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Engineering three-dimensional stem cell morphogenesis for the development of tissue models and scalable regenerative therapeutics

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Abstract

The physiochemical stem cell microenvironment regulates the delicate balance between self-renewal and differentiation. The three-dimensional assembly of stem cells facilitates cellular interactions that promote morphogenesis, analogous to the multicellular, heterotypic tissue organization that accompanies embryogenesis. Therefore, expansion and differentiation of stem cells as multicellular aggregates provides a controlled platform for studying the biological and engineering principles underlying spatiotemporal morphogenesis and tissue patterning. Moreover, three-dimensional stem cell cultures are amenable to translational screening applications and therapies, which underscores the broad utility of scalable suspension cultures across laboratory and clinical scales. In this review, we discuss stem cell morphogenesis in the context of fundamental biophysical principles, including the three-dimensional modulation of adhesions, mechanics, and molecular transport and highlight the opportunities to employ stem cell spheroids for tissue modeling, bioprocessing, and regenerative therapies.

INTRODUCTION

The balance between stem cell proliferation and differentiation is tightly controlled by local cues present in the stem cell niche microenvironment.^{111,137} In response to chemical or physical perturbations, cells exit the niche and undergo differentiation processes,¹⁰² often to mediate regeneration or repair in pathological contexts such as hemogenic repopulation⁹² or wound healing.¹⁵⁶ One particularly dynamic example of stem cell microenvironment regulation occurs within the blastocyst-stage embryo, whereby a compact cluster of cells, known as the inner cell mass (ICM), develop into all somatic tissues and organs.⁶¹ During the early stages of pre-implantation development, the cells of the ICM undergo sequential specification, through which cells commit along the three germ lineages – endoderm, ectoderm, and mesoderm – and continue to make cell fate decisions in a spatially and temporally controlled manner, thereby providing a robust model by which to study cell plasticity and tissue formation. The patterning of cell fates is mediated by physical processes, such as proliferation⁶² and migration,⁵⁶ which occur concomitant with biochemical gradients,⁴⁷ thereby highlighting the need for novel technologies to recapitulate the multiparametric stimuli present within the tissue microenvironment. For example, during gastrulation, the prospective mesoderm cells undergo a dynamic epithelial-to-mesenchymal

transition (EMT) and migrate through the primitive streak.^{18,31} Similarly, collective cell migration of epithelial sheets has been implicated in processes such as branching morphogenesis.⁵⁰ Biophysical signals mediating the spatiotemporal dynamics of cell migration mediate the formation of functionally and structurally distinct, yet adjacent, tissue structures, such as heart, lungs and kidney, each of which is defined by precisely controlled, heterotypic multicellular organization. The precise presentation of biochemical and biophysical cues *in vivo* motivates the development of engineering approaches that recapitulate the stem cell niche in order to create functional heterotypic multicellular structures *in vitro* which are amenable to the replacement of damaged or diseased tissue through scalable bioprocessing and tissue engineering approaches, and offer new cellular platforms for high-throughput pharmaceutical screening and drug development.

In order to emulate *in vivo* tissue-scale morphogenic processes, *in vitro* platforms have been developed to present chemical and physical cues in three-dimensional configurations, analogous to the multicellular structure of native tissues. Early studies of pluripotent embryonal carcinoma cells created high-density cellular environments *in vitro*¹¹² by E-cadherin-mediated self-assembly of cells,⁹⁸ which alters the microenvironment through local 3D presentation of adhesive and biochemical signals, analogous to aspects of embryonic development. Similar approaches have been adapted to initiate differentiation of pluripotent stem cells (PSCs) and induced pluripotent stem cells (iPS) via 3D aggregation as embryoid bodies (EBs), either through forced aggregation in small media volumes (hanging drop, microwell centrifugation) or through self-assembly via random association in bulk suspension cultures.⁹⁴ More recently, the aggregation of multipotent stem and progenitor cells, such as mesenchymal stem cells (MSCs)⁷ and neural stem cells (NSCs),¹⁴ has also been explored, in order to provide physiologically relevant cell-cell and cell-ECM adhesions and to induce paracrine factor secretion, which together are hypothesized to support tissue maturation. Together, engineering approaches to systematically control⁸⁹ and perturb three-dimensional scaffold-free stem cell-derived microtissues enable new routes to study the expansion and differentiation of stem cells and have increasingly become more accessible and broadly applicable in the fields of stem cell biology and tissue engineering.

