cell cortex tensions and adhesive forces between cell types may contribute to regulating germ layer specification, gastrulation, and other early developmental processes (Hammerschmidt & Wedlich 2008).

In addition to mechanical properties that vary on a developmental timescale, cyclic strains are an important feature of many natural microenvironments that can also influence stem cell behavior. Stretching lung embryonic MSCs stimulates expression and nuclear localization of tension induced/inhibited protein-1 (TIP-1) and inhibits expression of TIP-3, thereby promoting myogenesis and inhibiting adipogenesis, respectively. These proteins have been shown to act as transcriptional coactivators that enhance histone acetyltransferase activity at histones H3 and H4 within myogenic and adipogenic promoters (Jakkaraju et al. 2005). Cyclic stretching also inhibits differentiation of hESCs through the upregulation of TGF $\beta$ 1, Activin A, and Nodal and subsequent phosphorylation of Smad 2/3 (S. Saha et al.

2008). By contrast, when a localized cyclic stress is applied by magnetically twisting a 4- $\mu$ m-diameter RGD-coated bead bound to the surface of mouse ESCs, expression of the pluripotency marker Oct3/4 is significantly reduced (Chowdhury et al. 2010).

**Shear flow.** Another form of dynamic stress application is shear flow, which is most often associated *in vivo* with the circulatory system. Whereas the effect of shear stress on vascular function and endothelial cell behavior has been appreciated for decades, shear stress more recently has been found to be important in regulating stem cell function as well. Early work demonstrated that shear flow promotes the maturation and capillary assembly of endothelial progenitor cells (Yamamoto et al. 2003). Subsequent studies have found that shear flow can induce differentiation of several stem cell types including murine MSCs (Wang et al. 2005) and ESCs (Illli et al. 2005; Yamamoto et al. 2005) into specialized endothelial or cardiovascular cells. One study identified a potential epigenetic mechanism, as laminar shear stress enhanced total nuclear levels of acetylation at H3K14 and methylation at H3K79 while upregulating transcription from the VEGF-2 promoter as well as other vascular system-related genes (Illli et al. 2005). Whereas this work demonstrates the importance of shear stress in vascular differentiation, two recent studies have specifically demonstrated the importance of shear stress in embryonic vascular development. North and colleagues (2009) demonstrated in zebrafish as well as mouse embryos that blood flow is necessary for the proper development of HSCs in the embryonic aorta-gonad-mesonephros (AGM) region. Activation of nitric oxide (NO) signaling was able to rescue hematopoiesis even in the absence of blood flow, which implicates NO as a mechanotransductive signal (North et al. 2009). Adamo and colleagues (2009) arrived at a similar result using a miniaturized *in vitro* flow chamber. Mouse ESCs cultured under shear flow expressed higher levels of CD31 and Runx1, proteins expressed in endothelial cells, and

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**TIP:** tension-induced/inhibited protein

**Aorta-gonad-mesonephros (AGM):** a region of embryonic mesoderm from which the first hematopoietic stem cells arise

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generated more hematopoietic colony-forming units. Inhibition of NO production abrogated this shear flow effect (Adamo et al. 2009).

## Topography

Mechanical properties such as elastic modulus, stress, and strain play clear roles in regulating stemness. However, biophysical properties also include structural characteristics such as topography, a material's surface profile and shape. Topographical structures such as grooves, ridges, and pits are present in many natural systems at the nanoscale, such as in the fibrous structure of collagen and other ECM proteins, as well as at the microscale, such as in pores in bone marrow and undulating basement membranes in the epidermis. The presence of topographical information in natural systems motivates the use of technologies such as soft lithography, microfluidics, electrospinning, and deposition of nanostructures (Khademhosseini et al. 2006, Pirone & Chen 2004, Yang et al. 2005) to engineer a material's topography to study stem cell responses to both nano- and microtopography.

