Progress and Prospects for Stem Cell Engineering

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Abstract

Stem cells offer tremendous biomedical potential owing to their abilities to self-renew and differentiate into cell types of multiple adult tissues. Researchers and engineers have increasingly developed novel discovery technologies, theoretical approaches, and cell culture systems to investigate microenvironmental cues and cellular signaling events that control stem cell fate. Many of these technologies facilitate high-throughput investigation of microenvironmental signals and the intracellular signaling networks and machinery processing those signals into cell fate decisions. As our aggregate empirical knowledge of stem cell regulation grows, theoretical modeling with systems and computational biology methods has and will continue to be important for developing our ability to analyze and extract important conceptual features of stem cell regulation from complex data. Based on this body of knowledge, stem cell engineers will continue to develop technologies that predictably control stem cell fate with the ultimate goal of being able to accurately and economically scale up these systems for clinical-grade production of stem cell therapeutics.

INTRODUCTION

mESC: mouse embryonic stem cell

Pluripotency: the ability to self-renew and differentiate into all cells of the adult organism

hESC: human embryonic stem cell

Induced pluripotent stem (iPS) cell: differentiated cells reverted to a pluripotent state by ectopic introduction of reprogramming factors

Embryonic stem (ES) cells: cells isolated from the inner cell mass of a blastocyst

Extracellular matrix (ECM): a scaffold of polysaccharides and glycoproteins that provide instructional cues and adhesive support for resident cells in tissues

HT: high-throughput

Stem cells are characterized by the abilities to proliferate while maintaining a primitive state (self-renewal) and to differentiate into one or more specialized lineages (potency). These cells exist throughout the adult body in numerous tissues including the brain, muscle, adipose/fat, and tissues of the hematopoietic system, where they were first discovered in 1963 (1). Such adult stem cells are multipotent, or capable of differentiating into multiple cell types that are generally restricted to those of their local tissue. In 1981, mouse embryonic stem cells (mESCs) were successfully cultured and demonstrated pluripotency, the capacity to generate all cell types of the adult organism (2), and in 1998, human embryonic stem cells (hESCs) were successfully derived from blastocyst-stage embryos (3). Most recently, induced pluripotent stem (iPS) cells with properties similar to embryonic stem (ES) cells were generated by the overexpression of four transcription factors that can collectively drive a differentiated cell back to a pluripotent state (4). iPS cells may bypass potential ethical challenges associated with hESC research because they are not derived from embryos.

Because they can be expanded and differentiated into cells of therapeutic interest, stem cells are highly promising for the development of cell-based models of human disease and for cell replacement therapies to treat such diseases. As an example of the former, iPS cells can be derived from skin cells of a Lou Gehrig's disease patient, then differentiated into neurons afflicted in Lou Gehrig's disease to study fundamental mechanisms of disease pathology and potentially serve as a screening platform for pharmacological and toxicity assays. For efforts in cell replacement therapies and regenerative medicine, stem cells can be isolated, expanded ex vivo, and differentiated to the desired precursor or lineage-committed cells prior to transplantation into patients. Bone marrow transplants were the first such successful therapies (5), and they are able to repopulate the hematopoietic tissues of cancer (leukemia) patients whose systems have been ablated through irradiation or chemotherapy. These highly successful therapies are now routine; however, progress in treating diseases and injuries of other tissue types requires overcoming numerous challenges. This review discusses engineering approaches that have been developed to surmount a major barrier in the field, the limited ability to control the behavior of stem cells outside the body.

In vivo, stem cells are regulated by specialized microenvironments that present them with numerous regulatory signals—in particular soluble signaling molecules, biophysical cues, cell-extracellular matrix (ECM) interactions, and cell-cell contacts—that are collectively referred to as the stem cell niche (6). Several engineering strategies have been developed to investigate mechanisms by which the niche controls stem cell behavior, including high-throughput (HT) technologies to identify factors and combinations of factors that modulate stem cell self-renewal and differentiation, as well as the development of mathematical models to elucidate fundamental mechanisms by which cells respond to microenvironmental signals. Furthermore, such basic information has been translated to create biomimetic or synthetic microenvironments to control cell behavior for biomedical applications, both at the laboratory and the bioprocess scale. This review therefore discusses recent progress in engineering efforts to discover, model, and manipulate stem cell regulatory mechanisms and behavior (**Figure 1**).

ENGINEERING HIGH-THROUGHPUT METHODS TO INVESTIGATE STEM CELL REGULATION

Two experimental approaches for in vitro exploration of factors that regulate stem cell fate are candidate analysis and unbiased library screens. The candidate approach—which investigates one or more factors likely to have an effect on cell function—has been prolific, but it is limited to known

