From Nano to Macro: Multiscale Materials for Improved Stem Cell Culturing and Analysis

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Stem cells respond to nanoscale, microscale, and macroscale cues, such as matrix, growth factors, and niche organization, which are difficult to physiologically recapitulate in culture. We discuss how utilizing bioengineering approaches to manipulate and integrate spatiotemporal cues across these discrete length scales can improve traditional methods for controlling cell fate.

Circumstances dictate an individual's sensitivity and responsiveness to his or her environment; this statement is equally true for a stem cell. In recent decades, pioneering research has elucidated how stem cells respond to their direct microenvironment. A key finding from these experiments is that stem cell behavior is dictated by the integration of signals that occur across multiple spatial and temporal scales (Figure 1). Despite numerous breakthroughs in our understanding of the biological cues that drive cell behavior, the commonly used in vitro platforms for culturing stem cells and studying their behavior have remained mostly unchanged. In this Forum article, we advocate for the integration and use of multiscale bioengineered constructs, which intentionally incorporate nanoscale, microscale, and macroscale features, into stem cell culture systems. Specifically, we argue that engineering specific aspects of the stem cell microenvironment across these length scales provides advantages for efficiently culturing stem cells and directing their behavior.

Advantages of Multiscale Control of Cellular Environments to Improve Stem Cell Cultures

Conventional in vitro experimentation involves the removal of stem cells from their natural and biologically complex environment and culturing them in artificial systems such as 2D tissue culture dishes or simple hydrogels, which lack the complexity of the endogenous stem cell niche. Most often, non-physiological concentrations of stem cells are seeded on top of stiff tissue culture plastics to form monolayers that are covered with a disproportional volume of culture medium, leading to rapid dilution of secreted factors. These artificial environments are prone to causing aberrant stem cell behavior, and many in vitro findings are unique to the experimental settings in which they are performed. For example, hematopoietic stem cells (HSCs) have the ability to self-renew extensively in vivo, while only having limited self-renewal capacity in vitro.

We argue that developing and integrating novel technologies to control cellular environments at multiple length scales will help to align stem cell behavior in vitro and in vivo. In vivo, stem cell behavior is directed across multiple length scales (Figure 1). For example, gradients of molecules, local substrate stiffness, nanoscale architecture of the surrounding matrix, microscale spatial arrangement of cells relative to their neighbors, and physiological crosstalk between organs jointly orchestrate cell behavior. Furthermore, stem cell microenvironments are naturally dynamic, necessitating temporal control to truly mimic niches in vitro. Thus, incorporating dynamically controllable multiscale features into in vitro environments provides significant opportunities to improve stem cell culture systems.

Although major advances in engineering materials for stem cell culture platforms have been reported, these approaches often focus on manipulating single and specific aspects of the microenvironment. For example, tunable hydrogels with user-defined stiffness for directing lineage commitment during differentiation, controlled-release approaches for delivering soluble factors, or patterned substrates to control cellular architecture offer useful solutions to specific questions, but provide limited control over other factors. Instead, integrating multiple approaches to coordinate multiparametric control of culture environments provides an enhanced ability to control stem cell behavior (Figure 2A). Although historically appreciated, such integration may finally be possible due to the many recent advances made in both the biological and bioengineering communities. Here, we provide our perspectives on the emerging directions provided by various multiscale bioengineering approaches, particularly with respect to advances in biomaterials and biofabrication techniques, which will enable the development of the next generation of stem cell culture platforms.

Materials for Integrating Temporal Control of Cellular Environments

Cells are presented with a relatively static environment in many tissue culture systems. This absence of temporal control provides challenges for recapitulating the dynamic nature of natural tissues in vitro. Recently, several extraordinary biomaterials whose properties, for

