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Context clues: the importance of stem cell-material interactions

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Abstract

Understanding the processes by which stem cells give rise to *de novo* tissues is an active focus of stem cell biology and bioengineering disciplines. Instructive morphogenic cues surrounding the stem cell during morphogenesis create what is referred to as the stem cell microenvironment. An emerging paradigm in stem cell bioengineering involves “biologically driven assembly,” in which stem cells are encouraged to largely define their own morphogenesis processes. However, even in the case of biologically driven assembly, stem cells do not act alone. The properties of the surrounding microenvironment can be critical regulators of cell fate. Stem cell-material interactions are among the most well-characterized microenvironmental effectors of stem cell fate, and they establish a signaling “context” that can define the mode of influence for morphogenic cues. Here we describe illustrative examples of cell-material interactions that occur during *in vitro* stem cell studies, with an emphasis on how cell-material interactions create instructive contexts for stem cell differentiation and morphogenesis.

Introduction

The derivation of biologically relevant cell types and tissues *in vitro* remains a challenge in stem cell biology and regenerative medicine. During early development, stem and progenitor cells differentiate into tissue-specific cell types and undergo morphogenesis (“creation of shape”) to generate organs with complex three-dimensional (3D) architecture. Since the isolation of human embryonic stem cells (hESCs),⁽¹⁾ tremendous progress has been made toward directing stem cell differentiation into more restricted somatic lineages. Such approaches generally use “morphogenic cues” that include soluble growth factors and small molecule agonists or antagonists of signaling pathways related to the developmental stage and tissue of interest. While these approaches have achieved greater than 90% efficiency directing differentiation toward certain lineages, (2–4) other cell types such as pancreatic islet cells⁽⁵⁾ have eluded efficient derivation by traditional methodologies. Traditionally, *in vitro* experimental systems do not faithfully recapitulate the complex and dynamic signaling present during natural morphogenesis. This limitation may explain, in part, the limited success in the derivation of some target cell types. There is strong interest in using materials

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to define *in vitro* stem cell microenvironments more reminiscent of native developing tissues.

The concept of “biologically driven assembly” is an emerging paradigm in regenerative medicine, wherein precursor cells self-organize into higher-order structures in the absence of exogenous patterning cues. This concept is in contrast to traditional “induced differentiation,” in which precursor cells are exposed to biochemical cues added to the environment to affect a specific biological outcome. Growing evidence suggests that certain tissue structures can simply coordinate their own assembly *in vitro*.(6–11) For instance, Eiraku et al. showed that hESCs cultured as embryoid bodies (EBs) in basal media differentiated with high efficiency into polarized neuroepithelia resembling the native architecture of early cortical tissue.(12) The same group more recently demonstrated that hESCs cultured as EBs in the presence of basement membrane proteins spontaneously formed fully stratified optic cup structures reminiscent of the post-natal retina.(13) A guiding premise of these studies and other similar reports was that cell populations could undergo complex morphogenesis processes on their own, without the aid of any particular scaffolding or intricate external control over cell signaling. However, it is also critical to note that biologically driven assembly processes do not occur in a biological equivalent of a vacuum. They occur in environments in which soluble and insoluble signals are not only present, but are further regulated by defined spatial and temporal constraints. Examples of critical parameters in biologically driven assembly approaches to date include the size and shape of a cell or cell aggregate, concentration gradients of soluble and insoluble molecules, and even the surrounding oxygen tension. These parameters are defined to varying extents in cell culture, and each have the ability to influence a morphogenesis process. Here we will refer to these characteristics of the surrounding environment as “context,” which can influence stem cell phenotype and biologically driven assembly in critical ways. It is important to emphasize that materials may help to define this surrounding context while still allowing for tissue assembly to be dictated primarily by the cells themselves. An illustrative example is the now well-accepted importance of EB size during hESC differentiation down the erythropoietic,(14) cardiogenic,(15–19) and ectodermal lineages.(20, 21) Presently, there is a significant opportunity to design bioinspired materials that define the context in the cellular microenvironment, while remaining permissive to biologically driven assembly. Here we review how material components interact to present distinct contexts that impact stem cell differentiation. The purpose of this review is not to cover material-dependent stem cell behavior comprehensively. Instead, we cover examples of cell-material interactions that help to illustrate how materials in the stem cell microenvironment can define the signaling context.

