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Microwell regulation of pluripotent stem cell self-renewal and differentiation

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Abstract

The fates of pluripotent stem cells (PSCs), including survival, self-renewal, and differentiation, are regulated by chemical and mechanical cues presented in the three-dimensional (3D) microenvironment. Most PSC studies have been performed on two-dimensional substrates. However, 3D culture systems have demonstrated the importance of intercellular interactions in regulating PSC self-renewal and differentiation. Microwell culture systems have been developed to generate homogenous PSC colonies of defined sizes and shapes and to study how colony morphology affects cell fate. Using microwells, researchers have demonstrated that PSCs remain in a self-renewing undifferentiated state as a result of autocrine and paracrine signaling. Other studies have shown that microwell regulation of embryoid body size affects lineage commitment during differentiation via cell-cell contact and expression of soluble signals. In this review, we discuss recent advances in the design and utilization of 3D microwell platforms for studying intercellular regulation of PSC cell fate decisions and the underlying molecular mechanisms.

Keywords

microwells; pluripotent stem cells; 3D microenvironment; cell fate

1 Introduction

The derivation of pluripotent stem cells (PSCs), which include mouse and human embryonic stem cells (mESCs and hESCs) [1-3] and induced pluripotent stem cells (iPSCs) [4], has permitted researchers to access early embryonic developmental events *in vitro* and to perform experiments to dissect pathways that regulate these events. In the developing embryo, cells experience a dynamic, three-dimensional (3D) microenvironment. This microenvironment regulates cellular processes such as survival, self-renewal, and differentiation by coordinated spatial and temporal presentation of molecular, structural, mechanical, hydrodynamic, and electrical cues. Thus, in order to fully understand how PSCs are directed towards specific lineages, systematic control over the microenvironment can be coupled with regulation of molecular signal transduction mechanisms and assessment of cell fate.

Researchers have primarily cultured PSCs on flat tissue flasks or Petri dishes because these substrates are inexpensive, simple to implement, and leave the cells easily accessible to observation, characterization, and presentation of soluble molecular cues. However, these culture systems lack specific extracellular matrix (ECM) architecture, and fail to control spatial interactions from cellular neighbors [5]. Thus, in order to predict stem cell decisions

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and control their behavior for drug screening, tissue engineering, and regenerative medicine applications, *in vitro* studies that take into consideration all components of the physiologically relevant 3D microenvironment will be necessary. While there are numerous reviews regarding the engineering of the stem cell microenvironment [5-10], this article will highlight how the microenvironmental contributions from the intercellular and autoregulatory signaling that occurs in PSCs and their derivatives can be unraveled by the development of 3D microwell cell culture platforms. In context of this review, a microwell is a structure with dimensions on the order of magnitude of 10s-100s μm designed to house 3D cellular aggregates of uniform, defined size and shape.

2 Endogenous, Intercellular Signaling in Pluripotent Stem Cells

2.1 Pluripotency and Self-Renewal

Successful PSC culture relies on both exogenous signaling factors and endogenously produced signals. Hallmark characteristics of pluripotent and self-renewing PSCs include expressing high levels of transcription factors like Oct3/4 and Nanog, and high levels of surface markers SSEA-3/4 in human PSCs and SSEA-1 in mouse PSCs. Further PSC characteristics include the ability to proliferate extensively *in vitro* while maintaining a normal karyotype and the ability to differentiate into derivatives of all three germ layers [11]. Conventionally, in order to maintain pluripotency and self-renewal, requisite exogenous factors include culturing in the presence of mouse embryonic fibroblast (MEF) feeder layers and using media supplemented with Knockout Serum Replacer and FGF-2 for human PSCs, and Leukemia Inhibitory Factor (LIF) for mouse PSCs [12]. Recently, better defined media that bypasses feeder cells and undefined components of animal origin have been developed such as mTeSR [13], StemPRO [14] and E8 [15] for human PSCs and 2i for mouse PSCs [16].

