Constructing stem cell microenvironments using bioengineering approaches

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Submitted 7 June 2013; accepted in final form 24 September 2013

Brafman DA. Constructing stem cell microenvironments using bioengineering approaches. Physiol Genomics 45: 1123–1135, 2013. First published September 24, 2013; doi:10.1152/physiolgenomics.00099.2013.—Within the adult organism, stem cells reside in defined anatomical microenvironments called niches. These architecturally diverse microenvironments serve to balance stem cell self-renewal and differentiation. Proper regulation of this balance is instrumental to tissue repair and homeostasis, and any imbalance can potentially lead to diseases such as cancer. Within each of these microenvironments, a myriad of chemical and physical stimuli interact in a complex (synergistic or antagonistic) manner to tightly regulate stem cell fate. The in vitro replication of these in vivo microenvironment will be necessary for the application of stem cells for disease modeling, drug discovery, and regenerative medicine purposes. However, traditional reductionist approaches have only led to the generation of cell culture methods that poorly recapitulate the in vivo microenvironment. To that end, novel engineering and systems biology approaches have allowed for the investigation of the biological and mechanical stimuli that govern stem cell fate. In this review, the application of these technologies for the dissection of stem cell microenvironments will be analyzed. Moreover, the use of these engineering approaches to construct in vitro stem cell microenvironments that precisely control stem cell fate and function will be reviewed. Finally, the emerging trend of using high-throughput, combinatorial methods for the stepwise engineering of stem cell microenvironments will be explored.

stem cell; microenvironment; extracellular matrix; biomaterials; cell-cell interactions; biophysical; high-throughput

UNLIKE THE FULLY DIFFERENTIATED and mature cells of the adult organism, stem cells are unspecified cells that possess the capacity to proliferate indefinitely and differentiate into various cell types that comprise the mature, adult organism. Therefore, stem cells provide a unique model system to study early human development as well as a ready supply of cellular “raw material” that can be used to generate mature and functional cells suitable for disease modeling, cell transplantation, and replacement therapies. In general, stem cells can been categorized into three different types, which have unique differentiation propensities: 1) totipotent stem cells [e.g., ground-state or naïve embryonic stem cells (ESCs) (124)], which are representative of the early blastocyst stage and all the cells of the adult body in addition to the extraembryonic cells, 2) pluripotent stem cells [including ESCs and induced pluripotent stem cells (iPSCs)], which can give rise to the hundreds of different cell types that comprise the adult organism but not the cells that comprise the extraembryonic tissues, and 3) multi- or unipotent adult stem cells, which are, by comparison, more limited in their differentiation potential and can only give rise to a specialized subset of cell types. Despite these differences, future scientific and clinical applications of totipotent, pluripotent, and adult stem cells will require a detailed understanding of the mechanisms and signals that maintain their undifferentiated, multipotent state or result in their differentiation to specific lineages.

In vivo, adult stem cell reside in multifaceted, local tissue microenvironments, termed niches, which tightly regulate and balance stem cell self-renewal and differentiation (123). The various niche components interact in a complex manner to prevent excessive cell proliferation that could lead to cancer while at the same time limiting cell differentiation, which could lead to the depletion of a tissue’s regenerative potential. The concept of the niche, which was first proposed by Schofield in 1978 (140), gained wide acceptance with the discovery and the characterization of the hematopoietic stem cell (HSC) niche (1). Additional stem cell niches have been identified and characterized in the mammary glands (mammary stem cells) (105), the base of the crypt of the intestinal epithelium (intestinal stem cells) (14), basal layer of the epidermis (epidermal stem cells) (33), subventricular zone of the lateral ventricle, and the subgranular zone of the hippocampus of the central nervous system [neural stem cells (NSCs)] (60, 61), the bulge region of the hair follicle (epithelial stem cells) (38), the basal layer of the seminiferous tubules (germline stem cells) (47), and under the basal lamina of myofibers (muscle satellite cells) (34, 35). Additionally, mesenchymal stem cells (MSCs), with their broad differentiation spectrum and enormous clinical
potential, have been identified in several tissues including the bone marrow, synovium, periosteum, adipose, and placenta (129). Recently, adult stem cells have also been identified in several adult epithelial tissues in the stomach, cervix, anus, testes, and lens, but their niches have yet to be extensively characterized (6).

One of the main challenges in the study and clinical application of stem cells is that although these cells are multipotent and highly self-renewing in vivo, they are difficult to maintain and expand in vitro. In fact, only a few adult stem cell populations, such as neural progenitor cells (NPCs), have been successfully cultured and propagated in vitro (61). Therefore, much effort has been invested in the stepwise engineering of in vitro stem cell microenvironments that mimic the in vivo niche and, therefore, can precisely control stem cell fate. The various tissue-specific niches share a common set of basic engineering components that interact in a complex manner to influence stem cell fate. These components can be organized into several categories: 1) immobilized protein factors [such as extracellular matrix proteins (ECMPs)], which act as cell adhesion substrates, 2) soluble chemical stimuli (such as growth factors and small molecules), which influence cell signaling pathways through extracellular or intracellular mechanisms, 3) biophysical stimuli (such as mechanical strains, topography, and stiffness), which can influence cell fate by mechanoreceptor signaling or modulation of the actin cytoskeletal network, and 4) cell-cell interactions (such as Notch or cadherin-mediated signaling).