Components of cell-material interactions

To begin a discussion of signaling context in the stem cell microenvironment, we first consider the material components that define it. These include components related to cell-material adhesion, such as mechanical stiffness, surface topography, and spatial confinement (i.e. “patterning”). These components are intrinsic, and therefore are present and persist as part of the signaling context from the onset of the experiment. In addition, cell adhesion components may include cell adhesion ligands or protease-degradable linkages, which enable cell-material adhesion and protease-mediated degradation. There is an added complexity here, as changes in material properties during degradation have been shown to influence stem cell fate.(22–24) Materials can also influence the soluble signaling environment in conjunction with soluble factors such as growth factors, cytokines, small molecule regulators of signal transduction, and oligonucleotides. Specifically, materials can modulate the effects of these soluble factors by temporally or spatially controlling their

delivery.(25, 26) Properties that regulate soluble factor context may be considered as extrinsic material components that help to define the soluble microenvironment.

Context introduced by stem cell adhesion

The cell adhesion process involves many aspects that introduce context into the stem cell microenvironment. These include adherent cell density and spatial arrangement, cell adhesion ligands that define the mechanism and specificity of attachment, and mechanics of the underlying material. As most stem cells require some level of adhesion for survival, individual aspects of cell adhesion have been thoroughly examined for their role in morphogenesis. Examples include arrangement and density of cell adhesion ligands, which have been shown to significantly influence stem cell fate and morphogenesis.(27–30) Recent studies have also demonstrated interplay between the individual aspects of adhesion. For example, the established influence of material mechanics on stem cell fate may also be dependent on spatial presentation and density of cell adhesion ligands.(31) The complex downstream effects of cell adhesion on cell signaling warrant a closer look at how material properties collectively define the cell adhesion microenvironment and influence stem cell fate.

Molecular mechanisms of cell adhesion

The molecular mechanism of cell adhesion typically involves binding of cell adhesion ligands to integrins,(32) which are heterodimeric cell-surface receptors. Perhaps the most well-studied cell adhesion ligand is the arginine-glycine-aspartic acid (RGD) sequence found in many native extracellular matrix (ECM) proteins, including fibronectin, vitronectin, and certain collagens.(33) Integrins function not only as cell adhesion receptors, but also serve as physical linkages between the ECM and the cell cytoskeleton. By way of these physical linkages, insoluble characteristics of the extracellular environment are transduced into changes in cytoskeletal organization that may influence intracellular signaling downstream of adhesion. However, specific adhesive interactions between integrins and a material may not only influence cytoskeletal arrangement, but may also participate in cross-talk with specific growth factor signaling pathways distinct from those downstream of adhesion-mediated signaling.(34) This cross-talk may be facilitated by adhesion-dependent activation of a growth factor receptor (GFR), a mechanism in which an integrin either interacts directly with a GFR or assembles a platform of signaling proteins that promote receptor activation.(35) Integrins have also been implicated in GFR endocytosis, and may alter downstream cellular responses by downregulating the number of specific receptors available at the cell surface.(36) Conversely, growth factors themselves may regulate the expression of specific integrins at the cell surface and thereby affect integrin-mediated signaling as well as GFR-mediated signaling.(37–40) Examples of such crosstalk have been observed for growth factors such as bone morphogenetic protein (BMP)-2,(41, 42) transforming growth factor (TGF)- β 1,(43, 44) and vascular endothelial growth factor (VEGF),(45, 46) and have more recently been implicated in influencing stem cell behavior. For example, Koepsel et al. modified self-assembled monolayers (SAMs) with varying densities of a BMP-receptor binding peptide (BR-BP) and a heparin proteoglycan binding peptide (HPG-BP) in combination with differing densities of the cell adhesion peptide GRGDSP. Results indicated that while increasing the density of GRGDSP alone led to higher surface coverage by human mesenchymal stem cells (hMSCs) and increased alkaline phosphatase staining indicative of osteogenic differentiation, effects of BR-BP on hMSC behavior were also dependent on the density of GRGDSP.(47) Specifically, no adhesion of hMSCs was observed on surfaces that presented BR-BP or HPG-BP alone, but BR-BP presented in combination with GRGDSP promoted hMSC surface coverage in comparison to controls presenting equivalent densities of GRGDSP alone. The results of this study were in line with previous work that suggested synergy between integrin signaling and BMP-

receptor activation, and demonstrate how the adhesive environment may potentiate effects downstream of specific growth factor signaling pathways. Adhesion-dependent growth factor signaling was also recently demonstrated in the context of angiogenic behaviors regulated by VEGF signaling. Combinatorial studies revealed an influence of cell adhesion peptide density on VEGF-mediated endothelial cell proliferation.(48) Here the authors investigated differences between soluble and insoluble presentation of a VEGF receptor binding peptide (VR-BP). The study identified additional context parameters in VR-BP induced proliferation, as VR-BP was only able to increase proliferation on surfaces with a sufficient density of the cell adhesion ligand GRGDSP. This observation is consistent with a series of studies demonstrating cross-talk between VEGF receptor activation and integrin-mediated adhesion.(45, 49, 50) The BMP and VEGF studies described here only serve as examples of a broader theme in the biomaterials literature. Specifically, there is important cross-talk between integrin-mediated signaling and growth factor signaling, and therefore cell-material adhesion is a critical element of context during growth factor induction of stem cell differentiation.