MSCs are likely to encounter and be influenced by these types of topographical cues in their tissues, and several studies using engineered ECM systems strongly support the concept that topography regulates cell function. Culture atop vertically oriented nanotubes of 70–100 nm (but not <30 nm) in diameter induces hMSCs to differentiate into osteoblasts in the absence of osteogenic media (Oh et al. 2009). It was hypothesized that the larger-diameter nanotubes would place adhesion clusters farther apart and thus require the hMSCs to stretch and generate high internal tension, analogous to the use of a broad ECM island (McBeath et al. 2004) or a stiff ECM (Engler et al. 2006). Interestingly, culturing hMSCs on nanopits of the same length scale as the nanotubes, approximately 100 nm, also induces osteogenesis in the absence of osteogenic media. This study also identified anisotropic, or disordered, presentation of the nanopits as necessary for osteogenesis (Dalby et al. 2007). The

disordered or asymmetrical nanopit presentation may be required for induction of cell polarity or of cellular heterogeneity within the monolayer culture, which could generate either intra- or extracellular gradients of soluble or cell surface signaling molecules, respectively.

Fibrous proteins such as collagen and laminin are also present in vascular basal lamina in the brain, which suggests that NSCs could also be responsive to nanoscale topography. Indeed, culturing adult rat hippocampal NSCs on laminin-coated synthetic polyethersulfone fibers with 280 and 1,500 nm diameters increases oligodendrocytic and neuronal differentiation, respectively, in differentiation-inducing media (Christopherson et al. 2009). In the presence of growth factors, NSC proliferation increases with decreasing fiber diameter. Interestingly, NSCs spread extensively on smaller-diameter fibers, raising the possibility of cell shape regulation of NSCs as previously observed for MSCs (McBeath et al. 2004). Collectively, these studies suggest that nanoscale topography may act through regulation of the spatial presentation of ligands and regulatory factors, or altering cellular morphology or mechanics, to modulate cell function, thus representing another example of the interplay between biochemical and biophysical cues.

At the microscale, NSCs are exposed to numerous topographical features in the brain, including many crevasses and undulations as well as intersections of layers of different cell types. Mimicking this topography, adult hippocampal NSCs have been cocultured with astrocytes on micrometer-scale grooves etched into polystyrene substrates by photolithographic and reactive ion-etching techniques. The NSCs aligned with the grooves and subsequently generated higher percentages of neurons on grooved compared with control flat substrates (Recknor et al. 2006). Several potential mechanisms may sense these topographical cues including actomyosin and RhoA signaling, which also have been implicated in regulating micropost inhibition of fibroblast proliferation (Thakar et al. 2008), in modulus sensing for NSCs and for MSCs (Engler et al. 2006) as



discussed earlier, and in cell shape-mediated effects on MSCs (McBeath et al. 2004).

A more specialized neural precursor, an oligodendrocytic progenitor cell (OPC), is also sensitive to topographical cues, which indicates that progenitors can be topographically sensitive at multiple stages of specialization. Rat OPCs *in vivo* have been observed to differentiate at approximately postnatal day 8, a phenomenon traditionally thought to be regulated by an intrinsic timer. However, *in vitro*, OPCs differentiate at a rate dependent on cell density, not absolute time. Rosenberg and colleagues (2008) hypothesized that this effect was not due to increased paracrine signaling or cell-cell contacts with increasing cell density but that it was a physical, steric effect. To test this hypothesis, rat OPCs were cultured with polystyrene beads that were biochemically noninteractive with OPCs. Interestingly, beads of intermediate size, 20  $\mu\text{m}$ , were observed to induce oligodendrocytic differentiation, whereas 5- and 100- $\mu\text{m}$  beads were not, which indicates that OPCs sense topographical cues on the size scale of the OPCs themselves ( $\sim 20 \mu\text{m}$ ) (Rosenberg et al. 2008). Despite the differences in the length scales of the topographies for this and the above examples, nano- and microscale topographies appear to induce some analogous changes in cell shape and morphology and thus may act through common signaling pathways, such as Rho GTPases, to regulate stem cell behaviors. Systems engineered to investigate the relative effects of different length-scale topographies as well as biochemical ligands patterned on different size scales may help elucidate common mechanisms.

The numerous examples above of stress and strain in stem cell systems have identified several signaling pathways that mediate mechanotransductive responses. However, as mentioned throughout this review, the observed effects of a particular cue may be partially or fully dependent on the presentation of other microenvironmental signals or conditions, and a recent study that investigated the interplay between topology and mechanics further illustrates this point. hMSCs were cultured

on polydimethylsiloxane membranes micropatterned with grooves to align the hMSCs in one direction. When 5% strains were applied at 1 Hz parallel to the grooves, hMSCs upregulated the smooth muscle cell marker calponin 1 and downregulated chondrogenic and osteogenic markers, as well as increased their proliferation. However, when the topographical cue was altered so that hMSCs were aligned perpendicular to the strain, most of these observed phenotypes were no longer induced (Kurpinski et al. 2006).