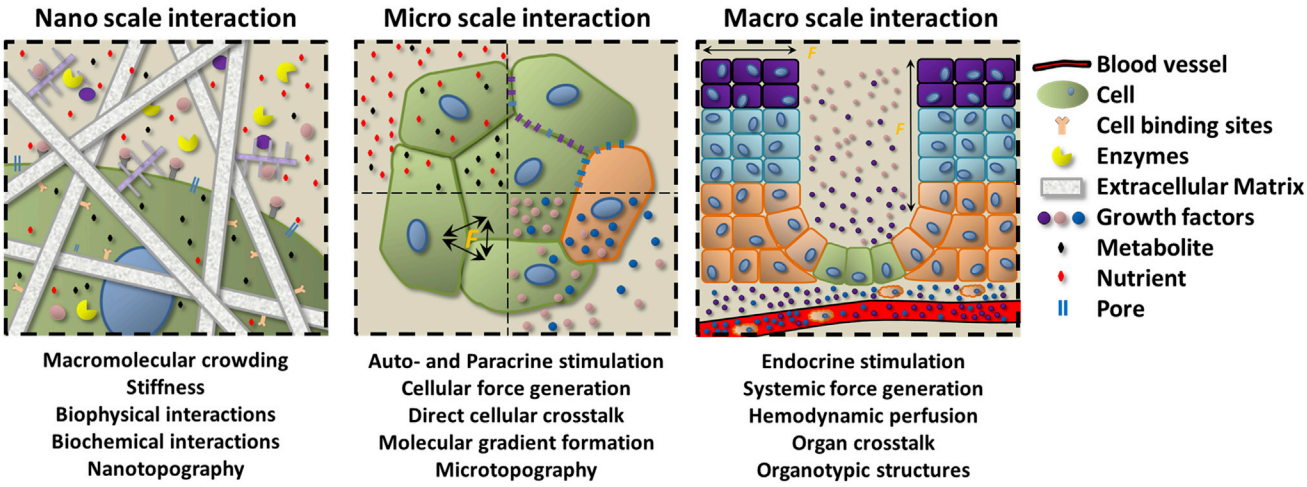

Figure 1. Schematic Depiction of Nanoscale, Microscale, and Macroscale Interactions, which Are Temporally Regulated

example stiffness, can be altered dynamically have been reported. These biomaterials are useful tools for addressing unresolved questions about the influence of microenvironment stiffness on the behavior of stem cells during wound healing, embryonic development, or cancer progression. For example, a recent study reporting on the development of a hydrogel with temporally controllable stiffness revealed that stem cells possess a mechanical memory of their past physical environments, which affected their future cell fate decisions (Yang et al., 2014). When tuned to possess a stiffness of 10 kPa, this culture substrate activated yes-associated protein (YAP) in human mesenchymal stem cells and induced osteogenic induction. YAP nuclear translocation was reversed when the hydrogel was photolytically degraded to provide a softer elasticity of 2 kPa. However, prolonged culturing on the stiff hydrogel led to irreversible YAP activation. This remarkable finding emphasizes that continuous control over the stem cell's environment is highly desirable to achieve more predictive and controllable outcomes. This discovery also indicates that some of the currently used in vitro stem cell culture systems may intrinsically alter cell fate directions. For example, isolating stem cells by adhesion to plastic culture dishes could potentially alter their behavior. Thus, hydrogels with dynamically adjustable stiffness present an exciting opportunity to continuously and optimally direct stem cell behavior.

Exciting advances in dynamic regulation of other factors in stem cell culture systems have also been recently reported. Novel biomaterials have been recently developed that allow induced presentation of peptides, following exposure to an external stimulus such as a change in temperature, electromagnetic field, or light. Recently, the inducible expression of the adhesion peptide RGD was achieved via a chemical modification with photolytic 3-(4,5-dimethoxy-2-nitrophenyl)-2-butyl ester, which effectively caged the peptide until exposure to UV (350–365 nm) light (Lee et al., 2015). As a demonstration of the applicability of this system, the caged RGDs were incorporated into a hydrogel that could be transdermally activated to uncage the RGD domains (Figure 2B). Following subcutaneous implantation, dynamic presentation of RGD by the biomaterial promoted enhanced cell adhesion at wound sites, as well as vascular invasion, and mitigated fibrous encapsulation of the implanted material that might otherwise block regenerative responses. The ability to dynamically regulate the expression of bioactive factors represents a major advancement toward engineering dynamic stem cell environments. Numerous protocols, such as the differentiation of induced pluripotent stem cells (iPSCs), typically rely on multistep and time-sensitive sub-protocols that require subjecting cells to a regime of timed growth factor supplementation. This approach is effective for cell monolayers but less so for 3D cultures due to lower rates of diffusion, and it is generally incompatible with in vivo manipulation of experimental cell populations. Therefore, temporal control through inducible expression of bioactive elements could drive the translation of current 2D protocols into clinically relevant 3D approaches. Furthermore, this methodology for dynamic control of cell culture components could be expanded beyond peptides to release other types of molecules, for instance drugs, or to regulate gene expression through activation of RNAi and CRISPR-Cas9 systems.