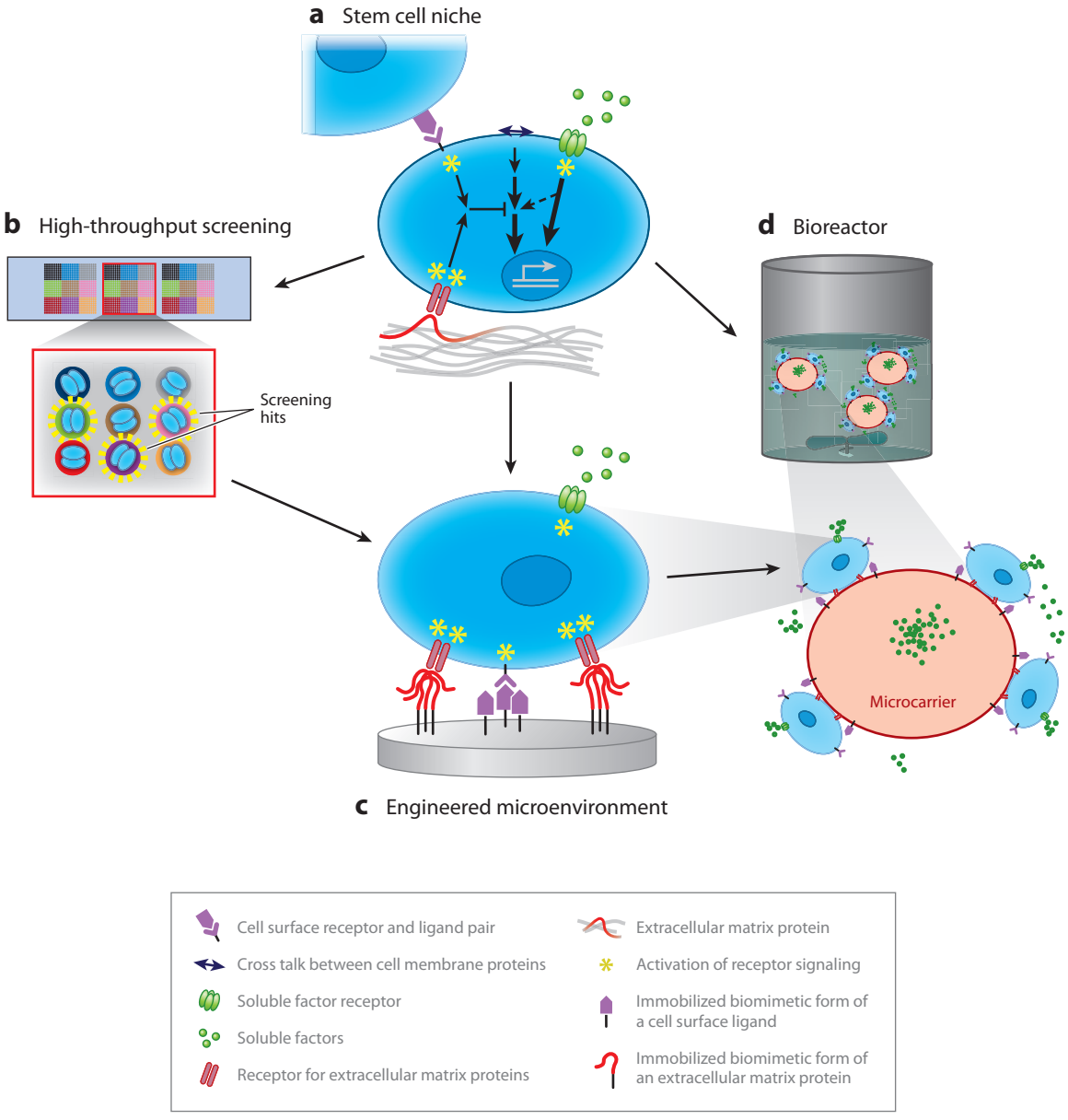

Figure 1

Engineering approaches for stem cell biology and therapeutics. Stem cells (*a*) process both biochemical and biophysical signals from adjacent cells, the extracellular matrix, and the soluble medium in their niche. The complex signal transduction and genetic networks (*black and gray arrows* inside cell, respectively) that process these microenvironmental signals to regulate self-renewal, death, or differentiation behaviors can be mathematically modeled to facilitate our understanding of stem cell biology. High-throughput screening technologies, such as seeding stem cells on arrays of micropatterned extracellular matrix proteins or synthetic polymers (*b*), promote the discovery of regulatory factors that can be applied in engineering synthetic microenvironments (*c*) to study and control stem cell behavior ex vivo. Knowledge gained about stem cell biology and microenvironmental factors from modeling and the use of engineered microenvironments will facilitate the design of bioreactors (*d*) for large-scale and clinical-grade stem cell therapeutics.

NSC: neural stem cell

factors, typically explores a relatively small set of candidates, can be laborious, and can require relatively large amounts of materials. In contrast, library approaches can explore the effects of numerous known and unknown factors as well as complex combinations of factors analogous to those encountered in endogenous stem cell niches, though they require the implementation of HT methodology. As described in the section below, microarrayed and microfabricated platforms, i.e., micropatterned surfaces and microfluidic devices, have HT potential while using minimal amounts of reagents (7).

Microarrays

Microarrays were developed as a platform for HT analysis of gene expression more than two decades ago (8). Since then, microarray technology has been used in many facets of cell biology and has served as the basis for live-cell microarrays, in which cells are cultured on top of microscope slides that have been patterned with high-density and typically robotically arrayed spots of target molecules (9). For example, lentivirus-infection cell microarrays are patterned arrays containing upward of ~5,000 different lentiviral vector clones, each encoding a unique cDNA or short-hairpin RNA sequence, and have been developed for HT genetic library screens in primary cell types (10). Although this technology has not yet been applied to genetic library screens in stem cells, it represents a promising HT method for elucidation of intracellular signaling factors that regulate stem cell fate.

In addition, cellular microarrays are powerful HT platforms for reductionist approaches that explore the combinatorial effect of specific microenvironmental factors on stem cell fate. For example, to investigate the effect of ECM proteins on hepatic differentiation, the Bhatia group (11) differentiated mESCs on arrayed combinations of ECM proteins spotted on cell adhesion–resistant, acrylamide hydrogel–coated slides. Automated data acquisition and analysis of the microarrays revealed that culture surfaces composed of collagen I and fibronectin vastly enhance early hepatic differentiation of mESCs. Extending ECM microarrays to investigate the effect of ECM protein–coated surfaces on hESC proliferation and pluripotency, Brafman et al. (12) discovered that culture surfaces coated with proteins adsorbed from equimolar solutions of collagen I, collagen IV, fibronectin, and laminin are both necessary and sufficient to support extended culture of hESCs. Finally, consistent with the previous study and recent efforts to develop fully defined synthetic culture surfaces for hESC long-term culture (see section on Engineering the Stem Cell Microenvironment below), Yang and colleagues (13) combined HT microarray screens, which explore the ability of libraries of acrylate-based polymers to support hESC culture, with HT surface characterization techniques (e.g., time-of-flight secondary ion mass spectrometry, water contact angle, atomic force microscopy) to elucidate structure-function relationships between chemical moieties in the acrylate-based polymer and the polymer's ability to maintain hESC pluripotency in culture. Using this HT screening and characterization platform, they recently demonstrated that specific acrylate-based polymer structures, which readily adsorb vitronectin from serum-containing culture medium, could support clonal expansion of hESCs (14).

For further recapitulation of the multifactorial complexity of endogenous stem cell niches, microarray platforms have also been developed to simultaneously test the effect of ECM proteins and soluble growth factors on stem cell fate. For example, microarrays of ECM proteins, soluble growth factors, and recombinant cell adhesion molecules were used to investigate signaling pathways that regulate the neuronal differentiation of bipotent human neural stem cells (NSCs). Interestingly, Wnt3a signaling was found to be neurogenic, and Notch signaling drove glial differentiation; yet, Notch ligands and Wnt3a in combination apparently offset one another's effect and maintained NSCs in an undifferentiated state (15). More recently, the Bhatia group (16) adapted

their ECM microarrays to a microwell format that facilitated parallel assessment of mESC cardiac differentiation in 240 unique ECM protein/growth factor microenvironments. These studies not only supported the established cardiogenic effect of Activin-A/bone morphogenic protein-4 media conditions, but they also revealed nonintuitive agonistic and antagonistic cross talk between ECM protein and growth factor signaling pathways. Given the complex results that multifactorial microarrays can yield, computational modeling approaches may be valuable tools for investigating underlying signaling mechanisms (see section on Modeling Stem Cell Behavior below).

Microfabrication Using Soft Lithography

Similar to microarray technology, the development of soft lithographic techniques has spurred the creation of numerous micropatterned and microfluidic devices for miniaturization of cell biology assays. Soft lithography techniques, pioneered by the Whitesides group (17, 18), are a set of tools for engineering micrometer-scale patterns of complex biochemicals or cells on substrates using elastomeric materials—created with the aid of silicon-based photolithography—as pattern transfer agents. Soft lithography techniques are inexpensive, facile, and can be used to develop a variety of HT cell culture platforms. Their application to stem cell investigations is thus likely to expand.