In this review, the engineering approaches for mimicking these key components of the stem cell microenvironment will be discussed (Fig. 1). Although many of these technologies have been applied to pluripotent stem cells, this review will focus on the applications with adult stem populations. Finally, future application of high-throughput and combinatorial technologies to assemble in vitro microenvironments will be discussed.

CHEMICAL CUES TO CONTROL STEM CELL BEHAVIOR

It is well established that soluble factors including growth factors, cytokines, and small molecules can exert potent and specific effects in stem cell microenvironments. For example, both canonical and noncanonical WNT signaling has been implicated in maintaining stem cells in the hematopoietic niche (108, 148). Likewise, small cell-permeable molecules such as oxygen precisely regulate HSCs within the niche (52). To that end, several approaches have been used to study the effect of chemical stimuli on stem cell fate.

Soluble Factors to Influence Stem Cell Fate

Because of the relative ease of investigation, the influence of soluble factors added directly to the media has been exten-

![Fig. 1. Engineering approaches used to study the stem cell microenvironment. The stem cell consists of several components such as chemical stimuli, extracellular matrix, biophysical cues, and cell-cell interactions, which act individually and in concert to regulate stem cell fate. Engineers are applying several novel approaches to dissect each of these components and understand how each regulates stem cell behavior.](http://physiolgenomics.physiology.org/doi/10.220.33.6.onJune17.2017)
Engineered gradients of heparin-binding EGF to precisely control lineage [132]. Along similar lines, Miller et al. [119] cultured off the BMP2 pattern differentiated toward the myogenic lineage on the BMP2 substrates, cells grown on the BMP2 substrate (132). Indeed, when MDSCs were cultured simultaneously toward the osteogenic and myogenic lineages on the same substrate (132). Moreover, exogenous addition of platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β), and FGF have been used to modulate differentiation and growth of MSCs [126].

Immobilization of Growth Factors to Regulate Local Concentration and Long-range Signaling Ability

In vivo, soluble signaling factors are often bound to the extracellular matrix (ECM), which regulates their diffusion and specifies local concentration gradients. In fact, several growth factors interact directly with the ECM or are bound to the ECM through heparin binding domains [137]. For example, several FGFs, PDGFs, vascular endothelial growth factors (VEGFs), and TGF-β have heparin binding domains, which bind them to the ECM, thereby increasing local concentrations and establishing local concentration gradients [16, 26]. Along similar lines, it has been shown that sonic hedgehog (SHH) binds directly to the ECM matrix, which modulates SHH long-range signaling activity [134]. To that end, engineers have developed several methods to immobilize growth factors on culture substrates to present them in a more biologically relevant form. Willerth et al. [161] immobilized neurotrophin-3 (NT-3), SHH, and PDGF in fibrin and heparin substrates to induce neuronal differentiation of mouse NPCs. Immobilization of these signaling molecules provide precise control over NPC differentiation by allowing for the dose-dependent modulation of the relative percentages of neural progenitors, neurons, oligodendrocytes, and astrocytes in differentiating cultures [161]. A similar study demonstrated that immobilization of SHH enhances differentiation of bone marrow-derived MSCs [78]. Specifically, polymer substrates functionalized with SHH support increased osteogenic differentiation of bone-derived MSCs compared with cells cultured on similar surfaces with soluble SHH added directly to the media at the same concentration [78].

Engineers have also taken advantage of emerging technologies such as inkjet bioprinting to create spatially defined patterns of immobilized signaling molecules to control stem cell fate. In one such study, inkjet printing was used to print bone morphogenetic protein-2 (BMP2) on fibrin substrates with the intent of directing muscle-derived stem cells (MDSCs) simultaneously toward the osteogenic and myogenic lineages on the same substrate [132]. Indeed, when MDSCs were cultured on the BMP2 substrates, cells grown on the BMP2 pattern differentiated toward an osteogenic lineage, while cells cultured off the BMP2 pattern differentiated toward the myogenic lineage [132]. Along similar lines, Miller et al. [119] engineered gradients of heparin-binding EGF to precisely control the proliferation, migration, and differentiation of MSCs.

Finally, in a recent study, immobilization of growth factors on bioactive beads was used to investigate asymmetrical stem cell division, a fundamental property of stem cells in which one cell gives rise to one daughter cell that is a stem cell and another daughter cell that has a more specialized fate [73]. In vivo, asymmetrical presentation of signaling molecules has been shown to regulate asymmetrical stem cell division. However, with current technologies asymmetrical stem cell division has been difficult to study in vitro. To that end, Habib et al. [73] immobilized WNT proteins on beads and introduced them to ESCs in culture. Using single-cell imaging, the authors found that the WNT-proximal daughter cell expressed higher levels of pluripotency genes, whereas the distal daughter cell acquired markers indicative of differentiation. Such novel approaches could be readily applied to study cell division in adult stem cell populations. Together, these studies demonstrate the effectiveness of immobilized growth factors to influence and study stem cell phenotypes in a manner that is not readily achievable by traditional methods.