PSCs, in particular, exhibit striking examples of morphogenesis upon three-dimensional assembly and differentiation,¹³⁴ thereby providing an intriguing model system amenable to studying and perturbing physiochemical elements mediating cell fate. For example, induction of Rx+ neuroepithelium in 3D PSC spheroids resulted in spatially distinct patterns of differentiation, with the resulting neo-tissues exhibiting phenotypic markers and architecture reminiscent of the native optic cup.^{46,115} Interestingly, the dynamic structural changes, including evagination of epithelial vesicles and subsequent invagination, yielded distinct layers of neural retinal and retinal pigment epithelial (RPE) cells that were directed solely by cell-intrinsic morphogenic processes and self-organization of cells in 3D, thereby highlighting the ability of stem cell-derived microtissues to recapitulate aspects of tissue development *in vivo*.⁴⁶ Similarly, simple biochemical induction of differentiation directed the morphogenesis of 3D microtissues comprised of organized enterocytes, goblet, Paneth and enteroendocrine cells, thereby establishing an *in vitro* organoid model of intestinal structure and function.¹⁴⁹ Another model exhibiting self-formation of complex cerebral structures⁹⁷ was developed to study the pathogenesis of human microcephaly using iPS cells. Moreover, similar approaches have yielded functional anterior pituitary,¹⁵¹ thyroid,⁴ and hepatic,¹⁵⁴ structures which exhibit secretory functions when transplanted *in vivo*. Together, 3D aggregation of stem cells provides opportunities to study tissue morphogenesis (Fig. 1) across a wide array of cell phenotypes and microtissue structures. In this review, we discuss the biophysical basis and engineering opportunities to control and perturb 3D pluripotent stem cell morphogenesis, as well as the potential applications of PSC-derived

microtissues as valuable tools for developmental biology, drug discovery and regenerative medicine.

3D STEM CELL MORPHOGENIC PROCESSES

Intercellular adhesion organization in 3D

The formation and morphogenesis of three-dimensional cellular aggregates is a dynamic process regulated by differential cellular adhesions, matrix synthesis, and remodeling. In contrast to the cell-matrix adhesions that mediate cell seeding on scaffolds or within hydrogels, the rapid formation of 3D aggregates (12–24 hours) is primarily directed by cellular adhesion molecules (CAMs) such as cadherins and connexins.⁶ CAMs are also key regulators of self-renewal and the induction or maintenance of pluripotency^{26,148} with pluripotent cells being sensitive to disruption of cell-cell contacts and dependent on CAM-mediated interactions for survival. Therefore, the central role of CAMs in both regulating pluripotency and 3D aggregate formation highlights opportunities to study the kinetics and mechanisms of CAM biology in regulating stem cell morphogenesis.

Owing to the central role of CAMs in mediating the rapid transition from single cells to neotissues, spheroid cultures offer unique opportunities to study the adhesive signatures characteristic of the earliest cell fate decisions and developmental morphogenesis. CAM expression within pluripotent aggregates is altered temporally during differentiation, with undifferentiated PSCs highly expressing E-cadherin. In contrast, E-cadherin expression is often decreased upon differentiation, concomitant with differential expression of other CAMs during lineage commitment. For example, Stankovich et al. identified the CAM profile (i.e. E-cadherin, Cldn4, ZO-1, ZO-2, Cx43) related to hematopoietic and endothelial cell subpopulations.¹⁵⁰ Further, modulation of CAMs, via knock-down of E-cadherin and connexin 43 within PSCs led to decreased efficiency of hematopoietic differentiation,¹⁵⁰ indicating that intercellular adhesions play a critical role in regulating stem cell differentiation.

In addition to physically connecting adjacent cells, intercellular adhesions also influence local cell signaling. Consistent with the loss of E-cadherin during primitive streak migration in embryonic development,¹⁸ cadherin-mediated assembly of PSCs alters the temporal expression of E-cadherin, and influences the downstream signaling within the associated Wnt/ β -catenin pathway.⁹⁰ Simply altering the kinetics of aggregate assembly, therefore, results in modulation of Wnt-dependent mesoderm differentiation, with precise regulation of Wnt signaling required for cardiac differentiation.¹⁶² Similarly, hanging drop formation of stem cell aggregates also modulates the extent of cardiomyocyte differentiation, likely due to similar mechanisms downstream of intercellular adhesions.^{132,178} In addition, controlling the cellular composition (cells per aggregate) also modulates cardiac differentiation efficacy, which is thought to arise due to changes in the surface area-to-volume ratio, which alters the cell polarity and distribution of CAMs.¹² Together, these examples illustrate the interrelationship between CAMs and developmentally-relevant signaling, which can be manipulated to engineer stem cell microenvironments by controlling cell aggregation and spheroid formation.

Extracellular matrix deposition and remodeling in 3D stem cell spheroids

In addition to intercellular adhesions, stem cells are also in contact with the extracellular matrix (ECM), a complex three-dimensional network of macromolecules that provides distinct biomechanical, biophysical, and biochemical cues.³⁶ Production of the ECM is a dynamic cyclical process comprised of matrix synthesis, deposition, organization, degradation and remodeling;³⁸ if not properly regulated, alterations in ECM processes contribute to disease or pathological states.^{34,120} ECM synthesis and assembly are regulated

through bi-directional signaling between the cells and the underlying matrix via integrins.¹⁷³ Concomitantly, physical signals transmitted via integrins affect the intercellular actomyosin network, which induces ROCK-mediated proliferation, ultimately leading to fibronectin fiber assembly and cleft stabilization.³⁹ These examples demonstrate how extracellular cues initiated through cell-ECM interactions influence intracellular signaling that mediate morphogenic processes.

