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Emerging Strategies for Spatiotemporal Control of Stem Cell Fate and Morphogenesis

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Abstract

Stem cell differentiation is regulated by the complex interplay of multiple parameters, including adhesive intercellular interactions, cytoskeletal and extracellular matrix remodeling, and gradients of agonists and antagonists that individually and collectively vary as a function of spatial locale and temporal stages of development. Current approaches to direct stem cell differentiation focus on systematically understanding the relative influences of microenvironmental perturbations and simultaneously engineering platforms aimed at recapitulating physicochemical aspects of tissue morphogenesis. This review focuses on novel approaches to control the spatiotemporal dynamics of stem cell signaling and morphogenic remodeling to direct the differentiation of stem cells and develop functional tissues for in vitro screening and regenerative medicine technologies.

Directed stem cell differentiation

Pluripotent stem cells, including both embryonic (ESC) and induced pluripotent stem cells (iPSC), as well as multipotent mesenchymal (MSC) and hematopoietic (HSC) stem cells are of immense interest to the biological and biomedical communities due to their potential impacts across broad scientific and technical applications. The ability of stem cells to differentiate into a spectrum of relevant cell phenotypes has enabled their use in pharmacological screening platforms [1], to modulate endogenous regeneration [2], and for direct transplantation to restore cellularity to damaged tissues [1,3]. Directed differentiation approaches have traditionally focused on the delivery of soluble morphogens and/or the manipulation of culture substrates in two-dimensional, monolayer cultures, with the objective of achieving large yields of homogeneously differentiated cells. While such approaches can be directly implemented in high throughput screening of defined single or combinatorial environmental perturbations [2,4], a more complete understanding of stem cell niche complexity [5] motivates tissue engineering approaches to inform the development of physiologically relevant, biomimetic models of stem cell differentiation [6]. The capacity of pluripotent and multipotent stem cells to simultaneously differentiate toward multiple tissue-specific cell lineages (Figure 1A), has prompted the development of new

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objectives aimed to guide complex morphogenesis (Figure 1B) of functional tissue structures for the replacement or regeneration of damaged tissue [7]. This review focuses on novel strategies to direct stem cell differentiation via the development of technologies to create defined microenvironments with precise spatial and temporal control of signaling and morphogenesis. Such technologies are specifically focused on controlling the spatiotemporal delivery of exogenous factors, as well as manipulating the dynamics of endogenous cell signaling and morphogenic remodeling responses (Figure 2).

Temporal dynamics of stem cell differentiation

Most established protocols to direct the differentiation and subsequent maturation of stem cells rely on a series of morphogen addition steps, coincident with analysis of cell state via phenotypic marker expression [8]. Many such differentiation methods have been largely derived through the understanding of morphogen secretion and signaling dynamics that occur during embryonic development. For example, mimicking aspects of the HSC niche via hypoxia-driven differentiation toward endothelial and hematopoietic cell phenotypes elucidated the mechanisms whereby the developmental-stage specific levels of vascular endothelial growth factor (VEGF) and the receptors VEGFR1/2 (Flt-1/Flk-1) direct the specification of hemogenic mesoderm, which ultimately enables engineering approaches to control the differentiation of Flk-1+ populations in normoxic stem cell microenvironments [9]. Recent reports highlight the importance of manipulating signaling pathway dynamics, specifically through temporal control of pathway activation and inhibition during different stages of stem cell differentiation. For example, the specification of ESC-derived mesoderm toward cardiac lineages can be perturbed via temporal control of transforming growth factor β (TGF β) signaling; mouse ESCs exhibited a biphasic response, whereby early inhibition of TGF β signaling by proteasomal degradation of the receptor TGF β R2 inhibited cardiac differentiation and receptor degradation between days 3–5 enhanced cardiomyocyte specification [10]. The temporal perturbation of TGF β signaling demonstrated a previously unknown role of TGF β as a repressor during specific stages of cardiomyocyte differentiation. A similar biphasic regulation of Wnt signaling has previously been implicated in cardiac differentiation [11]. More recently, a systematic study of temporally controlled Wnt signaling indicated that the Wnt pathway is both necessary and sufficient for cardiac differentiation [12]. Early induction of Wnt signaling via glycogen synthase kinase 3 (Gsk3) inhibition, followed by subsequent shRNA mediated inhibition of β -catenin signaling enabled the production of differentiated cardiomyocytes with high efficiency (98% cardiomyocytes) and yield (>15 cardiomyocytes per input ESC). Taken together, the increasing comprehension of the signaling dynamics accompanying stem cell differentiation highlights opportunities to engineer strategies for controlled temporal administration of morphogens to direct stem cell fate more efficiently and effectively.