Material stiffness

Mechanical properties of the material to which cells are adhered also impact morphogenesis through a process called mechanotransduction.(51–55) Briefly, the mechanism of mechanotransduction is mediated through focal adhesions (FA), which are large protein complexes containing integrins, adapter proteins, and kinases. FAs are extracellularly attached to cell adhesion ligands presented by a material and intracellularly attached to the actin cytoskeleton. Rearrangement of actin filaments attached to FAs occurs as the cell interacts with the material, and establishes a physical connection between extracellular mechanical properties and the cytoskeleton. Changes in cytoskeletal organization in response to mechanical cues are mediated by myosin motor protein activity, contracting and generating tension along actin filaments. This FA-mediated cytoskeletal tension is at the core of the mechanism that transmits mechanical information from the extracellular material to the cell. Tension generated by myosin on actin leads to changes in activity of cell cortex-associated enzymes such as focal adhesion kinase (FAK). Rho-associated kinases (ROCKs) are also involved in this process, as their control of myosin phosphorylation dictates in part the amount of cytoskeletal tension generated. The overall process is mediated through a complex signaling cascade involving FAKs, ROCKs, and downstream changes in nuclear membrane organization that may impact transcriptional regulation. While the complete details of mechanotransduction are a more complex and active area of study, they are out of the scope of this review and are reviewed elsewhere.(56)

The elastic modulus (E) of the material to which stem cells are adhered is a notable mechanical property, and is now known to strongly influence stem cell fate (Fig. 1A–C). Engler et al. elegantly demonstrated this significance.(57) They showed that hMSC lineage commitment toward neuronal, skeletal muscle, and bone phenotypes was strongly influenced by the stiffness of the underlying substrate. Specifically, they cultured hMSCs on poly(acrylamide) (PAAm) substrates that approximated the native E of brain, skeletal muscle, and osteoid (non-mineralized bone) tissues, and found increased propensity of hMSCs to differentiate into neurons, myoblasts, and osteoblasts, respectively. They modulated the stiffness of the substrates by varying the volume percent of a bis-acrylamide crosslinker, and characterized differentiation by measuring changes in cell morphology, tissue-specific cell surface markers, and expression of master regulatory genes for myoblasts (*MyoD*)(58, 59) and osteoblasts (*CBF α -1/RUNX2*). (60) Treatment with blebbistatin, a pharmacological inhibitor of myosin II ATPase activity, showed that stiffness-mediated lineage specification was dependent on non-muscle myosin II (NMMII). Disruption of NMMII contractility downregulated expression of *MyoD* and *CBF α -1* and ultimately

prevented stiffness-dependent lineage specification. Multiple studies have more recently extended from the initial work by Engler et al. and demonstrated stiffness-dependent self-renewal⁽⁶¹⁾ or differentiation of adult stem cells into adipogenic,⁽⁶²⁾ chondrogenic,⁽⁶²⁾ osteogenic,^(63–65) myogenic,⁽⁶⁴⁾ neural,^(64, 66) and smooth muscle⁽⁶²⁾ lineages. The concept of stiffness-dependent lineage specification has also been extended to 3D cell culture in synthetic extracellular matrices. Mooney and coworkers cultured murine MSCs and hMSCs in alginate hydrogels of varying *E* and observed differences in osteogenic differentiation.⁽⁶⁷⁾ Interestingly, they observed maximal osteoinduction in hydrogels of intermediate stiffness (22 kPa). These conditions of intermediate stiffness correlated to maximal cell-mediated deformation of polymer chains and clustering of RGD cell adhesion ligands, as measured by Förster Resonance Energy Transfer (FRET). Myosin ATPase inhibition with (2,3)-butanedione-monoxime (BDM) inhibited stiffness-dependent lineage specification and reduced ligand clustering, signifying a relationship between MSC osteogenic differentiation and a cell's ability to generate traction forces.

In a separate study, Musah et al. modulated the stiffness of an underlying substrate to maintain pluripotency and prevent lineage commitment of hESCs.⁽⁶⁸⁾ The authors explored the influence of matrix mechanics on hESC pluripotency using PAAm hydrogels of varying elasticity derivatized with a glycosaminoglycan (GAG)-binding peptide sequence derived from vitronectin. In contrast to hydrogels of low (0.7 kPa) and intermediate (3 kPa) stiffness, hydrogels of comparatively high (10 kPa) stiffness supported proliferation, formation of robust colonies, and long-term maintenance of pluripotency. This study indicated that stem cells may sense and respond to the mechanical environment through adhesions between the ECM and cell-surface GAGs, thereby adding potential complexity to the more well-established mechanism of cellular mechanosensing through integrin-based adhesions.