During early stages of development, ECM is spatiotemporally regulated to ensure proper induction of developmental processes. For example, branching morphogenesis of the submandibular gland requires the presence of laminin $\alpha 5$ to stimulate integrin $\beta 1$ -mediated MAPK signaling induced proliferation and bud formation.¹²⁶ Similarly, as stem cells differentiate, the ECM composition changes,¹¹⁴ leading to distinct profiles throughout different stages of tissue morphogenesis. The loss of polarity and cell-cell adhesions mediates cell migration⁷⁴ characteristic of EMT via ECM molecules such as glycosaminoglycans (GAGs)¹⁵⁸ and proteoglycans¹⁰⁶ and remodeling of the native ECM by matrix metalloproteinases (MMPs).¹¹⁹ Three-dimensional culture of stem cells as EBs *in vitro* recapitulates aspects of EMT,²⁵ including alterations in ECM composition and cellular organization as a function of differentiation. For example, GAGs such as hyaluronan and versican are increasingly synthesized with EB differentiation and co-localize within mesenchymal regions of the EBs.¹⁴³ GAGs are known to sequester and bind growth factors within the extracellular matrix to facilitate the local presentation to cells,¹⁸⁰ which reflects the ability of ECM to regulate biochemical signals in addition to providing physical cues. In addition to GAGs, other fibrillar ECM molecules such as collagen I and IV, fibronectin, and laminin are observed throughout EBs,^{63,113,128} while generally in lower abundance within pluripotent aggregates compared to mature tissues *in vivo*, ECM synthesis and deposition may play an important role in early stem cell morphogenesis. While three-dimensional culture of PSCs recapitulates many early developmental events, the specific role of extracellular matrix in PSC morphogenic processes remains largely unknown due to the limited techniques for achieving spatial and temporal precision similar to developmental processes, as well as the complexity associated with studying such multivariate processes in three-dimensional multicellular models.

Cellular mechanotransduction and stem cell phenotype

Three-dimensional remodeling of intercellular adhesions and ECM modulates the intracellular architecture, which is responsible for transmitting forces within and between cells.⁷⁹ The mechanical structure of individual cells is often described by the “tensegrity” model,⁷⁸ in which interior compressed elements (i.e. microtubules, microfilaments) are balanced by opposing elements (i.e. contractile actin cytoskeleton) in tension.⁵² Cellular forces are also opposed through extracellular factors,¹⁴⁵ such as ECM molecules, which impart dynamic feedback to mediate reorganization of the cytoskeleton¹⁰⁸ and associated cell-cell and cell-matrix adhesions.¹⁰⁵

Approaches to manipulate cell shape have been extensively explored in monolayer cultures, as the regulation of inter- and intra-cellular tension mediates stem cell responses, including proliferation¹¹⁶ and differentiation.¹⁰⁹ Engineering approaches aim to control stem cell mechanics (Table 1) by changing adhesive ligand coating density^{48,159} or through manipulation of substrate cross-linking,⁴⁹ independent of ECM concentration.⁵⁸ Downstream signaling of the Rho associated protein kinase (ROCK) pathway is often implicated in mediating cytoskeletal reorganization and stiffness-mediated phenotypic changes.¹⁰⁹ For example, EMT occurs within regions of high cellular tension, and is abrogated by perturbation of the ROCK pathway.⁶⁴ Similarly, MSC differentiation can also be patterned by controlling the geometry of multicellular sheets, with increased osteogenesis

in regions of high tension.¹²⁷ In addition, defective mechanotransduction has been linked to decreases in the expression of nuclear proteins, such as nuclear lamins,⁹⁶ which have also been implicated as markers of hPSC differentiation.³² Therefore, in addition to adhesive and cytoskeletal changes, nuclear shape may also play a key role in mediating and responding to cellular tension and directing phenotypic changes,³⁷ thereby demonstrating multiparametric effects of the extracellular environment on cellular tension and stem cell fate.

The role of mechanics in mediating three-dimensional stem cell differentiation, however, remains less well defined. Given the dramatic changes in cellular organization and adhesive profiles when stem cells are assembled as spheroids,³⁶ the cytoskeletal organization, and thereby biomechanics, are fundamentally distinct from properties measured in monolayer cultures (Fig. 2). While integrin adhesions and ECM impart the primary forces opposing cytoskeletal tension in 2D, the cells themselves take on load bearing processes in 3D, with the network of cell-cell cadherin adhesions and cytoskeletal associations regulating cellular tension. Moreover, the orientation and composition of extracellular and pericellular matrices vary spatially and temporally during differentiation,¹¹⁴ and play an active mechanical role in mediating changes in adhesive signature or clustering.¹²² Therefore, due to the established role of biomechanics in mediating stem cell phenotype, opportunities remain to develop methods to monitor and perturb local tension in 3D aggregates as a novel approach to direct spatial patterns of differentiation and morphogenesis.