## Dimensionality

A wealth of cell biological knowledge has emerged from studying cells in 2D cell culture systems; however, the topographical studies discussed above, although not fully 3D, hint at the importance of 3D features in regulating stem cell behavior. Although 2D-like cellular structures are present *in vivo*—including epithelial sheets, endothelial layers, and epidermis—these as well as organs, tissues, and niches generally occur in a 3D context. Three-dimensional culture presents several important differences and considerations including slower diffusive transport of soluble factors, the natural or engineered formation of gradients of signaling factors, and spatial presentation of regulatory factors from all directions. Thus, studying stem cells in 3D is arguably one of the most important future directions for stem cell research.

Stem cell systems that are already traditionally grown in 3D include hESC colonies and embryoid bodies (EB). hESCs typically are cultured in cell clusters more than 100  $\mu\text{m}$  thick, adhered to feeder cells or Matrigel on 2D substrates, whereas EBs are aggregates of differentiating cells grown in suspension. Three-dimensional culture may in some ways recapitulate early stages of embryonic development in which the establishment of spatial gradients of factors, owing to the transport limitations of soluble factors, functions to pattern early tissue structures. For example, mouse EBs cultured in serum-free medium have been

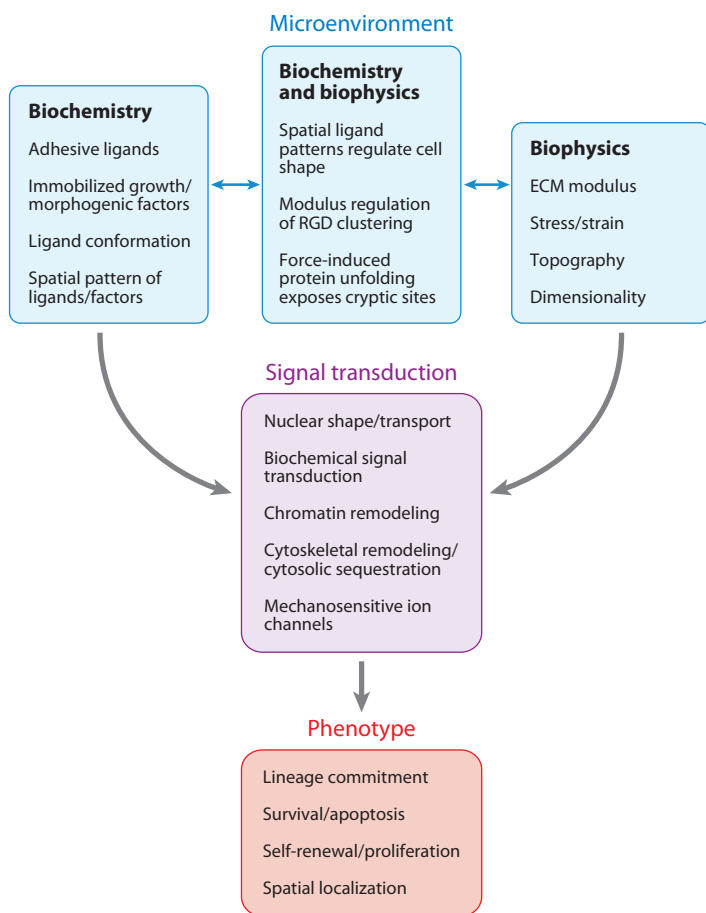
found to spontaneously form patterned and polarized neural tissue mimicking the temporal and spatial patterning in natural developmental corticogenesis (Eiraku et al. 2008). In serum-containing conditions, mouse EBs exhibit

gastrulation-like patterning dependent on Wnt signaling (ten Berge et al. 2008). Interestingly, both studies found that controlling cluster size and generating relatively homogeneously sized EBs simply by aggregating single cells on low-adhesion plates or in hanging drops improves the efficiency of pattern formation and controls the rate of differentiation, respectively. Engineered systems have been developed to study the effects of cluster size more precisely. Microwells fabricated via lithography and polymer-casting techniques allowed for generation of distinct EB sizes of 150 and 450  $\mu\text{m}$  (Hwang et al. 2009). Intriguingly, small EBs express higher levels of Wnt5a, and large EBs express higher levels of Wnt11. EB size control over Wnt signaling, in the context of Wnt signaling driving gastrulation-like patterning of EBs (ten Berge et al. 2008), suggests that EB size may result in differential gradients and molecular transport of signaling morphogenic molecules, thereby influencing patterning.