Another recent study suggested the role of substrate mechanics may involve not just mechanotransduction, but also stiffness-dependent protein conformation on a substrate. Trappmann et al. observed stiffness-dependent differentiation of hMSCs on PAAm hydrogels—consistent with previous studies—but not on polydimethylsiloxane (PDMS) substrates of comparable *E*.⁽²⁷⁾ They concluded that the effects of different PAAm hydrogels on cell behavior were mediated not by mechanotransduction, but instead by ECM organization. Specifically, the authors found that decreasing *E* of PAAm corresponded to an increased hydrogel porosity that changed the density of ECM proteins bound to the surface. Collectively, these studies implicate multiple mechanisms for substrate-induced changes in stem cell differentiation, including mechanotransduction, cellular clustering of cell adhesion ligands, and material-dependent presentation of ECM proteins to cells. There is a continuing need for robust, well-defined experimental systems to understand how surrounding mechanical properties interact to influence stem cell fate.

Spatial influences on stem cell adhesion

The spatial presentation of adhesion ligands on materials is also emerging as an important parameter of the adhesion environment, as it impacts cytoskeletal organization. In particular, one could envision that cytoskeletal arrangement would be greatly dependent on the geometric or spatial context in which a material is presented to the cell. Organization of integrins would differ between a two-dimensional (2D) substrate and a 3D matrix, as well as in response to differing concentrations, gradients, or arrangements of cell adhesion ligands presented by the material. Influences of material nanotopography serve as an example of the effect of integrin organization on stem cell fate at the nanometer length scale. McMurray et al. demonstrated that surfaces with “well-ordered” nanoscale topographies could be designed to maintain multipotency of hMSCs (Fig. 1B).⁽⁶⁹⁾ They suggested that topographical features served to influence integrin organization at the cell-material interface,

and that this process induced downstream effects on metabolism, transcription, and cytoskeletal organization that ultimately dictated cellular fate. Interestingly, similar studies by this group explored other distinct nanometer-scale topographies and found that “random” organization of topographical features had a substantial influence in directing MSCs toward osteogenic lineages.(29)

In another study, McBeath et al. microcontact printed cell adhesion ligands to confine hMSC spread area and explore cell spreading-dependent MSC differentiation. Differences in the degree of cell spreading, defined by the area of adhesive islands, resulted in differentiation down adipogenic or osteogenic lineages through a RhoA-ROCK mediated pathway (Fig. 2A–B).(70) A recent study from Kilian et al.(31) elegantly showed that cell shape can also influence stem cell phenotype, even without changing the total degree of cell spreading. They used spatially patterned cell adhesion ligands to impose various geometric confinements of hMSC spreading. These geometric confinements induced different lineage commitments, which could be particularly correlated with cell shape (Fig. 2C–D). An intriguing hypothesis presented in the Kilian et al. study was that different FA arrangements in the different geometries ultimately impacted cell contractility. Interestingly, their observed differences between adipogenic and osteogenic specification occurred with equivalent total area of cell spreading and on surfaces with equivalent *E*. Subsequent combinatorial studies from Kilian and coworkers investigated the influence of matrix mechanics, adhesion ligand identity, and cell geometry on hMSC differentiation. Briefly, PAAm hydrogels were modified with various ECM proteins through microcontact printing of geometrically confining patterns. Consistent with prior studies, the group observed increased neurogenesis on collagen-presenting hydrogels of low (0.48 kPa) stiffness(57), and increased adipogenesis on fibronectin islands that confined cell spreading. (70)Interestingly, hydrogels of equivalent stiffness presenting fibronectin instead of collagen favored adipogenesis over neurogenesis, and round, cell shape-confining islands presenting collagen in place of fibronectin favored neurogenesis over adipogenesis. This apparent interplay between stiffness, geometric confinement, and ligand identity further demonstrates the importance of context in the adhesive environment.(71)