Multicellular mechanics of 3D tissues and spheroids

Three-dimensional morphogenesis, often associated with tumorigenesis and embryonic development, is a fundamentally biophysical process,¹⁷⁵ as cellular migration and proliferation occur in conjunction with dynamic rearrangements of adhesions and the cytoskeleton. Mechanics have been directly implicated in mediating aspects of embryonic gastrulation,⁸² elongation,¹⁸² and dorsal and neural tube closure.⁵⁴ Moreover, the actomyosin cytoskeleton exhibits distinct dynamics during mesoderm migration accompanying gastrulation.⁸⁴

The association between morphogenic processes and cell plasticity suggests an active role for biomechanics in 3D stem cell morphogenesis. The stiffness of tissues has been related to force generation,¹⁶⁷ with mesodermal structures, which actively migrate through the primitive streak, exhibiting a 10-fold increase in stiffness compared to endoderm in embryonic explants.¹⁸² While direct experimental measurements of local 3D cellular tension are difficult to obtain with current approaches, computational simulations based upon mechanical models suggest that the homeostatic state of epithelial tissue structure is maintained by cellular tension, and accurately recapitulate experimentally observed cellular rearrangements upon laser ablation of individual cells.⁵¹ Similar methods have elucidated the biomechanical basis underlying the regulation of wing size in drosophila embryos;^{1,75} despite the presence of biochemical signals, cell growth is inhibited by cell compression at the center of the disc, which ultimately dictates the final wing size. Similarly, non-uniform proliferation has been linked to geometrical regulation of mechanical tension in cells,¹⁴² with proliferation occurring in regions of increased local tension.¹¹⁶ Moreover, Rho-mediated contractility has also been implicated as a mechanism underlying the dissociation of adherens junctions,¹³⁰ thereby demonstrating the interdependence of biochemical and biophysical processes in morphogenesis. Together, the mechanical mechanisms mediating proliferation and differentiation enable the formation of distinct shapes and cellular patterning to direct divergent lineages and form complex tissues.

In addition to the synergistic effects mediating cell phenotype, mechanical and biochemical signals are transmitted across different length scales in tissues.⁴⁴ For example, in spheroids, tension is transmitted between cells via the actomyosin-CAM network. Moreover, the

tension on cells at the exterior of tissues or spheroids differs compared to those residing inside the mass of cells.¹⁰⁷ Such transmission of force across and within spheroid structures may account, in part, for some of the differences in cell fate trajectories observed in aggregates of different sizes.^{11,12} In contrast, tissues comprised of large quantities of ECM or cells encapsulated in hydrogel materials restrict tension transmission, which potentially limits the spatial transduction of mechanical forces. Therefore, the dynamics of tissue remodeling during morphogenesis also dictates the local mechanical cues. While PSCs, in particular, mimic aspects of embryonic development on the biochemical level, opportunities remain to elucidate the biomechanical morphogenic processes in 3D stem cell spheroids, in order to draw from analogous *in vivo* events to direct cell fate by recapitulating physical cues present in embryonic microenvironments.

Diffusive mass transport of exogenous factors within 3D aggregates

Inductive cues, including oxygen, nutrients and morphogens, are important factors governing stem cell fate. Precise control of biochemical delivery within stem cell cultures is valuable for understanding cellular responses and critical for establishing reproducible stem cell *ex vivo* expansion and differentiation. In traditional 2D monolayer cultures, exogenous soluble factors or cell-secreted endogenous factors diffuse freely throughout the medium, and thereby reach an equilibrium in which all cells are exposed to similar biochemical environments.⁵ In contrast, in 3D aggregate cultures, a concentration gradient is established between the surrounding culture environment and the interior of the spheroids.¹⁶⁵ The distinct cellular dynamics in 2D and 3D stem cell culture^{136,170} as well as the heterogeneity within individual EBs most likely arise, at least in part, due to the aforementioned disparity in mass transport between the culture systems¹²⁸