Integrating Spatial Heterogeneity into Cell Culture Environments

In addition to temporal cues, spatial cues encompassing nanoscale to microscale features play a crucial role in tissue organization and behavior. While many 3D culture systems, such as hydrogels, provide environments that aim to mimic the chemical composition of the native extracellular matrix (ECM), these systems do not recapitulate the spatial heterogeneity of the cellular microenvironment. In vivo, tissue microarchitectures contain directionality, gradients, and unique compositions, often in a repetitive manner that regulates function. We therefore argue that the integration of spatial heterogeneity will yield more natural environments for directing stem cell fate decisions.

Bioengineers have developed several relatively facile approaches designed to control the spatial placement of biomaterials, bioactive cues, or cells. All of these approaches present environments that improve control over stem cell behavior.

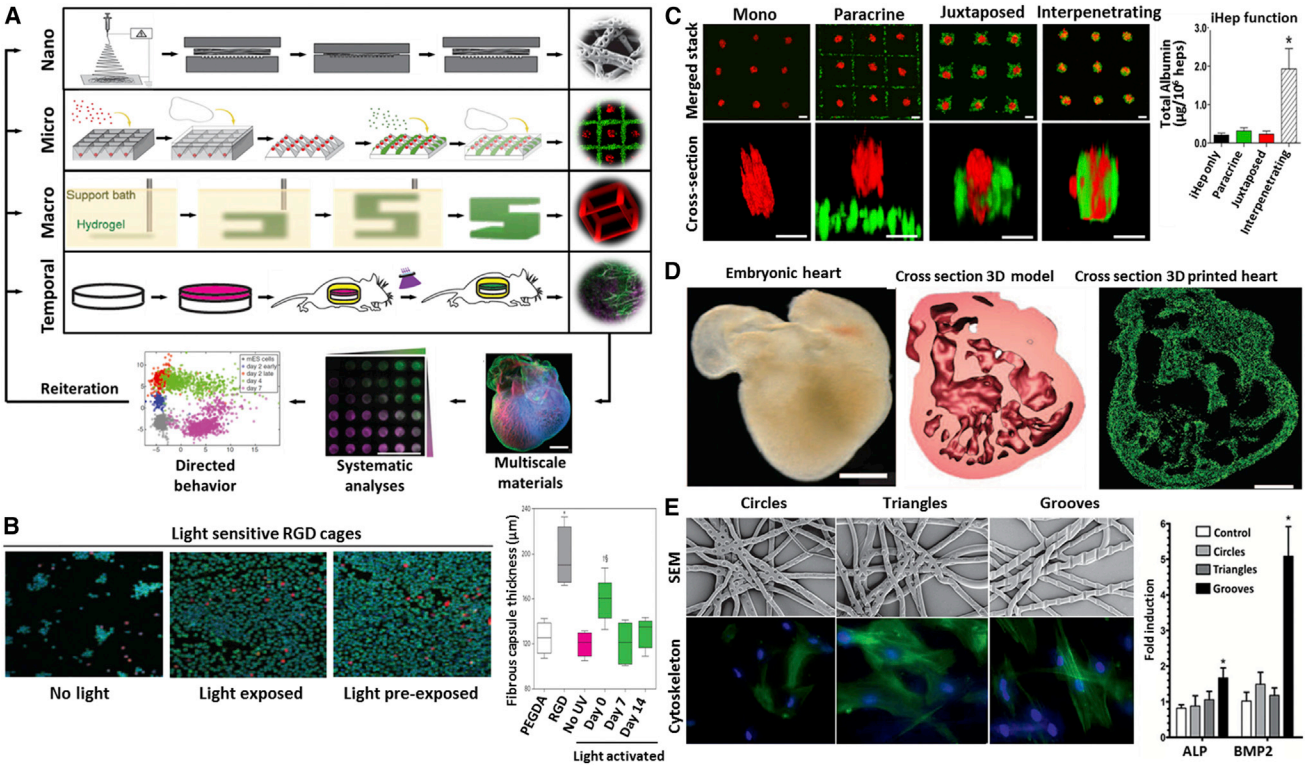

Figure 2. Multiscale Biomaterials to Control Stem Cell Behavior

(A) Integrating technologies and approaches for optimizing desired features across a range of length scales, while providing temporal control of selected parameters, will generate robust multiscale materials to improve stem cell culture platforms. High-throughput analyses to study how encoded features perturb cellular environments will enable systematic and rigorous investigations into stem cell behavior and facilitate iterative design of biomimetic materials. Specifically, cellular behavior can be guided using (B) temporal control, e.g., light-triggered expression of adhesion moieties, e.g., RGDs, to recruit cell populations such as neutrophils (green) and macrophages (red) in a temporal manner, which can control the formation of the fibrous capsule in vivo; (C) microscale control, e.g., micropatterning to harness the microtopography and cell placement in co-cultures to enhance the function of assembled microtissues such as albumin production by iPSC-derived hepatocyte-like cells (red) and stromal cells (green); (D) macroscale control, e.g., 3D printing to recreate organ shape and tissue function such as embryonic hearts with a complex internal trabecular structure; and (E) nanoscale control, e.g., nanotopographies to steer stem cell behavior such as the osteogenic differentiation of human mesenchymal stem cells on grooved electrospun fibers. Reproduced with permission (Gandavarapu et al., 2014; Hinton et al., 2015; Klein et al., 2015; Lee et al., 2015; Nandakumar et al., 2013; Stevens et al., 2013). Reprinted with permission from AAAS.