Like cell-material interactions, the context of cell-cell interactions can influence natural morphogenesis and functional maturation of tissues. For example, Bhatia et al. used photolithographic techniques to spatially pattern co-cultures of hepatocytes and nonparenchymal fibroblasts to control homotypic and heterotypic cell-cell interactions. This study systematically modulated spatial patterns to deconstruct the effects of cell type ratio and degree of heterotypic cell-cell contact on hepatocyte function. Maximizing heterotypic interactions between the two cell populations resulted in increases in urea and albumin synthesis, consistent with enhanced hepatocyte function.(72) More recent studies have utilized lithographic patterning techniques to explore the influence of homotypic cell-cell interactions on the propensity of hMSCs to differentiate down adipogenic and osteogenic lineages.(73) Tang et al. patterned isolated microdomains of an RGD-presenting substrate to restrict the extent of contact between neighboring hMSCs, and semi-quantitatively characterized hMSC differentiation as a function of homotypic cell-cell contact area. They found that increased levels of homotypic cell-cell contact resulted in higher percentages of cells differentiating toward adipogenic and osteogenic fates in their respective induction media conditions. These studies exemplify the utility of cell patterning techniques as tools to better understand how cell-material adhesion and cell-cell interactions modulate specific stem cell fate decisions.

Context effects on soluble factor presentation

Materials may include components that either intentionally or unintentionally influence the concentrations of soluble factors, such as growth factors present in the local stem cell microenvironment. Many strategies are inspired by an increasingly detailed knowledge of key signaling pathways in early tissue development.(74, 75) Since these natural signals are restricted to precise spatial localization and timing, some emerging approaches aim to create tailored materials that present signals to stem cells in a spatially and temporally controlled manner.(76)

Local soluble factor concentrations

Local concentrations of “endogenous,” or cell-secreted, soluble factors are often regulated by sequestering in the microenvironment. *In vivo*, sequestering is governed by proteins and proteoglycans (PGs) found in the native ECM. These biomolecules sequester soluble factors through noncovalent interactions,(77–87) thereby regulating their local concentrations and, in some cases, influencing their binding to cellular receptors.(88) Regulation of endogenous growth factors in this manner is illustrated by the ECM protein fibronectin, which serves not only to engage integrins on the cell surface, but also binds numerous growth factors as well as GAGs and PGs.(89, 90) These GAGs and PGs are in turn capable of sequestering growth factors through electrostatic interactions mediated by the density and organization of charged groups on GAG chains. Some recent advances in material design attempt to mimic native sequestering by modifying materials with natural or synthetic protein-binding moieties (Fig. 3A).(77, 91) For instance, materials in which natural GAGs or PGs are modified and covalently linked to a polymer backbone have been used to control growth factor release rates, and to stabilize growth factors, thereby maintaining their bioactivity *in vitro*.(80, 92)

Endogenous growth factor regulation can also be engineered into materials by incorporating synthetic molecules, such as peptides, that selectively bind GAGs or PGs (Fig. 3B).(77, 93) Hudalla et al. used SAMs presenting a heparin-binding peptide to sequester serum-borne GAGs.(93) This approach leveraged the ability of heparin to bind fibroblast growth factors (FGFs) and BMPs to augment the effects of these growth factor signaling pathways on hMSC behavior. In particular, hMSC proliferation and differentiation down the osteogenic lineage were potentiated by heparin-mediated sequestering and amplification of endogenous FGFs and BMPs. Importantly, it was also possible to spatially pattern amplified hMSC proliferation by patterning regions presenting the GAG-binding peptide. Based on the tight spatial regulation present during natural tissue morphogenesis, control over this aspect of growth factor sequestering will likely be important to the advancement of materials that direct similar processes *in vitro*. Another study from Hudalla et al. further demonstrated that the same sequestering approach could be used to amplify the effects of recombinant FGF-2 supplement on human endothelial cell proliferation, resulting in a substantially lower amount of FGF-2 supplement required for cell expansion.(78) Kiessling and coworkers used GAG-binding peptide strategies to generate chemically defined substrates for growth and maintenance of pluripotency in hESCs, demonstrating that GAG-binding peptides can not only bind soluble biomolecules, but also GAGs present on the stem cell surface. Here substrates presenting low concentrations of a heparin-binding peptide, GKKQRFRRNRKG, were capable of maintaining pluripotency of eight hESC lines.(94) While PG- and GAG-based approaches can influence growth factor signaling, the interactions are not highly specific since many biomolecules harbor GAG-binding domains. Another approach that has been used to add specificity to growth factor sequestering involves using peptide ligands identified via phage display or designed *de novo* by mimicking growth factors or growth factor receptors (Fig. 3C–D). For example, materials incorporating receptor-mimicking peptides that bind specifically to VEGF have been used to

upregulate or downregulate VEGF signaling.(95–97) Systems that regulate specific binding interactions between soluble factors and their receptors may circumvent complexities introduced by the promiscuous binding interactions of GAGs.