The mass transport within EBs has been measured experimentally¹²⁸ and modeled mathematically¹⁶⁵ as a function of the EB size (radius), extracellular matrix composition, cell packing density and molecular uptake rate. Changes in EB size impact the surface area-to-volume ratio, which is inversely related to the aggregate radius; therefore, EB size is a primary critical determinant of mass transfer in and out of EBs. The partial pressure of oxygen in mouse PSC aggregates has been theoretically¹⁶⁵ and experimentally¹⁷⁰ studied, and confirm that the concentration is inversely related to aggregate size, with decreasing concentration from the surface toward the center of aggregates. The varying oxygen levels throughout PSC aggregates may favor differentiation toward specific lineages. For example, hypoxic conditions within aggregates enhances VEGF secretion, which influences the differentiation of PSCs towards hematopoietic lineages.¹²³ Mathematical modeling also indicated that hematopoietic capacity is dependent on the size of EBs, with a 6-fold increase in CD45⁺ cells from larger size of EBs compared to smaller EBs under the same soluble differentiation condition.⁶⁹ Additionally, oxygen gradients may be spatiotemporally modulated during stem cell differentiation, as the oxygen uptake rate varies depending on the cell lineages.² The concentration gradients of glucose and cytokines have also been calculated using the Thiele modulus.¹⁶⁵ Within hPSC aggregates, the critical limit for glucose concentration was mathematically determined to occur after 74h in culture,¹⁶⁵ which is longer than the conventional time between media exchanges (24–48 hours). The cytokine concentration was largely dependent on its depletion rate, with EB size-dependent cytokine gradients occurring at increased depletion rates.¹⁶⁵ In addition, the cellular composition (packing density), also modulates the mass transport, as the cells at the outer edge of EB consume nutrients or bind growth factors faster than the characteristic diffusion time, which can significantly reduce the mass transport in EBs with high cell packing density¹⁶⁵ or increased size during long-term culture.¹⁴⁷

In addition to EB size, other structural parameters, such as the extracellular matrix composition, may also influence the mass transport and ultimately affect the differentiation propensity or efficiency.^{11,12,19,110,117} A diffusive barrier comprised by the outer layer of differentiating day 7 EBs was first reported by Sachlos and Auguste;¹²⁸ the 20 μ m tri-layer shell structure consists of a superficial collagen type I outer layer, a squamous epithelial cell layer and a collagen type IV basement membrane.¹²⁸ The physical transport barrier posed by this structure significantly reduces molecular diffusion, and is likely more pronounced for growth factors of varying molecular weights, thereby creating disparate concentration gradients for different molecules.¹²⁸ Manipulation of the exterior spheroid structure using enzymes, such as collagenase, has been explored as an approach to promote increased transport and demonstrated enhanced efficacy for retinoic acid-mediated neural differentiation.¹²⁸ Additionally, biomaterial principles have been employed to successfully control the release kinetics and spatial delivery of exogenous factors (i.e. retinoic acid, BMP4) within PSC aggregates in order to overcome diffusion limitations.^{21,22,123}

Endogenous factor transport

Pluripotent stem cells endogenously produce many of the morphogenic growth factors, such as BMPs, Activin and Wnts, that can control primitive cell fate decisions.^{41,80} Similar to gradients present during embryonic development,¹⁴¹ transport limitations can lead to non-uniform distributions of endogenous factors in stem cell aggregates that contribute to morphogenesis and spatial patterning of distinct phenotypes.^{11,85} The size of PSC aggregates has been linked to specific differentiation trajectories, as lower gene expression of Wnt5A and enhanced cardiogenesis was observed in larger aggregates.⁷⁶ Similarly, a radial gradient of Oct4, Nanog and pSTAT3 was observed within PSC colonies in the absence of exogenous LIF, suggesting that spatially organized self-renewal is controlled by gradients of endogenous Gp130 ligands.⁴¹ The cell density and spheroid size also affect the interplay between endogenous stimulatory or inhibitory signals and exogenous factors,¹²³ as local cell density influences the relative concentration of endogenous factors, leading to enhanced blood progenitor differentiation efficiency in 100-cell aggregates compared to 10-cell aggregates.¹²³

While the aforementioned parameters affect the concentration profile of endogenous factors throughout PSC aggregates, the ECM can also sequester growth factors, in order to regulate the bioavailability and local presentation to cells, and therefore contribute to the endogenous molecular gradients within 3D aggregates. ECM proteins such as fibronectin, vitronectin, collagens, proteoglycans, and heparin can actively bind many growth factors, such as BMPs, TGF- β s, FGFs, HGF, and VEGFs.^{66,70,121,169,183} In contrast to soluble delivery, morphogens retained in ECM are presented to cells in a localized manner, which can regulate the spatial patterning of cell fate.⁷⁷ As reported during the gastrulation of *Xenopus* embryos, PDGF-AA secreted from the ectoderm binds to heparin sulfate proteoglycan (HPSG)-modified, fibronectin-rich ECM and generates a gradient to direct mesendoderm migration.¹⁵³ Engineering strategies inspired by such biological phenomena have been developed to modulate the spatial presentation of extracellular environmental cues by incorporation of ECM-based microparticles into PSC aggregates.¹⁵