Endogenously produced signaling factors play a major role in regulating the stem cell microenvironment. The localized effects of autocrine regulation and intercellular communication either by direct cell-cell contact or paracrine signaling, help control the self-renewal, pluripotency and differentiation of stem cells. These specialized and localized microenvironments are referred to as “niches” *in vivo* and many multipotent stem cells exhibit niche dependent cell fate control [17]. PSC niches contain several autoregulatory mechanisms. For example, in a LIF-independent manner, mESCs express gp130 ligand that promotes autocrine and paracrine-induced pluripotency [18]. Similarly, Oct4⁺ pluripotent hESCs secrete GDF3 to suppress Smad1 signaling and promote maintenance of undifferentiated hESCs [19]. Even upon withdrawal of pluripotency-promoting exogenous cues, hESCs retain Oct4 expression in regions of high localized cell density. Fluid flow over mESCs cultured in a microfluidic device depleted the cell-secreted autocrine factors and caused the mESCs to exit their stable self-renewing state [20]. It was then determined that perfusion removed ECM-remodeling protein matrix metalloproteinase 2 (MMP2), preventing proper matrix remodeling and inducing an exit from the self-renewing state. In a related study, Moledina et al. directly manipulated the flow of local secreted factors and corroborated the results with a model predicting the endogenous, gp130-mediated intracellular STAT3 signaling of mESCs [21]. Though there were some discrepancies between the predictive model based on endogenous signaling with the empirically measured levels, the work firmly establishes the importance of endogenously produced signaling factors on cell fate.

In another study, hESC aggregates were encapsulated in low viscosity alginate acid and gelatin beads. The beads were 50-80 μm in diameter, with each bead typically encapsulating multiple hESC aggregates described as “closely packed.” In this format, pluripotent hESCs could be cultured in excess of 260 days without passaging or enzymatic and mechanical

manipulation [22]. Also, when mESC colony size and separation was precisely controlled using microcontact patterned ECM islands, the percentage of Oct4+ cells could be directly modulated by taking advantage of the endogenous gradients formed by mESC autocrine signaling [23]. These studies demonstrate that microscale modulation of cell-cell contact and intercellular signaling can directly affect PSC pluripotency.

2.2 Embryoid Body Differentiation

Intercellular and autoregulatory signaling drive the well-organized, dynamic, and intricate tissue development and rearrangement that occurs during embryogenesis and organogenesis. 3D aggregates of differentiating PSCs, termed embryoid bodies (EBs), have been used as a model of early embryonic development. However, EBs cannot replicate all aspects of embryonic progression, especially the later, more complex events that entail extensive structural organization or maturation [5]. Nonetheless, their autoregulatory processes are not entirely disorganized and EBs can generate descendents from all three germ layers. Upon initiation of EB differentiation, an outer layer of primitive endoderm forms, then this layer further differentiates into visceral and parietal endoderm and deposits a basement membrane. This basement membrane separates the primitive endoderm from the inner mass of undifferentiated cells and promotes the survival of adjacent cells, leaving the cells not in contact to undergo apoptosis, which contributes to the formation of a cystic cavity in the center of the EB [7].

The inner EB cells form columnar epithelium that resembles the pre-implantation epiblast. The ingression and subsequent development of mesoderm is caused by the interplay of the Wnt signaling pathway and the TGF β superfamily, beginning a gastrulation-like process [24]. An Activin A/Nodal gradient influences the expression of primitive streak associated transcription factors [25]. These early events trigger a cascade of a myriad of self-reinforcing, self-regulating, cross talking signaling pathways. Global gene expression analysis showed that this enables EBs to express genes in a temporal manner that recapitulates the sequence observed during normal development: primitive ectoderm formation, gastrulation, and eventual early cell specification prior to organogenesis [26, 27].