Micropatterned surfaces synthesized using soft lithography techniques have been used as HT genetic library screening platforms and as culture surfaces that facilitate execution of single-cell or low–cell number experiments in an HT fashion. Unlike features created by microarrayers, soft lithography-patterned features can be generated on the micrometer scale and thereby facilitate the synthesis of micropatterned surfaces for HT biological studies at the single-cell level (**Figure 2*a***). For example, Ashton et al. (19) used microcontact-printed arrays of cell-adhesive

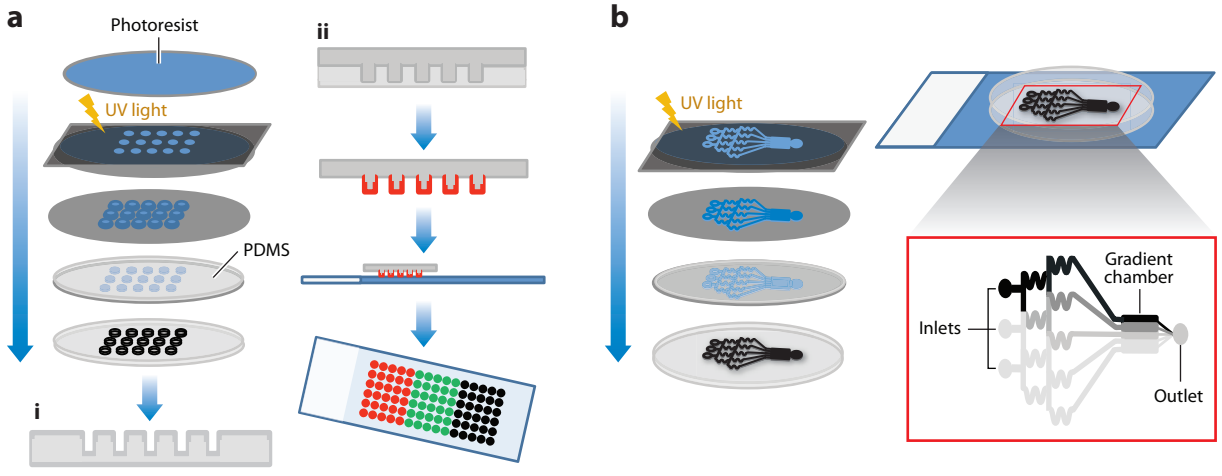

Figure 2

Soft lithography in stem cell research. Investigation of the myriad factors that influence stem cell fate can be enhanced through the use of soft lithography techniques. For example, microfabricated polydimethylsiloxane (PDMS) molds/stamps with micrometer-scale patterns can be used in soft lithographic techniques as pattern transfer agents to modify biosurfaces (microcontact printing) and regulate fluid flow (microfluidics). In brief, PDMS molds are fabricated by an initial lithography step that patterns photoresist onto a silicon wafer (*a*). Next, PDMS is cured on top of the patterned silicon wafer to create a soft, or elastomeric, micropatterned mold. The mold can then be used either directly as a microwell platform (*i*) or to form other PDMS stamps by replica molding, which can further transfer the patterns to culture surfaces by microcontact printing (*ii*). Finally, PDMS molds can be used to synthesize microfluidic devices, which could be used to generate microscale gradients of soluble factors (*b*) (18).

islands (20-μm diameter) to generate clonal microarrays—arrays of clonal cell populations derived from individual stem cells—for HT screening of genetic libraries in stem cells. As a proof of principle, clonal microarrays were used to demonstrate that overexpression of *Akt* increased NSC proliferation and to screen the NSC transcriptome for novel genetic sequences that regulate NSC proliferation.

MSC: mesenchymal stem cell

In addition to helping screen the effects of many factors on cell fate, micropatterned surfaces synthesized using soft lithography techniques can enable HT data collection as a function of several microenvironmental properties. For example, the Chen group (20, 21) used microcontact printing of fibronectin islands of various dimensions (1,024 or 10,000 μm^2) to create high-density single-cell arrays for investigating how the cross-sectional area (cell spreading) of human mesenchymal stem cells (hMSCs) affects their differentiation. Analysis of stem cell fate on these arrays under various differentiation conditions aided in elucidation of the role of cell shape/spreading in biasing hMSC osteogenic versus adipogenic (20) and myogenic versus chondrogenic (21) differentiation. In addition, in non-stem cell work, Nelson & Chen (22) decoupled cell-cell contact interactions from cell density and cell spreading using a micropatterned surface, an experimental paradigm not feasible in standard cell culture. The ability to pattern features appropriately sized to generate clonal populations or single-cell arrays is uniquely convenient using soft lithography and is expected to be increasingly utilized in stem cell investigations.

Although microfluidic devices have not yet been widely used for HT screens of factors that regulate stem cell fate, application of these methods in cell biology has enormous potential (23). The Whitesides group (24) pioneered the use of soft lithography techniques for microfluidics by fabricating a soluble factor gradient generator that can create gradients of soluble molecules on the scale of hundreds of micrometers (**Figure 2*b***). Over the ensuing years, microfluidic designs have become increasingly more sophisticated and allowed for the creation of spatial gradients and transient soluble factor exposure regimens in HT, massively parallel, miniaturized cell culture platforms (9, 25, 26). For example, the Quake group (25) recently designed a PDMS-based microfluidic chip for long-term, miniaturized (60 nl), HT stem cell culture. This technology was applied to study the osteogenic differentiation of hMSCs. Through HT investigation of a range of morphogen exposure periods (0–168 h), it was determined that 4 days was sufficient to induce osteogenic differentiation (25). Although additional microfluidic chip designs are at the proof-of-principle stage, the flexibility that microfluidics permit for exploring complex soluble factor conditions in an HT fashion is powerful (9, 26).

MODELING STEM CELL BEHAVIOR

HT methods facilitate the discovery of new regulators of stem cell fate, but often a deeper understanding of the corresponding molecular and signaling mechanisms is necessary to translate these discoveries into future therapeutics. Toward this end, engineers have applied systems biology and computational modeling techniques to enhance our understanding of stem cell biology. A primary focus of such models is on intracellular signaling networks, the stem cell's "computer processor." Stem cell fate choice is governed by complex intracellular signaling networks that process input signals from the cell surface and relay those signals to the nucleus. These signaling cascades may contain nonlinear components such as signal amplification, oscillation, feed-forward or feedback loops, and cross talk between multiple pathways (**Figure 1**). Once inside the nucleus, signal processing continues with circuits of transcription factors that control the expression of one another in addition to genes regulating fate choice. The resulting network is a complex, nonlinear, multilevel cascade that is difficult to investigate and understand without the aid of systems-level analysis and mathematical tools.

Multiple classes of computational models have been used to analyze stem cell signal processing networks. Deterministic, stochastic, and attractor state models summarize our knowledge of the system into formal mathematical statements, thereby highlighting gaps in our knowledge and driving further experimentation. As complementary approaches, statistical methods such as Bayesian networks and principal components analysis (PCA)/partial least squares (PLS) regression mine large "-omic" data sets (e.g., transcriptomic, proteomic, kinomic) to identify genes and modules whose behaviors are correlated, thereby offering mechanistic hypotheses that can be further tested to deepen our understanding of these complex systems (27).