Endogenous growth factor regulation may also occur in materials that are not specifically engineered to sequester soluble factors. Recent evidence has indicated that simple organic functional groups can influence stem cell differentiation, perhaps due in part to biomolecule sequestering.(98) Benoit et al. showed that lineage-specific hMSC differentiation can be influenced by the type of simple chemical functional groups present in the cell's microenvironment.(99) The authors tethered methacrylate, amino, tert-butyl, phosphate, tetrafluorobutyl, and carboxylic acid groups to a PEG scaffold to mimic the hydrophilicity and charge of functional groups typically found in the extracellular environments of various hMSC target cell types. hMSC culture in hydrogels derivatized with these groups led to tissue type-specific ECM production and differentiation. Significantly, hydrogels presenting tert-butyl and phosphate groups preferentially induced hMSC adipogenesis and osteogenesis, respectively. As these experiments were performed in maintenance media rather than induction media, the results suggested an innate capacity of organic functional groups to influence cell fate. A subsequent study(98) has pointed to a role of these functional groups in endogenous regulation, as adsorption of serum components such as ECM glycoproteins to phosphate-modified hydrogels was sufficient to drive hMSC osteogenesis. Thus, the influence of simple chemistries on stem cell fate may warrant closer mechanistic examination, with particular emphasis on the possible role of molecular sequestering.

Gradients of soluble factors

Natural morphogenesis processes occur in environments where soluble growth factor gradients are under tight spatial and temporal regulation. To recapitulate aspects of this environment *in vitro*, recent studies have used materials as delivery vehicles to generate gradients of growth factors and small molecules in stem cell culture environments.(100) Such delivery strategies can create gradients within highly cellular 3D cultures of stem/progenitor cells, such as stem cell aggregates, in which soluble factor transport is restricted by aggregate size, tight junctions, and deposited ECM (Fig. 4A).(101) Recent advances in this area have incorporated small molecule- or growth factor-containing materials to achieve localized delivery of these molecules within stem cell aggregates.(25, 102) These materials can locally release soluble factors in a controllable manner, and thereby manipulate the soluble signaling microenvironment in 3D cell aggregates. For example, Carpenedo et al. used rotary suspension to incorporate poly(lactic-co-glycolic acid) (PLGA) microspheres into forming EBs.(103) Microspheres loaded with retinoic acid (RA), a small molecule morphogen, promoted formation of cystic EBs with a visceral endoderm-like outer layer, while delivery of soluble RA in the absence of microspheres generated only spheroidal EBs throughout a range of RA concentrations. Gene expression and immunostaining analyses further revealed that microsphere-treated, cystic EBs resembled mouse primitive streak-stage embryos in structure and phenotype. Using a similar delivery approach with heparinized gelatin microparticles, Purpura et al. optimized a multi-parameter system to promote differentiation of EBs into hemogenic mesoderm.(104) Microparticle-mediated delivery of BMP-4 and thrombopoietin into size-optimized EBs generated a 1.7-fold increase in the number of colony-forming cells derived per EB when compared to bulk soluble growth factor treatment, while requiring a 14-fold lower growth factor amount than was necessary when BMP-4 was added to the surrounding solution instead. Thus, materials can be used as delivery vehicles to locally concentrate morphogenic cues. It is noteworthy that experiments to explore spatiotemporal regulation introduce new variables (source, location, and timing) when compared to standard stem cell culture, which suggests a critical

need for high throughput approaches. One can think of these experiments as 4-dimensional, as the soluble factor source has a defined 3-dimensional location and time is a critical 4th dimension.(105, 106) Thus, emerging high throughput approaches are needed to efficiently explore spatial and temporal delivery of soluble factors to stem cell aggregates.

Size restrictions imposed on EBs can also control the presentation of soluble factor gradients. The importance of EB size is among the simplest, and most well-characterized, examples of context-dependence in stem cell bioengineering. Substrate micropatterning can confine ESC adhesion and subsequent colony growth to a size-restricted area, where the number of cells comprising the patterned region is dictated by spatial dimensions of the pattern and the cell seeding density.(107–109) Typically, patterns are generated by soft lithography techniques, wherein elastomeric stamps are used either to print cell-adhesive proteins in specified geometries or as molds to create arrays of microwells with defined dimensions.(15, 16) EBs formed in these systems have revealed that differentiation can be dependent on EB size. For example, a system in which EBs were generated from 2D colonies patterned onto Matrigel demonstrated that smaller EBs favored a higher ratio of endoderm- to neural-associated gene expression in comparison to those generated from larger colonies.(110) Similar work with micropatterned or microarrayed substrates has shown EB size-dependent differentiation toward erythropoietic,(14) cardiogenic,(15, 16, 18, 19) and ectodermal lineages.(20, 21) Based on these studies and others, it is evident that EB size has some influence on ESC differentiation; however, no clear trend has emerged correlating EB size to specific differentiation lineages. Nonetheless, these studies emphasize that EB size is important, and have led to additional studies to understand the mechanistic origins of size-dependence. For example, mass diffusion models revealed significant differences in oxygen and soluble factor concentrations throughout EBs as a function of EB size.(101) Thus, diffusional limits inherent to EBs likely create significant gradients of soluble factors that affect downstream differentiation. In support of this notion, recent studies indicate that differential levels of Wnt signaling occur in EBs of different sizes.(16, 18)