Microfluidic Devices to Regulate the Soluble Signaling Microenvironment

In the native microenvironment, the temporal and spatial distribution of soluble signaling factors is tightly regulated in a way that is not achievable with traditional static cultures. Microfluidic devices, manufactured using etching or soft lithography approaches, have allowed for the culture of stem cells in a microenvironment that mimics the dynamic physiological soluble signaling environment [170]. For example, adipose stem cells (AdSCs) proliferated at a higher rate in microfluidic devices that provided a continuous exposure of EGF compared with static cultures in which EGF had no effect on AdSC growth [156]. Along similar lines, microfluidic devices have been used to generate stable gradients of signaling molecules allowing for increased NSC proliferation [32] or differentiation [131]. More recently, microfluidic devices have enabled the culture of endothelial cells (ECs) and MSCs in a three-dimensional (3D) microenvironment representative of the perivascular niche where the paracrine and autocrine signaling is tightly regulated [29].

Modulation of Paracrine Signaling to Investigate the Influence of Factors Secreted From Neighboring Cells

Within the niche, factors secreted by neighboring cells can modulate stem cell fate. For example, differentiated neurons in the olfactory bulb secrete a soluble factor that inhibits generation of new neurons by the resident NPCs of the olfactory epithelium [166]. Similar paracrine effects have been studied in vitro. For example, trans-well culture chambers were used to investigate if mature chondrocytes provide inductive signals for osteogenesis [67]. With this system, it was demonstrated that chondrocytes express soluble BMPs that selectively induce osteogenesis in MSCs [67]. Moreover, this effect was not mimicked through the exogenous addition of BMP. Another study showed that coculture of HSCs with MSCs increased HSC proliferation to levels not achievable through soluble cytokine addition [89].
ECM REGULATES STEM CELL FATE

The ECM consists of a complex mixture of proteins, proteoglycans, and polysaccharides that undergoes an iterative cycle of assembly, remodeling, and degradation (169). Importantly, the ECM provides adhesive interactions that are critical for stem cell maintenance within the niche (112). Furthermore, the ECM provides a range of structural signaling cues that regulate stem cell fate decisions (169). For example, laminin via a β1-integrin-dependent mechanism maintains spermatogonial stem cells (SSCs) within the germline niche of the testes (90). Therefore, precise engineering of the ECM will be critical in regulating stem cell fate in vitro.

Natural ECMs Recapitulate In Vivo Biological Activity

Natural ECMs from purified or recombinant sources are advantageous because they retain their activity and ability to be proteolytically degraded (110). Several studies have effectively used these natural ECMs as stem cell culture substrates. For example, laminin has been identified as the optimal substrate for long-term culture of NPCs (57). Along similar lines, a combination of collagen, laminin, entactin, and proteoglycans was developed as a neuronal ECM to support in vitro neural differentiation of NPCs (63, 64). Natural ECMs such as fibronectin and laminin have been used as cell culture coatings for the continued proliferation of primary muscle stem cells in vitro (163). Finally, native ECM produced by human MSCs in vivo was extracted in urea and coated onto tissue culture substrates. MSCs cultured on this substrate displayed enhanced proliferation, attachment, and multilineage differentiation compared with other commonly used ECMs such as collagen I (102).

Decellularized Matrices Recapitulate In Vivo Composition and Structure

Although natural ECMs can be readily used as stem cell culture substrates, they do not adequately mimic the spatial organization and mechanical composition of the native ECM. On the other hand, decellularized matrices, which can be obtained through the detergent treatment of isolated tissues, provide the mechanical and biochemical scaffold that can provide for the directed differentiation of stem cells to a specific cell type (49). Several studies have shown that decellularized matrices maintain the structural features of native tissue, therefore enhancing stem cell differentiation. For example, decellularized lungs maintained many of the structural features of native lungs such as intact vasculature and a scaffold rich in collagen I, collagen IV, laminin, and fibronectin. Furthermore, MSCs cultured on these decellularized lung scaffolds display morphologies of cells located in both parenchymal and airway regions (46). Similarly, culture of MSCs in a scaffold derived from rat liver improves differentiation of MSCs into functional hepatocyte-like cells (86). Finally, decellularized porcine brain matrix rich in glycoaminoglycans, collagen I, collagen III, collagen IV, collagen V, collagen VI, perlecan, and laminin improves neuronal differentiation of NPCs (50). In sum, these studies demonstrate that decellularized scaffolds provide both structural and biological cues that effectively control stem cell fate.

Natural Biomaterial Scaffolds to Modulate Stem Cell Fate

Naturally occurring biomaterials including fibrin, silk, and several polysaccharide-based substrates have been for the culture and differentiation of stem cells. These naturally derived scaffolds are composed of proteins and polysaccharides commonly found in the native ECM and contain regions that allow for cell adhesion (162). Additionally, the biomechanical properties of these materials can be fine-tuned and these scaffolds can be readily fabricated into a variety of structures (162).

Fibrin scaffolds are most often generated through the polymerization of the protein fibrinogen obtained from blood plasma (96). Culturing of MSCs in fibrin scaffolds increases the chondrogenic differentiation of MSCs as measured by collagen and proteoglycans secretion (165). As such, fibrin scaffolds have been used to deliver MSCs to the location of lesions to promote tissue repair (18). Fibrin substrates have also been used to direct the differentiation of additional stem cell populations such as the neuronal differentiation of stem cells derived from adipose tissue and skin (65).