In one example, aggregates of induced pluripotent stem cell-derived hepatocytes were patterned in an array of pyramid-shaped microwells (Stevens et al., 2013). The resulting microtissues were retrieved when the microwells were filled with hydrogel, which was subsequently cross-linked, detached from the microwells, and cultured further, generating engineered tissues up to centimeters in diameter. Microtissues derived from different cell types can be layered to create complex tissues with additional levels of spatial organization, and these multiscale manipulations allow thorough investigation of the effects of cell placement and density on the function of the engineered tissues, as well as analysis of mixed, juxtaposed, and paracrine co-cultures (Figure 2C). These design parameters are inherent to stem cell microniches but were previously often difficult or labor intensive to investigate or even discern in vitro.

In addition to defined placement of cells, spatial positioning of distinct biomaterials around individual stem cells or cell aggregates can be used to introduce asymmetrical environments and induce changes in cell polarity and differentiation. This was demonstrated by encapsulating embryoid bodies at the interface of two distinct hydrogels (Qi et al., 2010) to induce discrete differentiation patterns in defined regions of the same cell aggregate. This approach can be used to model several embryological processes ranging from early events such as blastocyst polarization to later events such as limb formation.

Patterning ligands within a single biomaterial is another method for specifying spatial organization. This can be achieved by using patterned light to conjugate photosensitive molecules to materials or surfaces. Although this often generates a static environment, a recent study reported a reversible strategy for the spatiotemporal patterning of bioactive elements using an allyl sulfide modified hydrogel (Gandavarapu et al., 2014). This culture system allows the patterned attachment, removal, and reattachment of biochemical ligands. We predict that methodologies such as this, which integrate spatial and temporal cues, will likely prove instrumental to more precisely control stem cell fate decisions. In particular, this general approach will be of great value for studies that aim to explore changes in cellular behaviors or phenotypes, such as cancer development or stem cell differentiation. Importantly, it can be argued

that an engineered environment with tunable spatial and temporal control transforms the investigator from an observer into an experimenter with active control over the culture environment.

Cell cultures can also be patterned at the macroscale level, in addition to the patterning techniques discussed above. 3D printing is exceptionally well-suited to produce complex biological macrostructures (Figure 2D). Hydrogels can be deposited in patterns to form biological structures that would not otherwise be possible, such as trabeculated embryonic hearts (Hinton et al., 2015). By combining 3D printing with a multi-nozzle approach, complex co-cultures displaying anatomical organization can be created. For example, trabecular long bone containing stem-cell-laden bone marrow compartments might be produced with such approaches. Continued development and integration of multiscale patterning techniques therefore may provide rich opportunities to generate and study structures in vitro that anatomically, and perhaps functionally, resemble physiological organs.