The scope of the vast experimental space [13] and the precision required for developmental stage-specific pathway activation to direct stem cell differentiation motivates the development of strategies for screening multiple environmental variables concurrently with high temporal fidelity. Microfluidic platforms enable high-throughput screening with increased temporal and spatial precision compared to standard batch-based morphogen delivery methods, thereby providing new opportunities to more accurately probe the concentration and temporal dependent effects of morphogens on stem cell differentiation [14]. Microfluidic platforms are capable of precise morphogen delivery, including the formation and delivery of stable, spatially defined gradients, which have been employed to probe the real-time activation of Wnt/ β -catenin signaling in response to a range of morphogen concentrations in parallel [15]. Similarly, spatially defined delivery of multiple morphogens simultaneously via microfluidics can induce hemispherical patterning of individual stem cell aggregates, analogous to polarization events that transpire during early

embryonic development to specify contrasting cell populations as a result of morphogen gradients [16]. Moreover, microfluidic platforms can evaluate heterogeneous stem cell responses to physical or chemical environmental perturbations for isolated, parallel clonal growth of individual stem cells [17]. The juxtaposition of multiple signaling pathways in a temporal, developmental stage-specific context highlights the degree of spatial and temporal precision required to effectively direct stem cell differentiation in vitro. Further understanding the scope of such biological complexity affords new opportunities to employ microfluidic technologies to achieve increased throughput and spatiotemporal control of cell fate.

Delivering and presenting exogenous cues

Although soluble addition of morphogens affords uniform delivery within monolayer culture platforms, tissue engineering strategies which aim to expand and differentiate stem cells as multicellular aggregates or dispersed within hydrogel materials exhibit limited spatial fidelity upon soluble delivery, thus motivating the development of novel approaches for presenting exogenous cues in these three-dimensional formats. Biomaterial approaches are amenable to extensive modifications to integrate bioactive domains and control morphogen delivery with defined and tunable release characteristics in order to create a biomimetic stem cell milieu [18]. Delivery of VEGF from bulk hydrogels has been accomplished by immobilization throughout cell-encapsulated agarose hydrogels [19], or by delivery from poly(lactic-co-glycolic) acid (PLGA) microparticles incorporated within bulk cell-seeded dextran hydrogels [20]; biomaterial-mediated delivery resulted in increased efficiency of blood progenitors and Flk-1 expressing cells, compared to soluble VEGF delivery and spontaneous EB differentiation, respectively. Alternatively, microparticles incorporated within ESC [21] and MSC [22] spheroids can control local morphogen delivery within scaffold-free 3D cell constructs. Microparticle-mediated delivery within EBs revealed a previously unknown role of retinoic acid (RA) in mediating ESC morphogenesis and differentiation toward an epiblast/primitive streak-like phenotype, analogous to an E6.75 mouse embryo, which could not be recapitulated with soluble RA treatment over a broad range of soluble concentrations [23]. Similarly, the combination of microparticle delivery of hemogenic morphogens (bone morphogenetic protein 4, BMP-4; thrombopoietin, TPO) in concert with microenvironmental perturbations (5% O₂) to increase endogenous VEGF secretion demonstrated the increased efficacy of hemogenic, colony forming cell differentiation by engineering multiple parameters of the 3D stem cell microenvironment to control local growth factor delivery [24].

In addition to the utility of biomaterials to serve as molecular delivery vehicles, the properties of the materials themselves can be exploited to alter the biochemical and biophysical composition of stem cell aggregate microenvironments. For example, recent work indicates that different synthetic and natural microparticle compositions (PLGA, gelatin, agarose) with varying material properties can influence the relative proportions of stem cell differentiation when incorporated within the EB microenvironment, independent of morphogen delivery [25]. In addition, biomaterials offer opportunities to present immobilized ligands to direct stem cell fate decisions. One of the most widely studied mechanisms of ligand-based control of stem cell fate is that of cell surface Notch receptors [26], for which Delta/Jagged ligands can be presented via co-culture with transfected cells or coating of biomaterial surfaces [27]. Similarly, E-cadherin has been presented to cells via microparticle immobilization [28] and through transfected fibroblasts [29]; the presentation of E-cadherin promoted the neural differentiation of ESCs [29], which highlights future opportunities for modulation of cell adhesion and polarity via immobilization and presentation of cell surface receptor moieties. Signaling molecules, including leukemia inhibitory factor (LIF), have also been immobilized on synthetic surfaces for local

presentation of bioactive ligands, which may be broadly applicable to a range of factors implicated in maintaining ESC pluripotency or directing differentiation [30]. Biomaterial strategies capable of delivering morphogens and presenting ligands within the local stem cell microenvironment permit the establishment of physiologically relevant signaling within 3D structures through spatially defined delivery and local presentation of bioactive ligands.