Silk, secreted from insects and worms, has been used for the culture of a variety of stem cell types. Because of its unique biophysical properties, silk has been extensively applied to the engineering of musculoskeletal tissue types (162). To that end, silk scaffold have been used extensively for the differentiation of MSCs into cell types that comprise bone and cartilage (79, 80, 117). In fact, the extent of chondrogenesis, as determined by deposition of glycoaminoglycans and type II collagen, was substantially higher when MSCs were cultured on silk compared with collagen scaffolds (118). Furthermore, silk-MSC scaffolds had a stiffness that was more similar to that of native cartilage than that of collagen-MSC scaffolds (80).

Several polysaccharide-based materials have been used as scaffolds for stem cell culture. For example, agarose, which is isolated from red algae, has been used to promote the differentiation of MSCs and AdSCs into chondrocytes (9, 82). Along similar lines, alginate, which is derived from brown algae, has been used to promote the chondrogenic differentiation of MSCs and AdSCs (9, 88). Alginate scaffolds have also been used to increase the proliferation of NPCs compared with standard culture conditions (7). Finally, chitosan, which can be extracted from crustacean shells, has been used to improve the osteogenic (71) and the chondrogenic differentiation of MSCs (48). In fact, composite scaffolds composed of fibrin and chitosan improve the ability of seeded MSCs to repair in vivo cartilage defects compared with seeded scaffolds composed only of fibrin or chitosan (48).

Synthetic Materials for Stem Cell Culture

Although natural ECMs have been used widely for stem cell culture, these proteins are difficult to isolate, often biochemically heterogeneous, and subject to batch-to-batch variations. Moreover, these structurally complex molecules contain several receptor-binding motifs, making it difficult to elucidate their precise biological function. In contrast, synthetic biomaterials, such as polymers and peptides, are easily fabricated and represent a reliable alternative for in vitro stem cell culture. Furthermore, iterative engineering of these biomaterials allows for the identification of the substrate components that regulate signal transduction and stem cell response. Finally, the physiochemical properties of polymers and peptides can be clearly
defined, fine-tuned, and precisely controlled to determine the optimal matrix environment for the desired stem cell fates.

Several studies have reported the use of peptides adsorbed on tissue culture plates or embedded in an interpenetrating polymer network (IPN) for stem cell culture. Because numerous integrins (e.g., collagen and fibronectin-binding integrins) interact with RGD tripeptide active site (84), RGD-containing peptides have been used extensively for stem cell culture. For example, RGD-containing peptide substrates have been used for the adhesion and proliferation of NPCs (2). On the other hand, substrates with the laminin-binding sequence IKVAV induced rapid differentiation of NPCs into neurons while preventing astrocyte formation (143). More recently, a high-throughput bacterial peptide display technology was employed to engineer novel peptide containing biomaterials that supported NPC self-renewal and multilineage differentiation (103).

Polymeric biomaterials have also been used extensively to control stem cell fate in vitro. For example, a tantalum-coated porous biomaterial was used to support long-term HSC maintenance and multipotency without the need for exogenous signaling molecules (10). In another study, Curran et al. (42) used silane-modified surfaces to control MSC adhesion and differentiation. Interestingly, methyl functionalized surfaces maintained MSC multipotency, amino and silane modified surfaces promoted osteogenesis, and hydroxyl and carboxyl containing substrates stimulated chondrogenesis. In another example, poly-o-lysine-coated poly(styrene/divinylbenzene) scaffolds allowed for the culture and differentiation of NPCs (76).

Ceramics (e.g., bioactive glass and hydroxyapatite) are another set of synthetic materials that have been used to manipulate stem cell fate (127). Several studies have shown that ceramic materials such as porous calcium ceramic cubes can be used to promote the proliferation and differentiation of MSCs to osteogenic fates (74, 100). Moreover, ceramic materials have also improved the ability of MSCs to induce bone repair when tested in animal models of bone defects (5).

### BIOPHYSICAL REGULATION OF STEM CELL FATE

The complex network of signaling and matrix molecules is also subject to mechanical forces (such as pressure, fluid shear stress, and stretch), which play important roles in specifying embryonic polarity (93) and tissue development (27). These biophysical stimuli can be mediated through integrin-ECM adhesions (62) or mechanoreceptors (51) that perturb cellular behaviors such as proliferation or differentiation. The effect of biophysical stimuli on stem cell attachment, spreading, proliferation, migration, and differentiation has been exploited in a variety of systems to direct stem cell fate.

#### Mechanical Atrains

Mechanical strain, such as stretch or compression, occurs in several regions of the body. For example, compressive forces are common in the skeletal system as the result of contractions of muscles or tendons moving bones. Likewise, blood vessels are subject to cyclic tensile strain due to the pulsatile nature of blood flow. At a cellular level, this external mechanical force is transmitted through the actin cytoskeleton, which activates various biochemical stimuli and results in a cell response.