NOVEL APPLICATIONS ENABLED BY 3D STEM CELL-DERIVED MICROTISSUES

Tissue modeling

One significant advancement enabled by three-dimensional stem cell aggregation is the creation of *in vitro* microtissues for developmental studies,¹⁵⁷ pharmacological screening,¹⁶⁶ and disease modeling (Fig. 3).¹²⁹ Several methods have been explored to generate tissues

via aggregation of differentiated,^{83,174} progenitor,¹⁰³ multipotent,^{7,57} and pluripotent cells.⁴³ The simplest approach is to aggregate homotypic cell populations (primary somatic cells, stem cells, or differentiated progeny) to yield microtissues such as embryoid bodies, mesospheres, cardiospheres, or neurospheres. While functional microtissues have been achieved by homotypic seeding approaches, many researchers are also investigating heterotypic cultures as a means to more closely recapitulate the multicellular environment found in functional tissues. To achieve formation of 3D heterotypic structures, cells can be assembled together within a single aggregate^{42,45,131} or individual aggregates comprised of distinct homotypic cell populations can be merged, which enables spatial control and the formation of higher order structures.¹²⁵ For example, merging of aggregates has been accomplished by a side-by-side seeding approach within microwells,^{17,125} via manipulation using magnetic microparticles within PSC aggregates,¹⁶ or using microfluidic devices to force aggregates into close proximity and facilitate merger of distinct spheroids.¹⁵²

Parallel studies of embryonic development and stem cell differentiation have enabled increased understanding and control of the molecular mechanisms governing cell fate. For example, human PSCs have been successfully differentiated into neurons^{24,81} cardiomyocytes,^{95,104} hepatocytes,¹⁴⁴ and pancreatic endocrine cells,¹⁷⁹ largely via sequential administration of biochemical cues implicated in various stages of embryogenesis. While similar approaches to promote differentiation in 3D remain an active area of research, the multicellular assembly, including the formation of adhesions and matrix synthesis, are thought to support tissue maturation, and therefore are expected to enable the establishment of complex models of tissue structure *in vitro*. While complex morphogenesis occurs through self-organization and differentiation in 3D stem cell-derived organoid models,^{4,46,97,115,149,151,154} opportunities also exist to engineer heterogeneity into the systems, in order to inform aspects of mammalian embryonic dynamics, particularly during some of the earliest cell fate decisions of the pre-implantation embryo.

Due to the aforementioned progress toward generation of *in vitro* tissue models, aggregate cultures offer opportunities to model disease progression and screen pharmacological targets. Induced pluripotent stem cells (iPS) enable patient-specific studies of disease states and population heterogeneity.⁶⁵ The combination of iPS technology with 3D aggregate culture will enable the generation of tissue models that manifest cellular disease phenotypes and can be used for drug screens even if the genetic mechanisms are unknown. To further advance the drug screening potential, developments in microfluidics and high-throughput screening have resulted in methods to subject an array of microtissues to varying molecule types and gradients in order to define optimal treatments.^{30,71}

Bioprocessing

The expansion and differentiation of stem cells as multicellular spheroids is also directly compatible with suspension culture platforms, which enables the simple integration of three-dimensional microtissues within the pipeline of bioprocess development. Scalable bioprocessing platforms have been implemented for both expansion and differentiation of PSC spheroids,^{20,53,138,184} and are amenable to scalable culture of multipotent stem and progenitor cells.⁷ As spheroids maintained in suspension are not constrained by surface area limitations posed by monolayer or microcarrier cultures,⁸⁸ it is possible to produce large cell yields by increasing the throughput and batch size, which is advantageous for limiting batch-to-batch variability. Moreover, an important benefit of scalable bioprocesses is the capacity for automation, control, and monitoring of physiochemical environmental parameters, in order to precisely and reproducibly control concentrations of oxygen, nutrients, morphogens and metabolites.¹⁰

While scaffold-based platforms also benefit from dynamic and controlled bioprocess culture configurations,¹⁸¹ the differentiation of stem cells as three-dimensional aggregates, in particular, is ideal for scalable tissue production because it enables parallel processing of many individual multicellular structures within a single bioreactor volume. Microscale tissues are easily maintained within the size limitations of non-vascularized tissues,¹⁶⁵ in order to provide the adequate molecular transport required to maintain cell viability and limit morphogen gradients.¹²⁴ The agitation and physiochemical conditions in mixed bioreactor environments are easily tunable and can be refined in the context of different stem cell applications to control phenotypic parameters, such as proliferation.^{33,177} For example, the mode and frequency of media replenishment can be monitored and adjusted in real-time, based upon culture requirements.^{23,35,86} Moreover, in addition to the precisely maintained environmental parameters, microtissue control is afforded through manipulation of individual aggregate environments using microencapsulation.^{40,172} Together, control of stem cell macro- and microenvironments enables maintenance of homogeneity between individual stem cell aggregates,⁸⁹ which is important from the standpoint of quality assurance for industrial applications.