Future considerations: material applications in stem cell bioengineering

Stem cell bioengineers can now use a multitude of enabling technologies (summarized in Table 1) to define the cell's microenvironment, and probe the interactions that impact stem cell fate. Advances in synthetic chemistry and materials science have created materials with increased control over the microenvironment. While the ability of soluble factors to direct stem cell differentiation is well-established, intrinsic material components (adhesive, mechanical, and biophysical), regulation of local soluble factor concentrations, and user-defined cell patterning have more recently emerged as potent regulators of stem cell fate and tissue morphogenesis. The understanding of these components in their appropriate context will continue to be critical to the design of bioinspired microenvironments to direct tissue morphogenesis. Furthermore, the development of novel material technologies that allow user-defined control over spatiotemporal aspects of cell-material interactions is an emerging area that will push *in vitro* stem cell culture systems toward the degree of complexity found in biological systems. However, it is not yet evident to what extent the stem cell microenvironment must be defined in space and time, and a continuum exists between systems that exert rigorous control over spatiotemporal aspects of the microenvironment and those that are largely defined by components outside of user control. Thus, with advances in material technologies, it is conceivable that the level of control afforded by these culture environments may exceed that which is necessary to support morphogenesis processes *in vitro*. Indeed, it is not difficult to imagine that an *in vitro* morphogenesis process might occur optimally in a system in which microenvironmental control is exerted only to define a set of initial “boundary conditions” (cell types, adhesive and mechanical environment,

concentration gradients) that serve as a context permitting further morphogenesis to be directed by the cells themselves.

Examples of this concept are provided by systems that use microfluidics to establish these initial conditions. Microfluidic devices support laminar flow, which enables one to form controllable soluble concentration gradients by tuning geometry and fluid flow velocity (Fig. 4B). Analogous gradients may be created in 3D by combining microfluidic strategies with hydrogel materials in which these gradients are established via encapsulated depots of morphogens (Fig. 4C). Microfluidic devices have been used in proof-of-concept studies to pattern expression of a master regulatory gene and mimic pluripotency-differentiation boundaries that arise during early development. Zhang et al. used a mouse ESC line engineered with doxycycline-inducible Nanog expression to study stem cell lineage commitment along gradients of Nanog expression, leukemia inhibitory factor, and RA, separately and in combination.⁽¹⁰⁰⁾ This study was a proof-of-concept demonstration that microfluidic systems may be leveraged to pattern gene expression domains. One could envision that these gene expression domains would then establish an initial signaling context, whereby stem cells patterned and “primed” in their phenotype might interact with one another and with their adhesive, mechanical, and soluble environment to drive their own morphogenesis.

It is also noteworthy that *in vivo* counterparts of the cell-material interactions discussed herein are rarely, if ever, static during morphogenesis. Recent studies have extended cell-material interactions into more complex environments and begun to elucidate new contexts that are likely to be important. For example, Burdick and coworkers recently demonstrated that the ability for tractional forces to influence adipogenic or osteogenic differentiation of hMSCs in 3D hyaluronic acid hydrogels is dependent not only on *E*, but also on the cell's ability to degrade the ECM.⁽²²⁾ Interestingly, this phenomenon is seemingly not a factor in previous 2D analogues of this experiment.⁽¹¹¹⁾ Future studies of stem cell fate in degradable environments may need to account for myriad changes in the stem cell microenvironment caused by material degradation, including mechanical properties, soluble factors (e.g. degradation byproducts, sequestering), cell adhesion ligand identity/density, and cell-cell interactions. As these and other mechanisms underlying specific cell-material interactions and associated cellular decision-making continue to emerge, the pool of components considered in material design will continue to expand. Improved biological understanding coupled with new material technologies will push the field closer to highly effective bioinspired contexts.

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Key Words

Stem Cell Biology

The study of stem cell processes that govern cell fates including proliferation, survival, and differentiation.

Tissue Morphogenesis

The process in which stem cells proliferate, migrate, and differentiate into specialized cells to give rise to organized tissues. This process is governed by molecular and physical instructions referred to as morphogenic cues.