Because of their presence in connective tissue, MSCs have been studied extensively in mecho-related investigations. For example, Park et al. (130) explored the differential effects of equiaxial and uniaxial strain on MSC differentiation. The authors found that uniaxial strain (versus biaxial strain or no strain), which is the type of strain experienced by smooth muscle cells (SMCs) in vivo, increased MSC differentiation into SMCs (130). By comparison, application of cyclic biaxial strain resulted in osteogenic differentiation of MSCs via an ERK1/2-dependent signaling mechanism (144). Cyclic compression has been shown to influence MSC fate as well. Increased chondrocyte differentiation was observed in MSCs subjected to cyclic compression as evidenced by enhanced ECM deposition and increased glycoaminoglycans secretion (113).

#### Shear Stress

In vivo several tissues types are exposed to dynamic shear environments that influence cell behavior at the molecular level. For example, vascular and endothelial cells are continually exposed to shear stress associated with blood flow. In fact, disturbed or oscillatory flows, which results in abnormal shear stress patterns, can modulate endothelial gene expression and lead to changes in vascular behavior that result in atherosclerosis (41). Shear stress can influence cell behavior in nonvascular tissue as well. For example, the mechanical environment of the bone can cause local pressure gradients that via the interstitial fluid flow stimulate shear stress-induced cellular responses (72, 94).

The application of shear stress has been used for the differentiation of adult stem cells into the vascular and endothelial lineages. AdSCs acquired an SMC phenotype, characterized by the expression of smooth muscle alpha actin, calponin, and smooth muscle myosin heavy chain, more readily under pulsatile flow than static conditions (157). Additionally, 3-dimen-sional polyglycolic acid scaffolds seeded with AdSCs exposed to pulsatile flow displayed biochemical properties similar to those of small diameter blood vessels (157). In another study, shear stress significantly increased endothelial differentiation of mesenchymal progenitor cells (158). Shear stress upregulated the expression of mature endothelial markers and angiogenic growth factors, increased the formation of capillary-like structures, and downregulated smooth muscle-related growth factors. In addition to inducing vascular and endothelial differentiation, shear stress has been shown to induce osteoblast proliferation or osteogenic differentiation of stem cells. For instance, steady shear flow stimulated osteoblast proliferation and differentiation through a dual ERK- and nitric oxide synthase-dependent mechanism (91). By comparison, pulsating fluid flow increased osteogenic differentiation of adipose tissue-derived MSCs via a nitric oxide-dependent mechanism (94). This collection of studies demonstrates that shear stress can have a wide variety of effects on stem cell behavior through similar signaling mechanisms.

#### Substrate Stiffness and Elasticity

Tissues within the body have a range of stiffness ranging from soft brain matrix (0.1–1 kPa) to collagenous bone (100 kPa). It follows that various cell behaviors can be influenced by substrate stiffness. Although the precise biochemical mecha-
nisms have yet to be elucidated, cells “sense” the substrate elasticity through their integrin adhesion complexes and actin-myosin cytoskeleton (51). A cell can respond to changes in substrate compliance through cytoskeletal reorganization, which alters a variety of signaling pathways and, ultimately, cell fate (160). The precise tuning of substrate stiffness will be important in the lineage specification of stem cells.

The effect of matrix elasticity on stem cell lineage specification has been studied extensively in MSCs. MSCs cultured on soft matrices that mimic brain (0.1–1 kPa) obtain a neural phenotype, while cells cultured on stiffer matrices that mimic muscle (8–17 kPa) or bone (25–40 kPa) differentiate toward myogenic or osteogenic lineages, respectively (55). Importantly, specific differentiation into these various lineages was achieved at similar efficiencies to soluble growth factor-induced differentiation. Furthermore, specific inhibition of non-muscle myosin II B by blebbistatin treatment inhibited all matrix stiffness-directed differentiation, thereby providing mechanistic insight into how a stem cell could sense the elasticity of its surrounding microenvironment.

Substrate elasticity can modulate the differentiation of other adult stem cell populations as well. For example, Saha et al. (138) demonstrated that substrate modulus directs NPC self-renewal and differentiation. Neuronal differentiation, measured by β-tubulin III, peaked on substrates with an elastic modulus of 0.5 kPa, approximately the same stiffness as brain tissue. By comparison, harder substrates (1–10 kPa) favored glial differentiation. Along similar lines, identification of the appropriate substrate stiffness has allowed for culture of several stem cell populations, such as muscle and HSCs, that have been previously difficult to maintain and expand in vitro. For example, soft hydrogels (12 kPa) that had an elasticity similar to muscle were able to support the proliferation and self-renewal of muscle stem cells (MuSCs) (68). Importantly, unlike MuSCs cultured on rigid tissue culture plates (10 kPa), MuSCs cultured on soft hydrogels were able to regenerate muscle in vivo when transplanted into mice (68). In another study, culture of mouse or human HSCs on soft tropoelastin-coated plates led to significantly more cell expansion than HSCs on control plates (81).

In vivo, various diseases, such as muscular dystrophy, can alter the elasticity of tissues and influence changes in cell behavior (147). Technologies such as atomic force microscopy (AFM) have made it possible to precisely identify the changes in tissue stiffness that may occur with disease (54). To that end, Engler et al. (53) used AFM to measure the differences in stiffness between normal and dystrophic muscle. Based on these measurements, the authors generated collagen substrates of stiffness similar to normal and dystrophic tissue to investigate the influence of substrate elasticity on the growth of myotubes (53). Myotubes differentiated and formed actin-myosin striations when grown on substrates with elasticity similar to that of muscle. Conversely, myotubes grown on stiffer substrates that mimic those of dystrophic muscle did not striate. These studies provide insight into possible disease mechanisms and have important implications for translational therapies where cells would be introduced into diseased tissue with altered biomechanical properties (53).