3 Advantages of Uniform 3D Cell Culture on EB Formation

3.1 Heterogeneity of Traditional EB Formation

Static suspension culture protocols for producing EBs simply aggregate singularized ESCs in suspension in bacterial-grade dishes [28]. A similar method for forming EBs from hESCs, which cannot survive singularization without small chemical inhibition of Rho-associated kinase (ROCK) [29], involves transferring intact colonies or cell clumps into suspension culture in a low attachment culture plate [30, 31]. However, the EBs are heterogeneous as a result of varied sizes and shapes of the ESC colonies from which they are formed. If defined numbers of mESCs are suspended in specific volumes of medium as separate droplets from the inverted lid of a Petri dish, the gravity-induced EBs that form are more homogenous than their static suspension counterparts [32]. However, this hanging drop method is not amenable to scale up and the low volume of medium per droplet is difficult to manipulate and cannot sustain longer term culture. Furthermore, the hanging drop method has not been effective for generating EBs from human PSCs. To these ends, much effort has been invested in controlling the formation of EBs to minimize heterogeneity. Typical points of reference for comparison among methods are EB size, shape, homogeneity and scalability [7].

3.2 3D Tissue Formation

A notable example of the advantage of a uniform 3D cell culture system in studying cell-cell signaling is described by Eiraku et al [27]. Prior to implementing a method for more uniform aggregation, induction efficiency of cortical-type neurons was low due to the varied sizes of EBs [33]. To initiate 3D culture, a defined number of dissociated mESCs were cultured in each well of low adhesion 96-well plates. The mESCs quickly associated and formed EB-like aggregates that differentiated in a layer and region specific manner with greater reproducibility. In a subsequent study, further structural support using basement membrane components improved the efficiency of retinal induction and subsequent formation of the retinal primordium (optic cup) [27]. Presumably the dynamic patterning and formation was self-driven by a sequential combination of local and internal cellular signals and forces. The complex morphology of the optic cup and invagination of the neural retina could only be observed in 3D culture. Thus, the differentiation of some cells and tissues may require the combination of intercellular signaling, cell adhesion, cell-cell contact, and mechanical forces in 3D EBs. Therefore, 3D microwell cell culture platforms can generate homogenous PSC colonies and minimize EB heterogeneity to allow the formation of certain 3D tissue and also aid in delineating the microenvironmental contributions of intercellular and autoregulatory signaling. Representative images of 3D cellular aggregates formed from microwells of different shapes as well as cells cultured in microwells are shown in Figure 1.

4 Innovations in Microwell Design and Manufacturing

4.1 Photolithography and Soft Lithography

One of the earliest well-related strategies in generating homogenous PSC colonies was described by Ng et al [34]. By filling round-bottom 96-well plates with a defined number of cells (300 to 10000) and then centrifuging, hESCs aggregates of defined sizes could be reproducibly produced. EBs generated by forced aggregation are often termed “spin EBs”. Using microfabricated chambers, researchers can fit many hundreds of thousands of microwells in the space of a traditional cell culture plate. In order to fabricate features at the micron to millimeter length scale, the size domain encompassing individual cells and colonies, microfabrication techniques first developed for the semiconductor industry, such as photolithography, have been adopted by cell biologists [35]. Photolithography describes the general process of precisely designing micropatterns onto silicon wafers using a photomask that selectively exposes the wafer surface to UV. Depending on the photoresist chemical chosen to cover the surface of the wafer, the exposed surfaces are either hardened to render it insoluble (“negative” photoresist), or degraded to become much more soluble (“positive” photoresist). The near-permanent micro pattern can then selectively protect the silicon wafer from micromachining. Micromachining creates topological features on the surface by etching using liquid chemicals (wet etch) or ion plasmas (dry etch) [35].