Stem cell-derived factor manipulation

Paracrine signaling has traditionally been employed as a method of directing differentiation via co-culture of stem cells with differentiated cell types; however, the complex and often ill-defined milieu present in such conditioned media highlights new opportunities for controlling autocrine and paracrine signaling to direct stem cell differentiation. Understanding the co-culture signaling responses governing cell specification can instruct synthetic platforms to recapitulate native responses; for example, the maturation of stem cells to produce antigen specific T cells has been accomplished by providing major histocompatibility complex molecules from either stromal cells or as tetramers in solution [31]. Additionally, patterning approaches have demonstrated the ability to create complex co-culture platforms, and thereby may inform approaches to spatially control differentiation. For example, the differentiation of ESCs in co-culture with spatially defined monolayers comprising both extraembryonic endoderm and trophoblast stem cells, which resemble extraembryonic microenvironments, successfully recapitulated proximal-distal patterning by directing divergent ESC fate specification [32].

As noted earlier, microfluidic technologies can systematically manipulate extracellular environments to examine stem cell fate decisions, including manipulation of endogenous stem cell-derived factors as well as exogenous delivery of molecules. For example, the removal of diffusible autocrine and paracrine signals using continuous microfluidic perfusion has implicated specific factors, including endogenous fibroblast growth factor 4 (FGF4), which are required for neuroectoderm differentiation [33]. Combining similar microfluidic approaches with computational modeling has demonstrated the ability to predict the spatial and flow dependent changes in cell fate upon flow-mediated modulation of autocrine and paracrine signaling [34]. Ultimately, the ability of microfluidics to deliver exogenous signals with sensitive temporal and spatial resolution combined with perturbing autocrine signaling pathways offers novel approaches to evaluate the fine balance between stimulatory and inhibitory cues regulating stem cell differentiation fate decisions.

Additionally, biomimetic approaches to recapitulate the native composition of extracellular tissue microenvironments can exploit the capacity of proteoglycans to bind a variety of growth factors and cytokines; such natural materials may thereby significantly alter the concentration and presentation of morphogens within stem cell environments. The remodeling of proteoglycan-rich microenvironments by differentiating ESCs indicates the dynamic regulation of endogenously secreted molecules, such as hyaluronan and versican, by stem cells undergoing morphogenesis [35]. Further examination of the composition and functional properties of stem cell environments can be facilitated by the demonstration of methods to decellularize or devitalize EBs in order to capture the native ECM and associated growth factors [36,37]. The molecular composition of isolated stem cell microenvironments is expected to modulate the phenotype of stem and somatic cells and influence tissue remodeling and regeneration. Individual glycosaminoglycans, such as heparin, have been engineered into novel MSC culture platforms, in order to spatially control stem cell proliferation and differentiation via sequestration of growth factors such as FGFs and BMPs [38,39]. Heparin hydrogel technologies are extremely promising for the rational design of differentiation systems, to both induce secretion of and sequester growth factors [40]; for example, the combination of heparin hydrogels and fluvastatin delivery to stimulate BMP-2

secretion resulted in a hydrogel microenvironment which supported increased osteogenic differentiation of MSCs [41]. Moreover, heparin conjugated hydrogels have demonstrated the capacity to sequester factors secreted from co-cultured osteoblasts in order to increase the efficiency of MSC osteogenic differentiation [42]. Taken together, manipulation of stem cell signaling by microfluidics, engineering material properties, and combinations thereof can serve as novel routes to augment or attenuate the spatiotemporal presentation of factors capable of directing stem cell fate decisions.