Most studies investigating the role of substrate rigidity on stem cell fate have made use of polyacrylamide or polyethylene glycol gels with varied levels of cross-linker, which alters their mechanical stiffness. The problem with this approach is that different cross-linker levels influence not only bulk mechanical properties but also microscopic properties such as porosity and ligand-binding density. As a solution to this problem, Fu et al. (58) implemented a technology of arrays of pillars with varying flexibility, termed micropillar arrays. With this technology, the authors were able to study the effects of substrate rigidity independently from the surface adhesive properties on MSC fate. Specifically, cells cultured on arrays with longer, softer pillars had a rounded morphology and differentiated towards an adipogenic fate. On the other hand, cells grown on arrays with shorter, stiffer pillars displayed an elongated morphology and were biased toward osteogenic differentiation (58). Furthermore, using traction force microscopy the authors identified a correlation between traction forces and the final differentiation state of individual MSCs. Interestingly, osteogenic differentiation was favored by cells that had higher traction forces, while adipogenic differentiation was more pronounced in cells with lower contractile forces. In the future, micropillar arrays may serve as a high-throughput method to investigate the role of soluble molecule-substrate interactions in modulating stem cell fate (28).

**Topography**

In vivo, cells are exposed to a variety of micro- (e.g., projections from neighboring cells) and nanosized (e.g., extracellular matrix proteins) topographical stimuli that influence cell shape, migration, proliferation, and differentiation (43, 44, 115). Cells interact with the topographical microenvironment through focal adhesions that prompt cytoskeletal remodeling and changes in gene and protein expression (115). Topographical cues have been used to direct the fate of various stem cell populations. For example, nanotubular-shaped titanium oxide surfaces were used to induce MSC differentiation into osteoblasts without exogenous osteogenic inducing factors (128). Specifically, MSCs cultured on small nanotubes (30 nm diameter) promoted adhesion and proliferation, while larger nanotubes (70 to 100 nm diameter) resulted in pronounced cell elongation, which resulted in cytoskeletal stress and differentiation in to osteoblasts. In a similar study, the influence of surface topography on the osteogenic differentiation of MSCs was studied in a more comprehensive manner. MSCs were cultured on polystyrene methacrylate substrates functionalized with nanopits arranged in disordered and organized patterns (45). Minimal cell adhesion was observed on highly ordered or disordered nanotopographies, while substrates with slight deviations from ordered patterns, similar to those that exist in vivo, promoted extensive osteoblastic differentiation. In fact, MSCs cultured on these slightly disordered nanotopographies (in the absence of osteogenic stimuli) differentiated to osteoblasts at a similar efficiency as cells cultured with osteogenic media.

The influences of topographical features on cell fate have been studied in other stem cell populations as well. Tsuruma et al. (153) found that NPC differentiation, measured by Nestin and MAP2 expression, could be controlled by altering the pore size on honeycomb surfaces. Similarly, modulation of surface roughness alone directed the neuronal differentiation of NPCs (11). For future translational studies, nanotopography will be an important engineering consideration when designing im-
planted devices that require recruitment or delivery of stem cell populations for tissue repair.

Electrospinning, which uses an electrical charge to displace fine fibers of ECM from liquid preparations, has emerged as a method to generate scaffolds that have a topography similar to that of native ECM (101). Moreover, it has been shown that electrospun scaffolds mimic the scale and 3D arrangement of the collagen and laminin fibrils of basement membranes (101, 125). For example, electrospinning was used to generate meshes of laminin with fiber size, geometry, and porosity similar to that of the native basement membrane. These scaffolds increased AdSC attachment and allowed for their differentiation to neural like cells in the absence of exogenous factors. In a related study, Christopherson et al. (30) demonstrated that the fiber diameter influences the fate of NSCs. Specifically, small nanofibers, which mimic a glial-like morphology, result in a differentiation biased toward an oligodendrocyte fate. On the other hand, scaffolds composed of larger fibers, which restricted cell spreading along a single fiber, result in more neuronal differentiation. In another study, Chua et al. (31) demonstrated that electrospun nanofiber scaffolds, with a topography similar to that of bone marrow, significantly improve the expansion of HSCs compared with culture on tissue culture polystyrene. Interestingly, scanning electron microscopy revealed discrete colonies of HSCs proliferating and interacting with the nanofiber scaffold in a manner that was not observable on standard tissue culture polystyrene (31).

Unlike other methods to generate stem cell attachment substrates, electrospinning enables the deposition of fibers in highly aligned patterns. This is particularly advantageous for efforts in engineering stem cell-based anisotropic tissues such as skeletal muscle, ligaments, cartilage, and blood vessels. In fact, electrospun scaffolds have proven an effective means to stimulate stem cell alignment as well as directionality of cell growth in stem cell-seeded scaffolds (101). For example, fiber-aligned scaffolds improved the chondrogenic differentiation of MSCs as assayed by higher glycoaminoglycans and collagen II expression (164). Additionally, such aligned scaffolds resulted in in vivo tissue regeneration with improved mechanical properties (87, 142). Along similar lines, electrospun nanofibers have been used as substrates to improve the cardiac differentiation of MSCs (151).