Of the microwells used for PSC culture, most integrate photolithography into the manufacturing strategy, as exemplified by Mohr et al [36]. Once microwells etched into silicon wafers were produced, Mohr et al. replicated the microstructures using a transparent silicone rubber, poly(dimethylsiloxane) (PDMS). The PDMS replicate molds (with the inverse of the features from the silicon wafer) can be easily reproduced and re-used. This process is referred to as soft lithography. Mohr et al. used the PDMS replicates to mold polyurethane substrates with the same features as the initial silicon microwell template. The surfaces between the microwells were then functionalized with a protein resistant, self assembling monolayer to confine cells to the interior of the wells. Conversely, Khademhosseini et al. designed microwell features to be replicated on PDMS, which was subsequently used for cell culture [37]. The size distribution of EBs from microwell-cultured hESCs was narrower and correlated with the dimensions of the microwells from which they

were formed. By using the PDMS to mold cell repellent poly(ethylene glycol) (PEG), Karp et al. demonstrated mESC aggregation and EB formation [38]. Moeller et al. improved upon this technology by, among other things, modifying the molecular weight of PEG to minimize non-specific cell attachment [39]. Microwells for cell culture can also be achieved by “stencils”. By laying down a thin PDMS stencil with “micro-holes” onto collagen coated glass, Park et al. localized mESC adhesion within these microwells [40]. These construction methods provide tight control over PSC colony and EB size.

Many microwell strategies, by nature of what is most feasible, have straight, vertical walls. However, some lithography techniques provide the opportunity for angled and curved microwell architecture. In order to scale up the process of making spin EBs, Ungrin et al. microfabricated pyramidal microwells that insert into standard sized cell culture plates [41]. By adjusting the cell density, they controlled the number of cells seeded per microwell and demonstrated the feasibility of generating millions of homogenous EBs per cell culture plate. In a microwell platform termed “Tapered Soft Stencil for Cluster Culture (TASCL)”, Yukawa et al. implemented micro-stereolithography to fabricate a master mold that would make curved, smoothly tapered array of PDMS microwells [42]. The researchers used TASCL as a stencil by the designing the PDMS mold to have a circular aperture at the bottom of the microwells. The openings on the bottom allowed the cells to cluster and aggregate on any sort of surface like ultra-low attachment plates or ECM-coated substrates.

4.2 Microwells Manufactured without Hard Lithography

Though the aforementioned microfabrication techniques have been widely adopted, photolithography requires access to a clean room to minimize environmental pollutants and particles. Thus, there has been effort directed toward fabricating microwells without photolithography and micromachining. A commonly used route, illustrated by Kang et al., employs a photomask to directly pattern microwells when UV-cross linking PEG hydrogels [43]. One interesting strategy implemented by Nguyen et al. to bypass photolithography altogether is laser-jet printed microwell patterns on pre-stressed polystyrene (PS). When the PS was heated to induce shrinking, round-bottomed molds were generated at a 60% reduction in in-plane size [44].

PDMS was then cast and used to culture cells and form EBs that were able to differentiate into cell populations containing beating cardiomyocytes. Choi et al. utilized vacuum and small holes to deflect thin PDMS to form convex microstructures. SU-8, a photoresist, was cast and used as a master for molding PDMS concave microwell arrays in PDMS [45]. When compared to similar scaled structures, with flat bottoms, mESCs aggregated much more efficiently and homogeneously in concave microwells. Sakai et al. implemented a programmable micromilling system to form concave microwells with diameters on the order of magnitude of 100 μm [46]. Selimovi et al. fabricated microwells on polyester film with laser ablation. The cross-section of microwells was generally conical with flat bottoms, which may aid oxygen and nutrient diffusion into the well [47]. Another interesting approach utilized dynamic, controllable materials for constructing microwells. Tekin et al. fabricated microwells with adhesive bottoms from PEG and a stimulus responsive polymer, poly(N-isopropyl-acrylamide) (PNIPAAm). PNIPAAm switches to being hydrophilic at temperatures below 32 °C and swells in water-based cell culture medium. The mechanical force caused by the swollen hydrogel expelled the cell aggregates out of the microwell [48]. While avoiding lithography has generated innovative and useful technologies, the microwell features are often less precise and subsequently fabricating complex features may not be feasible.