Although many current bioprocess configurations have been developed based on modifications from established bioprocessing industries, the distinct requirements and applications of stem cell aggregates provides opportunities to develop new platforms. In particular, bioprocesses can be developed in conjunction with additional technologies for upstream monitoring, processing, and sorting, analogous to industrial manufacturing pipelines, which require minimal manual manipulation. For instance, in addition to standard biochemical analytics, integration of bulk metrics based on cell dynamics,¹⁶⁴ physical characteristics¹⁴⁶ or metabolite/morphogen profile⁸⁷ will enable non-destructive monitoring and processing with high temporal resolution. Interestingly, many analytical approaches are being developed through microfluidic technologies, which provide opportunities to pair platforms across different volumetric scales, including the application of microfluidics upstream or downstream of bioreactor cultures.⁵⁵

In anticipation of scalable stem cell expansion and differentiation, laboratory-scale culture techniques need to incorporate bioprocess-relevant environmental parameters. For example, even relatively subtle changes in mixing dynamics can dictate changes in differentiated phenotypes.^{89,133} As multiple parameters (i.e. mechanical cues, molecular transport, cell adhesion kinetics) are simultaneously modulated in response to environmental perturbations, it is traditionally difficult to dissect or systematically perturb the relative influence on stem cell fate.⁸⁸ In addition to environmental changes, the precision of physiochemical control in bioprocess platforms may alter the temporal kinetics or concentration of morphogens required to achieve the same results compared to a batch-fed, static culture system.³⁵ Therefore, due to the promise of three-dimensional stem cell aggregates as scalable therapeutics, bioprocess design should be a consideration, even in laboratory-scale experiments, when developing approaches intended to control stem cell expansion and differentiation.

In vivo delivery

Stem cell delivery *in vivo* is a critical step to translate the regenerative potential toward clinical applications. Several factors should be taken into consideration prior to delivery and post-delivery, including therapeutic dose (i.e. cell number), delivery format, and cell survival, retention and engraftment. The unique features of stem cell aggregates provide several advantages as an *in vivo* delivery platform compared to conventional delivery of cell-populated 3D scaffolds or single cell suspensions.

Cell transplantation for regenerative therapies typically requires on the order of several million cells per kilogram of the patient.⁹⁹ Delivery of therapeutically relevant cell doses in small volumes is a key constraint for clinical practice, especially for direct intramyocardial implantation with typical injection volumes between 0.1 mL and 0.5 mL.^{3,161} High cell densities are difficult to achieve using 3D scaffolds, which are often on the scale of 10^5 cells per cubic mm.¹⁵⁵ In contrast, cell aggregation in a scaffold-free format can concentrate cells on the order of 10^6 cells/mm³ upon aggregation.^{9,89,101,140} Additionally, by varying formation conditions, the diameter and cell composition of stem cells aggregates can be highly controlled,^{76,89} which enables customization for specific clinical applications.

Implantation of 3D cell/scaffold constructs via invasive surgical procedures is an unavoidable caveat of most current tissue engineering approaches.¹³ In contrast, scaffold-free cell aggregates are readily injectable and therefore compatible with minimally invasive delivery techniques. Unlike single cell suspensions, which can also be easily injected, enzymatic dissociation is not necessary for cell aggregates, which reduces cell handling and preparation time, and avoids associated decreases in cell viability,⁶⁸ ultimately supporting enhanced cell viability and engraftment *in vivo*. Cell aggregates ranging from 100 to 1000 μ m in diameter have been injected intramyocardially, subcutaneously, or intramuscularly to induce myocardial,^{9,101} adipose,²⁹ or bone¹⁴⁰ tissue regeneration, respectively. Cell aggregates can be passed through standard needles without clogging and retain their native spheroid morphology.^{9,29} While it has been reported that single cells suspensions exhibit more than 30% acute cell death due to the mechanical disruption caused by extensional flow, the viability of aggregates remained unchanged after passing through a 30G needle.¹⁰

The functional benefits of current stem cell-based therapies have been limited due to the low survival, retention and engraftment of implanted cells. Upon implantation, donor cells are immediately exposed to the microenvironment of the injury site, which is often not favorable for donor cell survival and induces cell death.^{160,176} Various cytoprotective efforts have been explored for pre-conditioning stem cells before implantation.⁶⁷ Alternatively, multicellular aggregation creates a transferable microenvironment amenable to enhancing donor cell survival and engraftment post-implantation. Implantation of multicellular aggregates has demonstrated significantly higher cell survival (80%) in comparison to single cell suspension (40%) 4 days post-implantation following cardiac injury.³ Similarly, intramyocardial injection of human amniotic-fluid stem-cell aggregates resulted in 50-fold and 20-fold increases in cell retention compared to single cell treatments after 24 hours (50%) and 4 weeks (20%) post-implantation, respectively.¹⁰¹ The improvement of short-term cell survival and cell engraftment is a prerequisite for long-term functional enhancement.⁷²

The increased retention and engraftment of stem cell aggregates can be attributed to cell-cell interactions and enriched extracellular matrix (ECM). Cell-cell interaction is essential for mediating many cellular processes, including cell survival,⁸ proliferation²⁷ and differentiation.⁹¹ In the case of single cell suspension, this cellular interaction is completely abrogated by dissociation, whereas it is preserved in the cell aggregates.^{8,9,67,72} As previously discussed, cell aggregates also retain endogenous ECM (collagen III, fibronectin and laminin),^{29,100} which is transplanted as part of the spheroid microenvironment *in vivo* and supports cell survival, cell retention and functional enhancement after transplantation.⁷³ Finally, the composition of cell aggregates may shield the interior cell populations from the damage due to the influence of shear forces upon injection and enable increased retention due to existing cell-cell interactions.