HSC: hematopoietic stem cell

Deterministic Models

Deterministic models express molecular interactions among microenvironmental inputs and intracellular signaling networks as mass action expressions, and the outputs of the model are time trajectories of the concentrations of network constituents as well as steady state behaviors. Such models utilize and require detailed knowledge of most constituent molecular interactions, including the appropriate kinetic and binding constants. Because such data can often be limiting owing to a lack of measured constants, estimation of these constants from analogous systems is often required. Additionally, these models assume that reactants are abundant and thus use sets of continuous ordinary or partial differential equation formulations, which are often nonlinear and thus typically are solved numerically.

Deterministic models have highlighted intriguing and unintuitive network behaviors in stem cell systems. For example, stem cells execute all-or-nothing fate decisions in response to microenvironmental cues. One network behavior that could mediate such a decision is bistability, in which an analog change in an input parameter results in an all-or-nothing binary change in an output parameter. These bistable networks also exhibit hysteresis, making them resistant to noise in the input signal and thus avoiding rapid or indecisive switching between cell states at levels of an input signal close to the threshold for switching network state. Bistability has been studied in several signaling networks that regulate stem cell fate, including the Sonic hedgehog (Shh) signaling pathway's (28) regulation of developmental pattern formation and adult NSC proliferation (29), the GATA-binding factor 1 (GATA-1)-PU.1 transcription factor network's control of hematopoietic stem cell (HSC) fate choice (30), and the Octamer-binding transcription factor 4 (Oct-4)-Sex determining region Y-box 2 (Sox2)-Nanog transcription factor network's maintenance of ES cell pluripotency (31). These models can provide hypotheses to motivate experiments. Furthermore, as experimental knowledge of systems advances—such as recent work showing the pluripotency network to be larger than initially thought (32–34)—models can serve as a living summary of current knowledge that can be progressively refined.

Additionally, other intracellular signaling cascades have also been described by deterministic models. Recent work investigated the dynamics of the Notch signaling pathway (36), which in some contexts functions as a switch to drive boundaries in developmental patterns (37, 38) and in other contexts as an oscillator that can contribute to somitogenesis during organismal development or adult NSC maintenance (39, 40). A mathematical model demonstrated that the Notch circuit can operate in either of these two paradigms depending on the value of a single parameter: the ability of a downstream transcription factor (Hes1) to repress expression of target genes including itself (36). The mitogen-activated protein kinase (MAPK) pathway downstream of neurotrophin-3 in ES cell–derived neural progenitors (41) and the Jak/Signal transducer and activator of transcription 3 (STAT3) pathway downstream of leukemia inhibitory factor (LIF) in ES cells have also been deterministically modeled (42, 43). The latter example highlights the potential of mathematical models to identify critical, potentially nonintuitive control points within a signaling network that

could be manipulated to improve ex vivo production of cellular therapeutics (43; see 44 for a review).

Stochastic Models

Deterministic models assume that system states are uniquely determined by parameters of the model and the quantitative values of previous temporal states. However, many biological systems such as cells are characterized by slow biochemical reactions and/or low concentrations of reactants, resulting in a greater influence of fluctuations or noise (stochasticity) on signaling behavior. Early stem cell researchers studied the apparently stochastic nature of some stem cell fate choices (45–47); however, only recently have studies begun to apply molecular stochastic simulations to investigate the behavior of gene networks and biochemical reactions (48), and interest in the field has grown steadily (49, 50). As mentioned above, the Shh signaling pathway demonstrates bistable behavior. In addition to a deterministic model, stochastic simulations were also used to investigate the effects of noise near bifurcation points (28). This work demonstrated the ability of the Shh signaling 'switch' to resist noise and reliably direct stem cell fate.

Recent experimentation has shown that stochasticity may be important for cell fate determination. In fact, some stem cells seem to exist in multiple metastable states, and they are capable of switching between these (51), as investigated in both mESCs and HSCs. In one study, 80% of ES cells in culture expressed the transcription factor Nanog, and these cells were more resistant to differentiation. However, this same 80/20 distribution was reestablished when cells expressing low levels of Nanog were separated and cultured in isolation (52). A similar effect was seen upon separating HSCs into high and low Sca-1-expression populations, as after several doublings the initial Sca-l expression profile was reestablished. Although the precise molecular mechanism underlying this behavior is unknown, mathematical analysis indicates that stochastic effects in gene expression likely play a role (53).

Attractor State Models

A third type of model, the attractor model, posits that there is a stable state (or states) toward which a mathematical set of equations will converge. A useful heuristic is a potential landscape, in which the wells represent attractors, or states of equilibrium, toward which the system will move (54, 55). Use of these models to analyze genetic networks involved in stem cell fate choice only recently began with the analysis of the genetic state of cells during neutrophil differentiation. The authors showed that the transcriptional profiles of differentiating cells followed different trajectories but converged to a relatively common state despite different external cues driving differentiation (56). Attractor states were also used to analyze the GATA-1/PU.1 system mentioned above. The result indicated that attractor landscapes are malleable and capable of changing throughout the differentiation process, resulting in the gain or loss of attractor states (35). However, subsequent modeling has shown that there is likely a third, unknown cofactor that is important for the switch-like behavior seen in the GATA-1/PU.1 network (57). Further use of this type of model should prove useful in understanding the networks underlying stem cell fate choice, particularly in iPS cells as they revert to an ES-like state.

Statistical Models

The above analyses are useful for studying cell responses to the microenvironment provided the responsible signaling pathways are well understood. However, in many instances a researcher is

faced with analyzing a large "-omic" data set with little or no knowledge of the critical signaling network(s). Statistical models such as Bayesian networks and PCA/PLS can mine these data sets to identify tractable candidate principal components, or signaling modules, that aid in data analysis and potentially highlight unintuitive network behaviors worthy of further experimentation.

Bayesian networks can help reverse engineer causal relationships between measured quantities in a large data set. This analysis results in a graphical map representing the likelihood of finding a species in a particular state given the states of the surrounding species. At times the resulting network yields results that would have been difficult to uncover through typical reductionist experimental approaches, and these results can drive more experimentation. For example, this technique has been used on an mESC proteomic data set consisting of the levels and phosphorylation status of numerous signaling molecules in response to varying levels of fibroblast growth factor 4 (FGF4), the cytokine LIF, and the ECM molecules laminin and fibronectin (58). Although no assumptions were made about the structure of the underlying signaling network responsible for transmitting signals from these microenvironmental cues, the Bayesian analysis highlighted the importance of the extracellular signal-regulated kinase (ERK), MAPK/ERK kinase, and LIF/Jak/STAT3 pathways and were thus in good agreement with prior knowledge of key ES cell signaling networks. Additionally, previously unknown molecular interactions were highlighted and subsequently confirmed by experiment, such as the importance of α-Adducin for differentiation (58).