4.3 Microwells Integrated with Microfluidic Devices

To minimize manual manipulation of homogeneous clusters of PSCs and EBs and to provide greater spatial and dynamic control over soluble factor presentation, microwells have been incorporated with microfluidic devices. When used in biological applications, microfluidic devices are commonly made out of PDMS [10]. To be termed “microfluidic”, the device has at least one dimension, such as channel width, in the microscale. Moreover, flow speeds and directions can be precisely designed to deliver cells or gradients of soluble signaling cues. Torisawa et al. demonstrated the formation of mouse EBs within a double microchannel set-up where fresh medium could be perfused through a semi-porous membrane into the channel containing the cells [49]. The lateral dimension of the microchannel (200 μm) constrained EB diameters. The researchers also modified the device for more efficient and homogenous EB formation by designing microwells side chambers off the primary microchannel. The mESCs would get trapped in the side chambers and aggregate to form EBs. In the microfluidic system designed by Kim et al., by modeling the motion of spherical particles in the laminar flow of microchannels, the researchers were able to trap embryonic carcinoma cells in an array of microwells [50]. Differentiation towards the neural lineage was demonstrated while the EBs were within the microwells. In another modeling inspired design, Khoury et al. calculated the convective diffusion of oxygen through a micro-cup design [51]. As a result, segments were designed to form an array of U-shaped microcups that could efficiently trap cells and allow for sufficient nutrient flow through the microcup. This design also demonstrated the ability to deliver biaxial gradient of nutrients to the developing EB. Kang et al. combined the concave microwells designed by Choi et al. with microfluidics into a two tiered device [52, 45]. The bottom half consisted of microfluidic channels leading to concave microwells where EBs could form. The top half was a flat, chamber with cell culture substrate so that, upon inverting the whole device, the EBs could plate and undergo further differentiation without manual manipulations from pipettes or interactions with neighboring EBs. Jeong et al. designed a microfluidic device about the size of a glass slide with a large number of ~ 700 μm deep concave microwells 300, 500 and 700 μm in diameter [53]. To aid in efficient seeding of mESCs, the back wall of each well slanted downwards and by adjusting the density of mESCs injected into the device, the researchers could control the size of the EBs that formed. Inverting the device permitted harvest of the EBs. Coupling microwells and microfluidics offers great potential for studying the effects of the 3D microenvironment on PSC fates. A summary of common methods used to seed and fabricate microwells are depicted in Figure 2 while Table 1 highlights key features from the examples discussed within this review.

5 Microwell Culture Effects on PSC Self-Renewal and Differentiation

5.1 Microwell Culture Effects on PSC Self-Renewal

Over the past decade the development of microwell culture systems has enabled generation of EBs of consistent and homogeneous size and shape. Importantly, these microwell culture systems have illustrated that 3D cell interactions contribute to PSC self-renewal and differentiation fates. Khademhosseini et al. employed microwell-patterned PDMS for co-culturing MEF cells along with hESCs [37]. The entire surface of their microwell array was coated with fibronectin and MEFs. ESCs were confined to the 200×120 μm wells by using medium flow to gently remove cells that had not settled within wells. The viability and pluripotency, defined by Oct4 expression, of hESCs cultured in microwells were reported to be similar to hESCs cultured in 2D. To further illustrate how intimately tied PSC fate decisions are with intercellular signaling from the 3D microenvironment, Mohr et al. cultured hESCs within 3D cuboidal microwells with dimensions ranging between 50 to 500 μm laterally and up to 120 μm deep [36]. The hESCs proliferated to fill the microwells and expressed pluripotency markers. In fact, after 2.5 weeks without passaging, the viability and

percentage of Oct4+ cells in the microwells remained similar to continuously-passaged hESCs but much higher than in the unpassaged 2D cultured control. At any time, the cells could be removed and continued to be passaged in a self-renewing, pluripotent state in traditional 2D culture. In addition, the size distribution of the colonies upon enzymatic removal from the microwells was substantially narrower than the size distribution of colonies expanded on Matrigel-coated 2D substrates.