FUTURE PERSPECTIVES

To date, many approaches have been developed or proposed as a means to precisely control the biophysical characteristics of stem cell aggregates, including high throughput platforms to control the spheroid size and homogeneity,^{60,135,139,163} microfluidic approaches to increase the precision and profile of molecular transport,^{28,59,93,118,168} and biomaterial techniques to deliver, present, and/or sequester inductive cues (physical, chemical) in a highly efficient and spatiotemporally controlled manner.^{21,123} However, as discussed throughout, the impact of biophysical cues are manifested through multiparametric, synergistic responses to simple perturbations. Therefore, opportunities remain to develop experimental and mathematical approaches to understand the complex interrelationship between cellular composition (adhesions, remodeling) and physical characteristics (mechanics, transport), particularly as a function of space and time during morphogenesis. For example, ongoing efforts should focus on establishing advanced mathematical models to simulate the dynamic changes in mass transport within EB culture as the function of time (differentiation state, phenotype) and developing sensitive experimental tools to sample and control the microenvironment within aggregates. Such advances will elucidate the relative influences of various biophysical factors in mediating developmental dynamics¹⁷¹ and ultimately inform engineering approaches to perturb and control stem cell morphogenesis to guide the formation of tissue-specific microtissues amenable to screening and therapeutic applications.

Due to the recent increased interest and rapid advances in self-organization and organoid formation from pluripotent stem cells, several questions remain regarding practical translation of such approaches for pharmaceutical and therapeutic applications. For example, the extent to which microtissues *in vitro* accurately recapitulate native tissue function and drug responses *in vivo* remains unclear. As high throughput testing technologies are developed, the minimal functional units necessary to recapitulate tissue-scale behaviors will need to be determined in parallel to establish reproducible, physiologically relevant screening platforms. In addition, modular tissue engineering approaches may enable the development of heterogeneous tissue structures comprised of heterotypic cell types and/or aggregates for both *ex vivo* tissue modeling and for *in vivo* regeneration; however, there is an inherent tradeoff between engineering and cell-mediated self-organization. Therefore, parallel studies will elucidate opportunities to guide self-organization via systematic perturbations to stem cell-derived microtissues. Ultimately, modular tissue engineering approaches will enable increased organization and complexity within single heterogeneous tissue units, which will fundamentally impact the future of pharmaceutical screening and biomedical therapeutics.

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Figure 1. Physical parameters influenced by 3D assembly of stem cells

Multicellular assembly of stem cells alters the cell-cell and cell-matrix associations, the mechanical tension resulting from cytoskeletal rearrangements, and the distribution of exogenous and endogenous biochemical factors. Adherent MSCs in monolayer exhibit isolated spread morphologies with pronounced stress fibers, whereas high-density 3D aggregates of ESCs and MSCs exhibit cortical actin distributions. Red = F-actin staining via phalloidin (AlexaFluor 546; 1:40). Blue = Hoechst nuclear counterstain (10 $\mu\text{g}/\text{mL}$). Scale bar = 100 μm .

Figure 2. Cytoskeletal structure in monolayer and spheroid formats

Pluripotent (mESC, hESC) and multipotent (hMSC) stem cell populations exhibit distinct morphologies when expanded in adherent monolayer cultures, with PSCs exhibiting tightly packed, rounded colonies and cortical actin structures, in contrast to the prominent stress fibers exhibited by spread, fibroblast-like MSCs. When assembled in 3D, however, MSCs exhibit similar actin structures compared to PSCs, thereby demonstrating the fundamental structural changes upon multicellular assembly.

Figure 3. Applications enabled by 3D stem cell expansion and differentiation

The three-dimensional assembly of stem cells enables scalable bioprocessing approaches, including microliter scale upstream processing via microfluidics, as well as milliliter/liter-scale manufacturing. In addition, the self-organization and developmentally relevant signaling in microtissues and heterotypic cellular assemblies enables the study of tissue models for pharmacological applications or to model disease pathologies. The modular nature of stem cell spheroids also highlights the utility for therapeutics, as materials amenable to direct injection for delivery *in vivo*.

Table 1

Stem Cell Response to Mechanical Cues

Mechanical Signal	Method of Force Delivery	Force Sensing Mechanisms	Cell Fate Response(s)	Reference(s)
Ligand presentation	Ligand density	CAMs	Limited spreading and increased differentiation on substrates with decreased ligand densities	48, 159
Substrate stiffness	Cross linking density, elastomeric micropost geometry	CAMs	Increased osteogenesis on stiff substrates, adipogenesis on soft substrates	49, 58
Cellular tension	Manipulation of cell shape via patterning cellular/multicellular geometry	Cytoskeleton	Increased proliferation, EMT and osteogenesis in response to increased cellular tension and spreading	64, 109, 116, 127
Cellular compression	Surrounding cells	Cytoskeleton	Decreased proliferation in regions of increased cellular compression	1, 75