**Dimensionality**

Because of the difficulty in recapitulating 3D cell microenvironments, most studies examining the influence of microenvironmental parameters on stem cell fate have used flat two-dimensional (2D) cultures. However, in vivo stem cells reside within a complex 3D niche that plays a significant role in regulating cell behavior (141). Signaling and other cellular functions, such as gene expression and differentiation potential, differ in 3D cultures compared with 2D substrates (39, 40, 133). Recent engineering advances have made it possible to mimic the in vivo 3D stem cell microenvironment in vitro.

Several studies have demonstrated that the proliferation and self-renewal is improved in 3D culture systems. For example, self-renewal of mouse testicular germ cells was enhanced when these cells were culture in a 3D soft agar matrix (111). Along similar lines, culture of NPCs in a 3D collagen gel allowed for long-term survival and proliferation (66). Moreover, addition of neuronal induction factors to this 3D culture system resulted in a neural-like tissue construct with a morphology and cell composition similar to that of native neural tissue. Recently, a novel 3D culture system allowed for the expansion of single LGR5+ intestinal stem cells into long-lived gastric organoids resembling mature pyloric epithelium (12, 13).

The influence of 3D culture systems on stem cell differentiation has been examined by several groups. For instance, chondrogenic differentiation and functional maturation of MSCs was improved in 3D agarose cultures (114). Furthermore, mechanical loading of MSCs in these 3D cultures improved glycosaminoglycan production and aggrecan transcription activity (113). Likewise, NPC showed increased differentiation in 3D spheroid cultures, which mimicked the in vivo tissue architecture, compared with NPCs cultured in traditional 2D systems (107).

Recently, technological advances in computer-aided scaffold design and 3D printing have allowed for the precise spatial placement of cells and protein in 3D structures that mimic the 3D in vivo microenvironment (19, 120). For example, 3D printing was used to generate homogenous layers of human cardiac-derived cardiomyocyte progenitor cells, which enhanced the expression of early cardiac transcription factors in these cells (59). Similarly, the printing of NSCs in a growth factor-containing 3D scaffold allowed for sustained growth factor release and improved NSC growth. 3D printing can also be used to engineer complex, multilayered tissue constructs composed of different types of cells. For example, Xu et al. (167) printed amniotic fluid-derived stem cells, SMCs, and ECs in a multilayer matrix as method to generate vascularized bone tissues.

**CELL-CELL INTERACTIONS INFLUENCE STEM CELL BEHAVIOR**

Stem cells can interact with the various cell types of the niche through physical interactions (e.g., tight junctions, cadherins, Notch signaling) and other paracrine signals (139). These cell interactions not only serve to physically retain the stems cells within the niche but also provide signaling cues that dictate stem cell fate (139). Recent studies in model organisms have shown that these cell-cell interactions within the niche regulate cell polarity, division, differentiation, and ultimately migration out of the niche (112). Several engineering approaches have been used to examine cell-cell interactions in stem cell cultures.

**Cell Density as a Mechanism to Instruct Stem Cell Fate Decisions**

The simplest way to examine cell-cell interactions in directing stem cell behavior is through modulation of the cell density. For example, Purpura et al. (135) found that osteoprogenitor self-renewal was enhanced at high plating densities. Along similar lines, osteogenic and adipogenic differentiation of MSCs was found to be regulated by cell-cell contact (149). Interestingly, novel imaging analysis revealed that the extent of differentiation was linearly related to the number of directly neighboring cells (149). In another study, cell density regulated the lineage specification of cortical stem cells. Interestingly, cortical stem cells, which typically differentiate to neurons, astrocytes, and oligodendrocytes in high density cultures, dif-

Physiol Genomics • doi:10.1152/physiolgenomics.00099.2013 • www.physiolgenomics.org
ferentiated only to SMCs when cultured at lower densities (152). Bioengineering approaches have also been applied to modulate the cell-cell interactions that occur in vitro. One novel approach was the use of silicon combs to precisely regulate the cell density and cell-cell contacts between heterogeneous cell types (83). It is anticipated that such technologies can be used to create more complex in vitro microenvironments that more closely resemble those that occur in vivo.

Notch Signaling as a Main Regulator of Cell-Cell Communication

Of the many cell-cell communication mechanisms, the Notch signaling pathway is one of the prominent and widely studied mediators of cell-cell interactions within the stem cell niche (25). For example, specification of neuronal ganglion cells from a multipotent stem cell population that resides in the retina is mediated by Notch signaling (8). Notch signaling also regulates stem cell behavior in the niches of the ovaries (146), dental pulp (121), muscle (36), brain (77), and bone marrow (159).

Notch-mediated cell-cell signaling occurs when the Notch receptor presented by one cell interactions with the Notch ligands (e.g., Delta-like ligand, Jagged) presented by a neighboring cell. Therefore, most approaches to examine Notch signaling in vitro have used techniques to immobilize Notch ligands on various substrates. For example, Liu et al. (104) engineered “artificial niches” that incorporate the active domain of various Notch ligands for culture of NPCs. In another study, Notch ligands were coupled to magnetic microbeads by streptavidin-biotin binding and antibody-antigen coupling (150). These beads were then used to efficiently generate T cells from HSCs.