PCA and PLS are also used to analyze large data sets (59). If each measured quantity within a data set is an axis of the signaling space encompassing the entire data set, then PCA reduces the number of axes to several key or principal components. Each principal component is a new axis representing a linear combination of the signaling axes that have the highest covariance with one another. This reduces the data space to only a few dimensions, which simplifies data analysis. Within the stem cell field, PCA has been used to analyze gene expression patterns in cells of varying potency, including ES cells during development (60) and NSCs undergoing differentiation (61). Each of these studies identified a principal component axis composed of a set of genes indicative of the cell's potency. Similar analyses of other stem cell types could reveal genes that were previously not known to play a role in stem cell fate choice.

PLS, an extension of PCA that predicts relationships between independent and dependent principal components, has also been used to analyze stem cell fate. Using the same data set as above (58), mESC differentiation and self-renewal were correlated to the phosphorylation state of multiple signaling molecules (62). This analysis indicated that protein kinase C (PKC)ε may have an effect on the proliferation of differentiated cells, a result that was previously unknown. The authors went on to experimentally confirm the effect of PKCε, which indicated that these statistical techniques can be powerful tools for identifying important molecular effectors by analyzing large data sets from stem cell signaling networks. These studies have thus opened fresh avenues of research that will lead to a better understanding of how stem cell fate decisions are controlled.

ENGINEERING THE STEM CELL MICROENVIRONMENT

HT technologies that can identify novel factors or combinations of factors that regulate stem cell function, and the development of models to describe or even predict stem cell behavior in response to key signals, are two areas in which numerous engineers have offered important insights into regulatory functions of the stem cell microenvironment. In parallel, engineering methods to qualitatively mimic or reconstruct the stem cell niche enable mechanistic analysis of how key features of the niche regulate cell fate as well as aid the creation of culture systems for biomedical application. Because individual stem cell microenvironments are biochemically and biophysically complex, the

development and design of systems to explore their structure-function relationships are challenging. Furthermore, these microenvironments are highly variable, as stem cells reside in different tissues during all stages of development, from germ layer segregation during embryogenesis and tissue formation during development to declining niche properties in aged tissues. In each niche, myriad ECM macromolecules and resident cells interact in unique ways to shape its biochemical properties—such as the identities of natural and synthetic ligands and their spatial/architectural presentation—as well as its biophysical properties—such as modulus, topography, dimensionality, and shear/strain. Researchers have recently engineered material systems with the capacity to quantitatively tune one or more of these regulatory features in a modular manner, thus enabling detailed mechanistic and reductionist biological investigation of how individual properties of complex stem cell microenvironments impact cell function.

Topography: surface shape and microscale geometrical features

hPSC: human pluripotent stem cell

Biochemical Regulation

In general, biochemical properties confer specificity to interactions in biological systems that are crucial for developing and maintaining the structure and function of organisms, tissues, and cells. Within the niche, these biochemical properties include the molecular identities of ECM components, soluble factors, or cell-surface factors. Past and recent work has elucidated the identities and roles of small, often soluble protein factors in stem cell systems, such as Wnt proteins (63), insulin and fibroblast growth factors (64), and cytokines (65, 66). This important work has been extensively reviewed elsewhere (67, 68). However, in addition to the identities of biochemical factors and their specific effects on stem cells, the contextual presentation of these moieties, including potential immobilization and spatial organization on scaffolds or particles, have been engineered into stem cell culture systems and shown to affect cell behavior.

Adhesive ligands. Signals that promote the anchoring or localization of stem cells to their proper niche are critical for maintaining their stemness. The importance of adhesive signals in vivo was observed in nonhuman primates when injection of blocking antibodies against $\alpha4\beta1$ integrin, known to be expressed on HSCs and to bind to fibronectin (69) and the cell-surface sialoglycoprotein vascular cell adhesion molecule 4 (VCAM-4) (70), mobilized CD34+ hematopoietic progenitors and granulocyte/macrophage-colony-forming cells to the bloodstream (71). Numerous in vitro studies have made considerable progress in examining the roles of adhesive ligands such as laminin, fibronectin, and collagen (15, 69, 71, 72). However, each of these proteins is highly intricate and often exhibits both multiple isoforms (e.g., laminin has at least 15 known trimer isoforms) and numerous cellular receptor binding motifs per isoform, making it difficult to elucidate precisely what biochemical information an ECM molecule is conveying to a cell. Increasingly, engineered systems have aimed to dissect specific cell-ECM interactions by incorporating individual ECM-based motifs or peptides, rather than full-length proteins, into synthetic materials.

For example, there has been considerable recent progress in the development of defined pluripotent stem cell culture systems. When they were first derived, hESCs and iPS cells were cultured on feeder cell layers, which provided complex and initially undefined components to maintain pluripotency. Subsequent progress has led to the current standard for cell culture, a defined liquid medium and a substrate coated with Matrigel (73), a highly complex mixture of mouse tumor-derived protein. Recent work showed that $\alpha V\beta3$, $\alpha6$, $\beta1$, and $\alpha2\beta1$ integrins functionally contributed to hESC attachment to Matrigel (74), and a subsequent study narrowed down the components of Matrigel required for supporting long-term human pluripotent stem cell (hPSC) cultures to the 511 isoform of laminin. Function-blocking antibodies further showed that culture

on laminin-511 was dependent on α6β1 integrin (72). Interestingly, cells cultured on laminin-511 formed monolayer cultures as opposed to colonies, while retaining their pluripotent properties, suggesting that adhesion to laminin-511 is stronger than that to surrounding cells and that this adhesion is sufficient to maintain pluripotency. hPSCs are also capable of long-term culture on other adhesion motifs, including vitronectin (14, 75) and vitronectin- and bone sialoprotein–derived peptides conjugated to synthetic polymer coatings (76), as well as bare synthetic ammonium- and sulfate-containing acrylate-based polymer coatings (77). Interestingly, the chemical nature of the synthetic surface can affect how biological adhesion motifs are absorbed or attached and thus impact the quality of adhesion and ability of completely dissociated hPSCs to survive (14), a challenge encountered widely in the field. Additionally, αVβ5 integrins were found to facilitate hPSC attachment to vitronectin (14, 75), suggesting either that ECM binding to at least a couple different adhesion receptors is capable of maintaining pluripotency, or that different cell lines utilize distinct integrins for adhesion and maintenance. Future studies comparing across multiple cell lines and ECM motifs may elucidate the combinatorial factors and receptors required, and indeed may demonstrate that multiple conditions are permissive, for maintaining pluripotency.

Immobilization of growth factors and morphogens. The ECM in most tissues and cell culture systems functions to promote cell adhesion to the solid phase, and growth factors, morphogens, and cytokines added in solution are typically thought of as signaling from the liquid phase. However, in many cases the latter naturally adsorb to the solid phase. In natural systems, proteins such as Hedgehogs (78), FGFs (79), transforming growth factors (TGFs) (80), and many others have matrix-binding domains, and even synthetic polymer coatings can selectively absorb ECM factors from serum (14). Therefore, in general the ECM presents an even more complex repertoire of biochemical signals. Several studies have immobilized growth factors and morphogens to synthetic matrices and thereby increased the potency of their effects on stem cells. For example, Shh covalently linked to a polymer hydrogel surface promoted the osteogenic differentiation of hMSCs (81), whereas LIF conjugated to thin film polymer coatings supported mESC pluripotency for 2 weeks without soluble LIF (82). Likewise, immobilized epidermal growth factor (EGF) sustained MAPK kinase-ERK signaling in hMSCs and promoted greater cell spreading and survival over hMSCs cultured on unfunctionalized substrates in the presence of greater (and saturating) levels of soluble EGF (83).