Biologically Driven Assembly	<i>In vitro</i> tissue morphogenesis that largely occurs in the absence of externally provided morphogenic cues. The premise is that the stem cells themselves provide and regulate the spatial and temporal presentation of morphogenic cues.
Stem Cell Microenvironment	The combined set of chemical, physical, and mechanical parameters immediately surrounding the stem cell that serve as morphogenic cues.
Mechanotransduction	A process by which mechanical properties of a material are transduced to the cytoskeletal components, ultimately affecting intracellular signaling and transcriptional activity.
Cell-Material Adhesion	The molecular mechanism of cell adhesion to materials, which impacts cell signaling processes through both extent of adhesion and molecular identities involved.
Embryoid Body	An aggregate of pluripotent stem cells used for the study of three-dimensional tissue development and stem cell differentiation.
Growth Factor Sequestering	The regulation of local concentrations of soluble growth factors through electrostatic and hydrophobic-hydrophobic interactions.

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Figure 1. Mechanical properties of the microenvironment

a) Resistance to deformation on stiff materials increases cytoskeletal tension of human mesenchymal stem cells (hMSC) through focal adhesion kinase (FAK) and Rho-associated kinase (ROCK) activity, leading to differentiation. b) Introduction of topography results in rearrangement of integrins at the cell-material interface, promoting topography-dependent hMSC proliferation, self-renewal, or differentiation. c) Substrate stiffness may impact differentiation of hMSCs, where certain ranges of stiffness may preferentially promote differentiation down a particular lineage. Specific stiffness ranges should not be thought of as restrictive to particular cell fates, however, as the influence of material stiffness on lineage-specific differentiation is highly context-dependent. For instance, material degradability, adhesion ligand identity and density, dimensionality of the matrix, as well as the presence and degree of order of nanotopographical features, can all influence stem cell fate on materials of identical stiffness.

Figure 2. Local geometry and cell shape direct stem cell differentiation

Human mesenchymal stem cells (hMSCs) confined to shapes of differing geometry preferentially differentiate down osteogenic or adipogenic lineages. hMSCs confined to small micropatterned islands a) are rounded in shape and exhibit low cytoskeletal contractility, while those adhered to large islands b) are spread and highly contractile. Shapes with high local curvature c) promote increased cytoskeletal contractility that drives hMSCs toward osteogenic fates, while those with low local curvature d) result in decreased contractility and adipogenic fates.

Figure 3. Molecular interactions on materials can regulate growth factor signaling

Engineered materials may incorporate a) covalently bound glycosaminoglycans (GAGs) or proteoglycans (PGs) or b) moieties that bind GAGs/PGs, which can in turn sequester growth factors from the stem cell microenvironment. Alternatively, materials may be functionalized with c) moieties that bind growth factors or d) moieties that directly interact with growth factor receptors (GFRs), to upregulate or downregulate GFR signaling. Finally, GFRs and their associated signaling pathways may synergize with e) integrin-mediated adhesion and signaling downstream of adhesion.

Figure 4. Gradients present in stem cell microenvironments

a) Stem cell aggregate size establishes new contexts of soluble factor and oxygen gradients across the exterior to interior of aggregates. b) Microfluidic devices employ laminar flow in divergent channels to generate defined and stable concentration gradients of leukemia inhibitory factor (LIF), doxycycline (Dox)-inducible Nanog expression, and retinoic acid (RA) to pattern pluripotency in mouse embryonic stem cells (mESC). c) Analogous to two-dimensional microfluidic gradients, spatially controlled morphogen gradients in three-dimensional matrices can be generated via morphogen diffusion from delivery depots.

Stem cell-material interaction	Enabling technologies	References
<i>Cell-material adhesion</i>		
Growth factor receptor-integrin crosstalk	Substrate modification with defined adhesion ligand density (e.g. SAMs)	[47,48]
Material stiffness influences on stem cells	Degradable or non-degradable hydrogels of tunable stiffness	[22-24,27,57,67,71,111]
Spatial influences on stem cells (e.g. ligand clustering, cytoskeletal arrangement, cell-cell interactions)	Nanometer-scale lithography-generated nanotopography; microcontact printing or photolithographic patterning of cell adhesion ligands	[29,69]; [31,70-72]
<i>Soluble factor presentation</i>		
Regulation of endogenous signaling (i.e. “sequestering”)	Material modification with natural or synthetic glycosaminoglycan- or proteoglycan-binding moieties or chemical functional groups	[77,78,95-99]
Manipulation of spatiotemporal morphogen gradients	Microfluidic devices; biomaterial-mediated local delivery of soluble factors	[100]; [25,26,102-104]