Biomaterials to Mimic Nanoscale Environmental Features

Despite the many advances in engineering biomaterials for culturing stem cells, most of these biomaterials do not mimic the nanoscale architecture of natural ECM. Membrane proteins enable cells to sense, interact, and respond to their biochemical and biophysical environment at the nanometer scale. In fact, stem cells have been shown to respond to features as small as 8 nm, and the absence of precise nanoscale cues in vitro may contribute to inefficiency in controlling stem cell behavior in such artificial environments.

The nanoscale elements of the ECM, such as directionality, orientation, and nanotopography of individual fibers, can direct stem cell attachment, alignment, migration, and differentiation through altering focal-adhesion-mediated mechanotransduction. This lesson can be applied to stem cell culture systems by incorporating organized arrays of micropatterns and nanopatterns on 2D substrates or 3D microfibers and nanofibers (Nandakumar et al., 2013) (Figure 2E). We anticipate that the use of engineered nanofibrilar materials constructed with molecular precision will enable a range of novel applications. Peptide structures can be engineered to promote self-assembly into nanofibers with tunable biomechanical and biochemical signaling properties in user-defined patterns, with nanoscale precision. For example, DNA nanotubes can be functionalized to present bioactive peptides. This approach allows the uncoupled tuning of the biomaterial's nano-architecture and peptide-based bioactivity to synergistically and simultaneously control and probe stem cell behavior (Stephanopoulos et al., 2015). Another emerging avenue of research is the generation of multiscale topographies at microscales and nanoscales. Such hybrid approaches provide another level of physical control over stem cell behavior through the integration of custom-designed features.

Incorporating Systematic and High-Resolution Analyses

The development of advanced tools that move beyond population-level analyses and provide information at the single-cell level are required for us to develop a deeper understanding of individual stem cell fate decisions. Integrating complementary techniques to gain multiscale spatiotemporal control over a single cell's microenvironment would allow controlled fabrication of complex, functional, and biomimetic tissues in which stem cell behavior could be studied at this depth of resolution. Doing so, however, demands systematic high-throughput analyses to assess large numbers of cells, due to cellular heterogeneity and the desire to study responses across a large number of integrated culture conditions.

Many high-throughput screening platforms rely on the formation of stem-cell-laden micromaterials using spotting, stamping, or microfluidic droplet-generating techniques. Although some advanced high-throughput screening systems can query stem cells within artificial environments with single-cell resolution (Gobaa et al., 2011), they analyze a relatively low number of biomarkers, which has limited our comprehensive understanding of individual stem cell behavior. To address this need, droplet microfluidics has been used to enable the affordable and simultaneous RNA-seq analysis of tens of thousands of individual stem cells (Klein et al., 2015). Genetic barcoding of cDNA isolated from each cell was accomplished in nanoliter droplets, and the resulting libraries were multiplexed and sequenced to provide detailed insights into stem cell behavior at extremely fine resolution. This approach might be adapted to simultaneously screen molecular responses of single stem cells in a library of multiscale microgels. This level of systematically mapping the effects of multiscale cellular environments at the single-cell level would represent a major breakthrough not only for regenerative medicine, but also for the in vitro testing of pharmaceuticals and many other applications.

Challenges to Adoption: Availability and Ease of Use

As more advanced tools and approaches to manipulate stem cells in vitro are developed, rapid integration and widespread adoption of such methodologies within the stem cell research community remain key challenges. This will be dependent on the acceptance and availability of such platforms and an understanding of the level of control and knowledge they can provide. Leveraging these platforms will require individuals and research teams to possess multi-disciplinary expertise in cell biology, chemistry, engineering, and materials science. To this end, the ongoing convergence of life sciences, physical sciences, and engineering will play a crucial role. Such shifts toward big science may benefit from the formation of larger and joint academic departments, research consortia, and funding opportunities.

Manufacturing many of the culture systems discussed in this Forum article currently requires acquisition or access to costly equipment or dedicated infrastructure. The transition toward cost-effective, robust, and facile approaches with low-cost thresholds is likely to expedite the rapid adoption of technologies that produce multiscale bioengineered constructs and promote their early adoption by a larger number of stem cell biologists. In turn, this may facilitate moving away from 2D culture platforms and usher in new methodologies for culturing stem cells, techniques for manipulating their fate, and directions for promoting regenerative medicine and human health.

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