Modulating intrinsic stem cell responses

ESCs exhibit the capacity for the endogenous cell milieu to direct complex migration and self-organization, including the induction of the epithelial-to-mesenchymal transition (EMT) [35], as well as morphogenesis to yield structures resembling optic cup [43,44] and anterior pituitary tissues [45]. Three-dimensional differentiation of stem cells has been demonstrated as a method to modulate pathways involved in cell specification; for example, ESCs exhibit increased E-cadherin expression and decreased Wnt signaling upon spheroid formation, which demonstrates the interdependence between cellular adhesions and developmentally relevant cell signaling pathways [12]. Intercellular adhesions and associated signaling mechanisms can be perturbed by simple alteration of spheroid composition; for example, aggregate sizes have been correlated with different relative efficiencies of differentiation [46,47] and the control of EB size has been established as a method for decreasing culture heterogeneity [48]. Although initiation of ESC differentiation via aggregate formation has been extensively explored, there has also been increasing interest in the three dimensional association of multipotent stem cells in spheroid cultures. For example, MSC spheroids exhibit enhanced differentiation [49], as well as increased secretion of immunomodulatory factors, compared to monolayer cultures [50,51].

In addition to the cell-cell and signaling responses of stem cell spheroids, the differentiation of stem cells in 3D also enables the secretion of endogenous ECM and tissue remodeling. MSC differentiation within hydrogel scaffolds has demonstrated the capacity of the cells to deposit endogenous ECM and remodel matrices composed of natural materials, such as hyaluronan [52] and chondroitin sulfate [53]. Additionally, tunable hyaluronan hydrogels have been developed, whereby adhesion and degradation properties can be modulated to support the formation of vascular networks [54] and to spatially control cell migration [55]. Ultimately, greater understanding of endogenous stem cell extracellular remodeling responses in response to various extracellular cues may enable rational approaches to initiating and mediating complex tissue morphogenic events. Engineering approaches for directing endogenous self-organization and remodeling responses of stem cells afford new opportunities for indirectly controlling the complex signaling pathways regulating differentiation.

Future opportunities

While significant recent advances have demonstrated new viable routes to control stem cell differentiation, many opportunities remain for the development of new technologies to achieve increased spatial and temporal control of stem cell microenvironments. Although microfluidic technologies are promising due to the high temporal resolution (seconds to minutes), and the flexibility of the platform to achieve configurations amenable to multiparametric and spatially defined screening [56], thus far relatively few microfluidic technologies have been implemented in the stem cell field. For example, opportunities exist to adapt temporally intensive, multi-step differentiation protocols to microfluidic platforms, in order to increase the precision of morphogen delivery and to further characterize the temporal dependence of stem cell responses to chemical stimuli. While microfluidics are a

promising screening mechanism, however, the limitations of such platforms which rely on two-dimensional culture in small volumes and impart fluidic stimuli, also warrants parallel studies to understand stem cell fate decisions in the context of physiologically relevant, scalable three dimensional cultures. Such methods for directing complex tissue morphogenesis will likely involve engineering approaches to perturb cell responses, while also employing an increased understanding of developmental biology principles to permit endogenous self-organization and remodeling.

Advances in material technologies are expected to enable the delivery of morphogens with high temporal fidelity to support the differentiation and maturation of stem cells. Opportunities remain to employ delivery vehicles capable of either simultaneous or sequential delivery of multiple morphogens, analogous to directed differentiation protocols currently employed in monolayer culture platforms; multifactor morphogen delivery from distinct microparticle populations has been explored in applications to promote angiogenesis [57] and bone regeneration [58] and compartmentalized particle technologies are promising for delivery of multiple morphogens [59]. Additionally, stimuli responsive, or “smart” biomaterial approaches may enable on-demand release of morphogens in response to cell-autonomous or environmental changes [60]. Synergistic combinations of engineering strategies with cell intrinsic responses could yield increased efficiency and reproducibility of differentiation, as the release of morphogens in response to biologically relevant stimuli could account for and adjust appropriately to changes in differentiation kinetics between different cell lines or subtle variations across experimental conditions and independent investigations.

Tissue engineering strategies for improving the controlled formation of 3D tissue structures [61] composed of cells and materials are likely to enhance the spatial fidelity of patterning morphogenic cues. While analogous approaches currently exist to spatially control patterning in 2D using microfluidics [62] or micropatterning [63], new technologies will likely be required to attain similar control in 3D multicellular aggregate or hydrogel configurations. Microwell surfaces have enabled the controlled and reproducible formation of multicellular aggregates from ESCs [64] and MSCs [49], and have demonstrated the capacity to homogeneously incorporate microparticles at controlled seeding densities [25]. However, the development of novel chemistries which enable patterning of hydrogels on the micrometer scale [65] offer additional opportunities for creating biophysically or biochemically distinct regions, in order to incorporate divergent instructive cues to induce tissue polarity and direct more complex stem cell morphogenesis.