EMERGING TECHNOLOGIES: THE USE OF HIGH-THROUGHPUT, MULTIFACTORIAL TECHNOLOGIES TO CONSTRUCT IN VITRO MICROENVIRONMENTS

The various microenvironmental components discussed in this review do not act individually but rather in a complex combinatorial manner to influence stem cell fate. Therefore, high-throughput, combinatorial technologies have emerged as a means to study and engineer complex in vitro microenvironments (Fig. 2).

Combinatorial protein microarrays have been used to investigate the complex interactions between immobilized ECMPs and soluble signaling molecules on stem cell fate (21). These microarrays consist of an inert substrate (e.g., poly-dimethylsiloxane or functionalized glass microscope slides) where micrometer volumes of biologically active molecules have been deposited with microcontact printing in specific locations. The arrays can then be seeded with stem cells and analyzed for changes in gene or protein expression. Combinatorial microarrays have been used to investigate the effect of combinations of microenvironment components on the maintenance and differentiation of ESCs (23, 24, 56), as well as proliferation and differentiation of NPCs (145), human mammary gland progenitor cells (98), and hepatic stellate progenitor cells (22). These studies revealed previously unknown interactions that occur between insoluble matrix proteins and soluble signaling molecules in regulating stem cell fate. For example, by using arrays of combinatorial signaling microenvironments, Soen et al. (145) identified previous unknown synergistic and antagonistic interactions between WNT, BMP, and Notch signaling in regulating NPC fate. Along similar lines, LaBarge et al. (98) used protein microarrays to determine that the effect of Notch signaling on mammary gland progenitor cells is ECMP dependent.

Biomaterial arrays have been effectively used to systematically identify specific biomaterials that influence stem cell fate. For example, an array-based biomaterial screen was used to identify specific chemical groups that direct MSC differentiation (17). Interestingly, using this approach Benoit et al. (17) determined that t-butyl-modified surfaces direct adipocyte differentiation of MSCs, while phosphate surfaces favor an osteoblast fate. Biomaterial arrays have also been used to screen libraries of polymers with varying wettability, surface chem-
Cytokines, and elastic modulus to develop substrates with the precise physiochemical properties to support the growth and differentiation of various stem cell populations. For example, Anderson et al. (4) used biomaterial arrays to screen a library of 3,456 unique polymer combinations to identify biodegradable polymers that support the growth and expansion of MSCs and NPCs.

Microwell arrays are an emerging technology that has been used to investigate the effect of multiple biophysical stimuli, cell-cell interactions, and soluble cues on stem cell fate in a high-throughput manner. Using soft lithography techniques, microwell arrays can be fabricated with hundreds to thousands of small wells with defined heights and diameters. The defined well size allows for precise control over the number of cells in each well (95). Microwell arrays have been used to precisely control the fate of several stem cell populations (15, 37, 85, 92, 97, 109, 122). For example, microwell arrays have been used to study the role of spatial interactions and cell density in determining HSC fate (97). Microwell arrays have also been used to study the combinatorial effect of cell density, substrate rigidity, and soluble proteins on HSC (109), MSC, and NPC fate (69). Using this technology, Gobaa et al. (69) were able to engineer specific microenvironments to direct the adipogenic or osteogenic differentiation of MSCs.

Several high-throughput microfluidic platforms have emerged as a means to investigate the influence of mechanical and chemical stimuli on stem cell fate (155, 168, 170). Tumarkin et al. (154) developed a microfluidic device that allows for the coencapsulation of stem cells and various growth factors. The authors used this technology to screen the effect of signaling molecules on the fate of blood progenitor cells. In another high-throughput application of microfluidics, Gómez-Sjöberg et al. (70) engineered a screening platform that allows for the simultaneous analysis of various cell densities and media composition on the proliferation and osteogenic differentiation of MSCs. More recently, a similar microfluidic device enabled the parallel analysis of 1,600 cell culture conditions on single HSC proliferation (99).

These emerging technology platforms have allowed for the high-throughput, combinatorial study of various components of the stem cell microenvironment such as the ECM, chemical stimuli, matrix stiffness, cell density, and dimensionality. Future development of high-throughput technologies will be important to understand the myriad of factors that govern stem cell fate. Moreover, these technologies will enable rapid construction of complex stem cell microenvironments that are critical to advancing stem cell research and current reductionist approaches.

CONCLUSIONS

To realize the broad scientific and clinical potential of adult stems will require methods to generate the mature cell types that comprise the adult human body. Generating these cell types will require precise control over the numerous physical, biological, and chemical factors that regulate their fate. The various engineering approaches presented in this review will allow for the dissection of the various microenvironmental components and signaling pathways that modulate stem cell fate. It is conceivable that in the near future that these systems will allow for the step-wise engineering of in vitro stem cell microenvironments that precisely mimic their in vivo niche counterparts.

GRANTS

D. A. Braffman was supported by funding from the UCSD Stem Cell Program, a gift from Michael and Nancy Kaehr, and the California Institute for Regenerative Medicine (RT2-01889 and RB3-05086).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: D.A.B. prepared figures; D.A.B. drafted manuscript; D.A.B. edited and revised manuscript; D.A.B. approved final version of manuscript.

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