Spatial presentation of regulatory factors. Immobilization of factors also allows for their micro- and nanoscale spatial organization, as seen in numerous signaling systems including the clustering of Eph/Ephrins (84), T cell receptors (85), Hedgehog proteins (86), Notch and its ligands (87), neuroligin (88), and others. One of the first examples of engineering spatial control was the clustering of factors with antibodies or by absorption onto beads. Such nanoscale clustering of ligands, and subsequently their cognate receptors on a target cell surface, may aid in receptors dimerizing with and transactivating neighboring receptors, in increasing local intracellular concentrations of signaling effectors, and in facilitating force transmission to membrane-bound proteins (89). In numerous stem cell systems, clustering of the Notch ligand Delta is necessary for Notch activation (90, 91). For example, in neural crest stem cell cultures, antibody-clustered Delta inhibited neuronal and promoted glial differentiation (92). Similarly, immobilization of Delta on a cell culture substrate or beads is necessary for downstream Notch signaling in other stem cell systems including T cell differentiation from HSCs (93) and the activation of hematopoietic cord blood progenitor cells for subsequent engraftment in bone marrow (94). Clustering may also enhance signaling, as observed with enhanced osteogenic differentiation and angiogenesis in the presence of higher valency forms of Shh molecules conjugated to polymer backbones (95).

In addition to nanoscale organization, microscale patterning of adhesive or signaling factors regulates cellular shape, cytoskeletal organization, subcellular localization of proteins, and organelle localization. Engineered systems based on technologies such as microcontact printing (see section on Microfabrication Using Soft Lithography above) have exploited this axis of control to alter stem cell shape. For example, Chen and colleagues (20, 96) patterned small and large islands of adhesive protein on a 2D surface and found that small, round hMSCs preferentially differentiate into adipocytes, whereas spread cells differentiate into osteoblasts (20); furthermore, early changes in cell shape and cytoskeletal organization are predictive of MSC-derived lineages (96). Interestingly, for multicellular stem cell aggregates, shape control regulates their spatial differentiation patterns through a mechanical mechanism. Cells on the convex edges of patterned aggregates experience high tension and differentiate into osteoblasts, whereas those on the concave or low-tension edges generate adipocytes (97). These studies demonstrate the interdependent nature of the geometry of presentation of a material's biochemical properties and the mechanical effects it can exert on stem cells, which is discussed in greater detail below.

Biophysical Regulation

Stem cell niches are incredibly diverse biochemically; however, there are many accompanying differences in the biophysical properties of niches. Most apparent are differences in stiffnesses and topographies of different tissues as well as the forces imparted during the natural motions of organisms including joint bending, muscle contraction, compressive impact and strain on tissues, and pulsatile flow of the circulatory system. Even early in development and embryogenesis, significant forces are generated during cell adhesion and migration (98). These observations strongly suggest that biophysical niche properties also regulate stem cell behaviors. Recently developed engineered microenvironments can qualitatively and quantitatively emulate many biophysical properties of natural microenvironments and enable reductionist studies of their effects on stem cell behavior.

Stiffness. Many of the first engineered microenvironments mimicking the high water content of natural tissues were hydrogels composed of natural ECM polymers such as collagen and hyaluronan. However, synthetic materials such as polyacrylamide and poly(ethylene glycol) provide several advantages over natural ones, including the ability to generate a wide range of possible stiffnesses (in 2D: 10–10^6 Pa) while maintaining constant biochemical properties and remaining nonfouling to noncovalently linked ECM motifs. In landmark work, Engler and colleagues (99) created collagen I–functionalized polyacrylamide gels that mimicked the stiffnesses of bone, muscle, or neural tissue, and hMSCs cultured on these gels preferentially differentiated into the corresponding specialized cell types. NSCs are also mechanosensitive, as they differentiate primarily into neurons on soft hydrogels and astrocytes on stiff ones (100). This finding has been extended to 3D, as NSCs embedded in an alginate gel of variable stiffness exhibited analogous behavior (67). These results indicate that stiffness is a design parameter that can be exploited in materials to control stem cell behavior.

Shear and strain. In addition to static biophysical properties such as stiffness, engineered microenvironments can also impart dynamic forces on stem cells. For hESCs cultured on elastic polymeric membranes, cyclic stretching inhibits differentiation through the upregulation of TGFβ1, Activin A, and Nodal and the subsequent phosphorylation of Smad 2/3 (101). In contrast, when cyclic stretch was applied locally to the surface of mESCs by magnetically twisting a 4-μm diameter arginine-glycine-aspartic acid-coated bead bound to the cell surface, expression of the pluripotency marker Oct3/4 was significantly reduced (102). Shear flow, most often associated in vivo with the circulatory system, is another form of dynamic force application. Recent studies

have shown that shear flow can induce differentiation of mouse MSCs (103) and mESCs (104, 105) into specialized endothelial and cardiovascular cells. Furthermore, shear flow is crucial for the proper development of HSCs in zebrafish embryos. North and colleagues (106) demonstrated that blood flow activated nitric oxide signaling necessary for hematopoiesis in the embryonic aorta-gonad-mesonephros (AGM) region of zebrafish. Moreover, in a miniaturized in vitro flow chamber, Adamo and colleagues (107) observed that mESCs cultured under shear flow expressed higher levels of CD31 and Runx1, proteins expressed in endothelial cells, and generated more hematopoietic colony-forming units. Similar to the in vivo zebrafish study, inhibition of nitric oxide production abrogated this shear flow effect.

Topography. In addition to mechanical properties such as stiffness, shear, and strain, other biophysical properties, including structural characteristics such as topography, also regulate stem cell behaviors. Topographical information in natural systems, such as the fibrous structure of ECM proteins and the pores in bone marrow, motivates the use of technologies such as soft lithography, microfluidics, electrospinning, and nanostructure deposition (23) to engineer a material's topography to study stem cell responses.

In one recent example, Oh and colleagues (108) deposited vertically oriented nanotubes and found that hMSCs cultured on top of nanotubes 70–100 nm in diameter but not <30 nm induced hMSCs to differentiate into osteoblasts in the absence of osteogenic media. Interestingly, hMSCs cultured on nanopits of the same length scale (~100 nm) also induced osteogenesis in the absence of osteogenic media (109). The specific distance between features may reflect the distance between adhesion clusters, with greater distances requiring hMSCs to stretch and generate higher internal tension, potentially mimicking the effect of a larger ECM island (20) or stiffer ECM (99). As discussed above, researchers have made significant advances in engineering both the biophysical and the biochemical properties of the microenvironment to regulate stem cell behavior at the laboratory scale. These engineered systems and the conceptual discoveries they have elucidated about stem cell regulation will likely aid in and inform the development of large-scale and clinical-grade bioreactors by providing both useful structure-function relationships and fabrication technologies that can be scaled up appropriately.