hESC colony sizes have also been controlled using microwells, which results in more homogeneous colony sizes compared with typical hESC cultures on 2D substrates (Mohr et al. 2006). Microcontact printing of adhesive islands also restricts hESC colony sizes as well as regulates differentiation, with smaller hESC colonies generating more endoderm over ectoderm (Bauwens et al. 2008). Thus, for both ESCs and EBs, the 3D size and shape of cellular assemblies likely regulate cell function through mechanisms relevant during organismal development—spatial signaling gradients, changes in the spatial presentation and identities of cell-cell contacts, and potentially mechanical asymmetries—and the controlled investigation of these effects on cell function represents an interesting avenue for future research.

## SUMMARY AND CONCLUSIONS

The recent rapid development of novel cell culture systems has greatly expanded the possible regulatory cues researchers can explore. These engineered microenvironments



**Figure 3**

Solid-state biochemical and biophysical microenvironmental cues are highly interdependent (blue). Microenvironmental cues may be sensed and transduced by numerous cellular mechanisms (purple) resulting in changes in stem cell phenotype (red). Engineered systems with the capability to regulate solid-state biochemical and biophysical properties, such as the spatial presentation of adhesive ligands and material modulus, will enhance research to identify interdependencies between regulatory cues, such as the regulation of cell shape by ligand patterning. Furthermore, these culture systems in combination with molecular cell biological techniques will elucidate signal transduction mechanisms, such as changes in nuclear shape or mechanosensitive ion channels, that sense microenvironmental cues and ultimately modulate stem cell fate choices such as lineage commitment, survival/apoptosis, self-renewal/proliferation, and spatial patterning/localization within tissues. RGD, arginine-glycine-aspartic acid; ECM, extracellular matrix.

have provided the tools needed to elucidate the importance of mechanical perturbations and solid-state biochemical and biophysical properties of materials in regulating stem cell behavior. Furthermore, pioneering studies are increasingly combining analytical cell biology techniques with these engineered systems to gain mechanistic insights. Future work will continue investigating novel mechanistic hypotheses, likely drawing on mechanisms found in differentiated cells as well as some stem cells, such as signaling through focal adhesions (Turner 2000), compartmental sequestration of transcription factors (Miralles et al. 2003, Sotiropoulos et al. 1999), and force-induced conformational changes of biomacromolecules (Johnson et al. 2007, Klotzsch et al. 2009).

Reductionist biology using engineered cell culture systems not only provides an opportunity to explore new biophysical and solid-state biochemical parameters but also allows for quantitative, graded, and temporal control over

these regulatory features. In addition, improving the ability to orthogonally vary microenvironmental parameters in engineered systems in the future will allow researchers to address complex mechanisms involving cross-talk between interdependent regulatory cues, to study the conversion and transduction between biochemical and biophysical signals, and to develop a more complete systems-level view of stem cell processes. As alluded to throughout this review, no stem cell process is regulated in isolation from other elements of the microenvironment, and a systems-level perspective may shed light on novel regulatory interactions and networks beyond those traditionally studied through biochemical signal transduction (**Figure 3**). Developing this mechanistic and systems-level understanding of stem cell microenvironments promises to inform future stem cell-based therapies as well as our understanding of human homeostasis and disease states.

## SUMMARY POINTS

1. Engineered materials and cell culture systems afford exquisite qualitative and quantitative control over microenvironmental cues regulating stem cell behavior.
2. Biophysical and solid-state biochemical cues often provide necessary or enhanced regulatory control of stem cell processes.
3. Use of engineered systems with analytical and genetic techniques can reveal diverse molecular mechanisms underlying microenvironmental control of stemness.
4. Engineered systems reveal that biochemical and mechanical microenvironmental cues are often interdependent and synergistic.

## FUTURE ISSUES

1. The continued development of novel engineered cell culture systems will aid a reductionist examination of novel types of regulatory cues.
2. Precise, orthogonal control over microenvironmental features will allow interdependencies of regulatory cues to be studied at mechanistic levels.
3. Knowledge garnered using engineered systems will advance stem cell biology as well as provide prototypes for tissue engineering and strategies for therapeutics.

## DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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