Understanding the precise dynamics of phenotypic specification and morphogenesis will inform novel approaches to integrate spatiotemporal control within stem cell differentiation platforms. The increasing reliance on fluorescent or bioluminescent reporter systems, as well as the development of high throughput platforms [66–68] for simultaneous screening to monitor cell signaling, pathway activity, and stem cell phenotype in real time are expected to improve the understanding of stem cell heterogeneity and to highlight the importance of precise stem cell signaling dynamics. Additionally, non-destructive monitoring of alternative variables in concert with stem cell phenotype may be critical for the development of platforms that enable dynamic control of stem cell microenvironments. For example, monitoring of the bioprocessing parameters, including cell loss, in ESC suspension cultures enabled the maintenance of predictable and reproducible cultures which exhibited increased efficiencies of definitive endoderm differentiation [69]. Ultimately, the increased understanding of stem cell differentiation dynamics are expected to inform engineering approaches to perturb endogenous signaling and guide morphogenesis in more efficient and efficacious manners.

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Box 1

Stem cell fate. Stem cell fate is often discussed in terms of discrete phenotypic stages, defined by expression of cell or tissue specific genetic markers. However, studies aimed to direct stem cell differentiation are often constrained by the limited understanding of fate decisions during the continuum of transitions between and along lineages. The fate of stem cells is attributed to a complex cadre of microenvironmental cues, including physical cues which perturb cytoskeletal tension or cell-cell and cell-matrix adhesions, as well as chemical cues, which encompass autocrine, paracrine, or exogenous soluble signals presented locally within the cell environment. For both types of stimuli, such perturbations are closely tied to signaling cascades, which elicit cellular responses, such as proliferation or differentiation. Importantly, the response of cells to microenvironmental stimuli is dependent upon the initial state of the cell and thereby highlights the importance of understanding and addressing temporal kinetics in the context of stem cell engineering. Additionally, the inherent heterogeneity that is often present within *in vitro* cell cultures complicates the understanding of stem cell fate decisions, due to the capacity for cells to simultaneously exhibit divergent responses to the same stimulus. Endogenous cues also influence cell fate in heterogeneous cultures since the local microenvironment is defined by the milieu of secreted factors and adhesions from adjacent, and often phenotypically distinct, cells. Altogether, the complexity of cellular responses and the continuous nature of stem cell fate decisions warrants engineering approaches to systematically dissect and perturb both the spatial and temporal cues present within the stem cell microenvironment.

Highlights

- !! The temporal dynamics of signaling and morphogen delivery alter stem cell fate
- !! Biomaterials enable 3D delivery of morphogens and presentation of bioactive ligands
- !! Cell signaling can be perturbed via modulation of transport and factor sequestration
- !! Perturbation of 3D cell-cell and cell-ECM adhesions induces morphogenic events

Figure 1. Stem cell fate decisions

(A) Stem cell phenotypes are often depicted at discrete stages of varying potency, as commonly shown by the hierarchical example of pluripotent embryonic stem cell differentiation to the three germ lineages and subsequent mature cell progeny of each. (B) In reality, stem cell fate decisions constitute a continuum of phenotypes, governed by the cellular responses to microenvironmental stimuli (physical and chemical) presented to cell populations at different stages of development and maturation.

A. Temporal dynamics

B. Exogenous cues

C. Stem cell-derived factors

D. Intrinsic stem cell responses

Figure 2. Novel approaches to direct stem cell differentiation via spatiotemporal control of the biophysical and biochemical stem cell microenvironment

Increasing appreciation for the precision required to deliver soluble morphogens have prompted the development of techniques to modulate (A) temporal dynamics and to (B) enable localized spatial delivery of exogenous factors. Additionally, the local environment can be controlled through (C) perturbations of endogenous secreted factors as well as by (D) altering intrinsic stem cell morphogenic and remodeling responses.

Table 1

Enabling technologies for engineering the stem cell microenvironment.

Enabling technology	Application	References
Microfluidics	Spatial development of morphogen gradients	[15,16]
	Spatial control of paracrine factor transport	[34,70]
Biomaterials (Microparticle incorporation, hydrogel encapsulation)	Spatial control of 3D morphogen delivery	[19,20,22–24]
	Sequestration of secreted paracrine factors	[38–40]
	Providing biomimetic extracellular environments	[52–55]
	Presenting bioactive ligands to perturb cell signaling, cell-cell interactions and polarity	[27,28,30]