STEM CELL BIOREACTORS

As discussed above, in recent years stem cell scientists have discovered a vast number of microenvironmental cues that modulate and control the expansion and differentiation of stem cells. However, the majority of studies have been performed at the research laboratory scale, and the basic information they have yielded must be translated toward the design of scalable, safe systems for clinical applications (110). Approximately 10^9 cells would be required to regenerate one patient's cardiac tissue after a myocardial infarction (111) or to convey insulin independence to a 70-kg diabetic patient (112). Following the guideline that standard suspension bioreactors can produce cultures of 10^6–10^7 cells ml^{-1}, culture volumes of hundreds of milliliters to one liter would be required per patient, assuming complete homogeneity of the product cell population can be achieved (113). To design bioprocesses capable of producing therapeutic cells at this scale for numerous patients in a cost effective, pathogen-free, and reproducible manner, it is imperative that materials used for stem cell culture and differentiation are fully defined and produced via synthetic or recombinant means, e.g., no feeder cell layers, conditioned media, or animal or human-derived serum or proteins (see section on Engineering the Stem Cell Microenvironment above) (110). In addition, stem cell bioreactors will require control of parameters not traditionally considered during bench-scale tissue culture, e.g., dissolved oxygen, pH, and agitation-induced

shear (113). For example, stem cells in the developing embryo and in the adult brain function at oxygen levels much lower than those of standard culture conditions, and oxygen levels are known to regulate stem cell proliferation and differentiation in vitro (113).

Stirred suspension bioreactors (SSBs) are the traditional workhorse of the biomanufacturing industry, and these have been utilized in several impressive stem cell bioprocesses, as recently reviewed (44, 113, 114). Although considerable advancements have occurred in single-phase SSB, which generally contain only culture media and cells, the inherent heterogeneity of local microenvironments inside cultured cell aggregates, e.g. embryoid bodies (EBs), remains a major hurdle for producing homogenous cultures of terminally differentiated cells. Therefore, it is not yet apparent whether the appropriate level of control over the fate of hESC or h-iPS cells, collectively called hPSCs, can be achieved using the standard single-phase SSB (113). There has also been recent progress in the development of microcarriers and hydrogels for hPSC culture, and such two-phase SSB systems offer the opportunity to present instructive cues from both the liquid and solid phase (115, 116). This section will discuss current SSB designs for hPSC culture and therapeutic cell derivation with emphasis on evaluating the bioreactor's suitability for clinical-scale cell production.

Large-Scale Production of Pluripotent Stem Cells

Unlike mESCs, which can be seeded into bioreactors as single cells (117, 118), most hPSC cell lines are characteristic of more mature mouse epiblast stem cells and as a result exhibit significant rates of apoptosis when cultured as single cells under standard conditions (119). Recently, inhibition of p160-Rho-associated coiled-coil kinase (ROCK) during the first six days of hESC single-cell suspension culture was observed to reduce apoptosis and permit subsequent cell proliferation and formation of EBs—spherical aggregates of PSCs often used in initial stages of differentiation—with culture cell numbers reaching ~65% of the initial inoculated cell number after six days of culture. Although a sizeable fraction of the seeded cells still underwent apoptosis with ROCK inhibition, in its absence cell survival dwindled, as only 7.7% of the initially seeded cells remained viable after six days (120). Several groups have utilized ROCK inhibition and demonstrated long-term expansion of hPSCs in single-phase suspension culture using media supplemented with animal-derived ECM proteins (121) as well as defined culture conditions (122, 123). These methods are significant advancements toward the development of single-phase bioreactors for large-scale production of PSCs; however, 30–50% of the cell culture is still lost during subculturing, which must be performed at least weekly to limit the development of larger cell aggregates (>500 μm in diameter) that result in spontaneous cell differentiation and promote cell death due to limited oxygen and nutrient diffusion (121–123). Additional progress in cellular engineering may further alleviate cell viability problems. Recent molecular interventions—specifically the inhibition of ERK and glycogen synthase kinase (GSK) 3β and stimulation with LIF and Forskolin (2i/LIF/FK)—have been shown to aid in the reversion of the human epiblast-like cells into a naive mESC-like state and thereby facilitate single-cell seeding and expansion of hPSCs in culture (119). Although the 2i/LIF/FK cues only transiently support the naive hPSC state (up to 15–20 passages), they could potentially serve as the basis for large-scale expansion of hPSCs in single-phase SSB systems.

Several groups have developed two-phase SSBs for hPSC expansion using cylindrical or spherical microcarriers, which increase the bioreactor's available culture surface area (113). Two-phase microcarrier bioreactors achieve high culture cell densities, e.g., 10^6 cells ml^{-1} (124), while maintaining the pluripotent state of the stem cells. However, currently the translational potential of such protocols may be limited owing to the need to coat microcarriers with ECM proteins such as collagen and Matrigel to promote cell adhesion. Similar to single-phase SSBs, two-phase microcarrier SSBs also suffer from loss of significant cell numbers during subculturing. Even when cells

are subcultured as small aggregates in microcarrier protocols, reseeding a new batch of microcarriers with cells can occur at a low efficiency, e.g., ~30% (124). Development of microcarriers with novel 2D synthetic culture surfaces that support long-term hPSC culture (see section on Engineering the Stem Cell Microenvironment above) and improvement of microcarrier seeding protocols may help alleviate these issues and facilitate development of fully defined two-phase microcarrier SSBs (**Figure 1**) (110, 125).

Although EB and microcarrier technologies have been widely investigated for PSC culture in SSBs, these methods expose the cells to the fluidic environment of the bioreactor and thus to shear stress and conditions that may permit aggregation of cell clusters. Increased shear stress or cell cluster aggregation can negatively affect proliferation rates, but reducing the agitation speed to avoid these outcomes compromises optimal gas and nutrient transfer rates (113, 115, 126). As an alternative to cell adhesion to the exterior of solid carriers, cell microencapsulation within hydrogels can physically isolate proliferating cell clusters from the bioreactor's fluidic environment, and several studies have explored their use for hPSC expansion (115, 127). Using conditioned medium, static cultures with hyaluronic acid–based hydrogels were found to limit EB formation and actively promote the pluripotent state of encapsulated hESCs (128). Also in static culture, alginate-based hydrogels were able to limit cluster size and maintain the pluripotency of encapsulated hESCs over extended periods in defined culture conditions (129). In SSBs using conditioned medium, agarose hydrogels supported the expansion of encapsulated hPSCs, which eventually outgrew the hydrogel's boundaries if permitted (115). Alternatively, alginate-poly-L-lysine hydrogels with a liquefied core permitted hPSC expansion while limiting cell aggregate size to space within the hydrogel capsule (127). The choice of microencapsulation technique may vary for different processes to achieve promising results, but regardless of the technique, hydrogel pore size, porosity, and mechanical properties will have to be engineered to achieve the desired hPSC growth profiles.

Large-Scale Differentiation of Pluripotent Stem Cells

Although stem cells can be effectively differentiated in 2D static culture, bioreactor protocols for derivation of therapeutic progeny from hPSCs typically employ EB cultures in single-phase SSBs because these 3D systems are more readily scalable (113, 130–132). However, EBs can become resistant to inward diffusion of morphogenic factors present in the culture media owing to development of an exterior epithelial-like cell layer that forms tight cell-cell junctions and deposits an exterior basal lamina (133, 134). As a result, hPSCs in EBs undergo spontaneous and relatively uncontrolled differentiation into cell derivatives of the three embryonic germ layers (endo-, meso-, and ectoderm), such as neural, hematopoietic, endothelial, cardiac, or pancreatic cells (130–132). Several recent studies have attempted to engineer increased homogeneity into the complex milieu of EBs to improve control over hPSC fate. For example, EB size has been shown to inherently bias germ layer–specific and even lineage-specific differentiation of constituent hPSCs (135–137). Several techniques—including EB formation on micropatterned surfaces (135–137), in hydrogel microwells (138), and by forced aggregation in welled plates (139, 140) or in soft-lithography fabricated microwells (141)—have been developed to create EB populations of low polydispersity within a predetermined size range.

In addition, methods for controlled release of soluble patterning factors from coembedded microparticles are also being developed to enhance the homogeneity of the EB microenvironment (142–144). In a powerful display of engineering spatial differentiation of hPSCs in EBs, controlled release of retinoic acid from embedded poly(lactic-co-glycolic acid) microspheres was shown to induce cavitation of EBs, resulting in a definitive epithelial cell layer encompassed by a visceral endoderm layer, a structure reminiscent of 3D germ layer organization during early embryonic

development (142, 143, 145). In single-phase SSBs, the combination of control over initial EB size and the release of morphogens from embedded microparticles may be sufficient to effectively instruct lineage-specific differentiation of hPSCs in EB cultures, as both of these technological advancements shift the efficiencies of EB-mediated differentiation protocols closer to the higher efficiencies achieved in static monolayer culture systems (146–148).

Differentiation protocols using two-phase microcarrier SSBs are a viable alternative to monolayer culture and EB protocols due to their scalability and efficiency. Two-dimensional monolayer cultures offer the benefit of exposing cells to more uniform conditions, and differentiation of hPSCs on microcarriers may combine this advantage with scalability. For example, recent microcarrier SSB protocols have been reported to differentiate hPSCs into definitive endoderm (124) and cardiomyocytes (126) at efficiencies of 80% and 20%, respectively, which is comparable with monolayer culture efficiencies (148, 149). Modification of microcarrier surfaces with synthetic polymers known to support hPSC culture would further enhance the scalability of two-phase microcarrier bioreactors for differentiation of hPSCs (14, 76, 77).

Finally, two-phase hydrogel microencapsulation SSBs may afford increased control of hPSC fate because hydrogels can be engineered to present fate-instructive ligands to encapsulated cells in 3D fashion, as compared with standard culture's 2D exposure of cells to ligands only on their basal surface. Differentiation of hPSCs encapsulated in alginate, hyaluronic acid, and agarose hydrogels into definitive endoderm (bench-scale) (150) and cardiomyocytes (in SSB) (127), endothelial cells (bench-scale) (128), and hematopoietic progenitors (in SSB) (115) has been demonstrated. However, because these hydrogels were not engineered to present adhesive ligands, morphogenic cues, and/or optimal pore sizes, the encapsulated cells grew as EB structures, and thus differentiation efficiencies in these protocols were similar to those achieved in EB cultures (115, 127). Further elucidation of endogenous niche factors that instruct lineage-specific differentiation of hPSCs will likely benefit such efforts. In particular, future development of microcarriers and hydrogels for hPSC SSBs will likely aim to create cellular microenvironments that more effectively control hPSC fate by incorporating controlled release of small molecules and morphogens, immobilization of ligands that activate efficacious cell signaling pathways, and materials designed to produce optimal mechanical properties (see section on Engineering the Stem Cell Microenvironment above).

CONCLUSION

As stem cell fields increasingly mature, political and economical considerations are progressively encouraging the translation of stem cell science toward biomedical technologies that increasingly support a stem cell–based regenerative medicine industry. Stem cell engineers are uniquely qualified to contribute to this challenge owing to their development of a broad array of tools to discover, model, manipulate, and scale up regulatory features of the stem cell microenvironment. Continued development of such tools will benefit basic research on as well as clinical applications of stem cells.

A continual hindrance to advances in stem cell biology and biotechnology has been the inability to directly compare and translate experimental results owing to the use of ill-defined culture reagents and substrates. However, with the development of fully defined, synthetic substrates and media for stem cell cultures, stem cell researchers increasingly should begin to conduct experiments in culture environments with precisely defined features of the stem cell microenvironment. As demonstrated by numerous studies in this review, cells sense and respond to features on the micrometer scale; therefore, homogeneity in the quantitative, qualitative, and temporal properties of microenvironmental factors between various experiments will facilitate data comparison and modeling efforts.

In addition, as HT platforms for exploration of the myriad factors that regulate stem cell fate become more prevalent, the utility of computational models for analyzing large data sets and extracting nonintuitive concepts of stem cell biology will become increasingly important. Current modeling efforts often focus on defining relationships between perturbations in microenvironmental factors and the resulting changes in intracellular signaling networks and stem cell gene expression, yet comparatively less attention is given to modeling the metabolic changes that will inevitably accompany differentiation. Future models of stem cell differentiation may benefit from inclusion of metabolic pathways. Furthermore, as technology permits the generation of complex tissues containing multiple differentiated cell types derived from hPSCs, models of population dynamics and intercellular interactions will become increasingly important to predict spatial and temporal perturbations in cellular microenvironments during tissue formation.

In closing, future research endeavors will undoubtedly necessitate collaborations between researchers in the natural sciences and engineers not only to develop technologies with more advanced experimental and analytical capabilities but also to engineer accessible, robust, and economically tractable discovery systems. These same criteria will be required in the translation of stem cell discoveries to treatments for patients, and communication of ideas and knowledge between stem cell engineers, biological manufacturing engineers, and medical professionals will be crucial for the successful realization of stem cell therapies in the future. Thus, for both basic research and clinical applications, advancements in discovery, modeling, and stem cell microenvironment engineering technologies will be indispensably intertwined.

SUMMARY POINTS

1. Engineered HT platforms, such as microarrays, micropatterned surfaces, and microfluidics, are extremely helpful for investigating the myriad factors that regulate stem cell fate.
2. Systems biology and computational approaches for modeling stem cells facilitate understanding these complex and nonlinear systems and elucidating nonintuitive interactions between intracellular signaling pathways.
3. Engineered materials provide exquisite control over the biochemical and biophysical aspects of the stem cell microenvironment, and they can be used to reveal the molecular mechanisms that regulate stem cell fate.
4. For clinical-scale production of stem cell–derived therapies, single-phase and two-phase bioreactor designs will need to incorporate the aggregate knowledge obtained from stem cell studies, assisted by HT screening, computational approaches, and engineered cell microenvironments, to control stem cell fate effectively.

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