Sakai et al. compared EBs from mESCs made from 3D microwells, 2D micropatterned adhesive islands, round-bottomed 96-well plates without forced aggregation and the hanging drop method [46]. EBs were allowed to form and differentiate for 7 days. In the hanging drop and round bottomed 96-well plate cultures, the majority of the EBs developed cystic structures in the center. The mESCs inoculated on the 2D chip adhered to the micropatterned gelatin spots, formed a monolayer, and then gradually proliferated and formed EBs. The EBs within the microwells never adhered to the surface. Interestingly, though the ESCs proliferated, the EBs formed from microwells and micropatterning remained closely packed without any cyst formation. Moreover, in contrast with EBs from hanging drop, 96-well plates, and micropatterned chips, the EBs within microwells exhibited greater expression of pluripotency markers and markedly less expression of mesoderm and endoderm lineage markers. This is consistent with a previous report that hESCs remain viable and undifferentiated for weeks within microwells [36]. While Sakai et al.'s culture conditions were designed to promote differentiation, and Mohr et al. provided self-renewal factors in the culture medium, these studies demonstrate that pro-pluripotency autoregulation can be accentuated in configured 3D culture such that the effects on the cell fate decisions of the whole PSC population are substantial.

5.2 Microwell Culture Effects on Differentiation

Hong et al. compared EBs generated from hESCs in suspension, hanging drops and forced aggregation in microcentrifuge tubes [54]. Examining the impact on hematopoietic output, they observed that the dissociation and re-aggregation of spin EBs did not have a negatively impact, but instead discovered that the consistent ability to fabricate larger EBs provided an advantage. In addition, Hong et al. sought to determine whether the endogenous factors diluted in culture medium of developing EBs could be responsible for hematopoietic output [54]. By comparing the generation of hematopoietic cells, multiple EBs cultured together significantly outperformed EBs cultured in isolation. Moreover, using medium conditioned with multiple developing EBs on individually cultured EBs rescued the hematopoietic potential, further exemplifying the strong effects of intercellular communication mediated by soluble factors in the microenvironment.

When Ng et al. first described forming spin EBs of different sizes, they found that initial seeding of over 500 cells/well generated myeloid and erythroid cells in over 90% of EBs [34]. Burrige et al. reported a correlation between EB size and cardiomyogenic potential in human spin EBs generated in V-bottom 96 well plates and induced to differentiate toward cardiomyocytes [55]. EBs between 250 and 350 μm diameter had the greatest likelihood of containing beating regions and expressed cardiomyocyte genes at higher levels than other EBs. In the microwell platform designed by Mohr et al., the 100 to 300 μm microwells produced EBs with higher cardiogenic potential than EBs from colonies cultured in larger microwells or from unconfined 2D substrates [56]. Using adhesive stencils with holes that form microwells on tissue culture substrates, Park et al. seeded mESCs into 100, 300 and 500 μm diameter microwells and allowed the mESCs to adhere to the substrate surface and aggregate into EBs [40]. EBs made from smaller stenciled microwells expressed higher levels of ectoderm-associated genes and proteins, while the EBs from larger microwells favored mesoderm and endoderm gene expression. Nguyen et al. utilized shrink-induced, concave-bottomed microwell arrays to form mESC aggregates about 65, 85 and 105 μm

diameter [44]. The larger EBs preferentially upregulated endoderm and ectoderm markers and tended to retain the larger size as the cystic center formed. In fact, Burridge et al. were able to integrate the efficient formation of uniform homogenous human EBs into cardiac directed differentiation protocol to generate over 90% beating EBs in just 9 days with cardiomyocytes comprising 60-90% of the cells, depending on the hESC line used [57]. These studies confirm the influence microwell culture has in directing the cell fate decisions during PSC culture, EB formation and differentiation. Together, these studies illustrate that culture of PSC colonies in microwells results in the different lineage commitment that colonies cultured on 2D substrates, and that EB size affects cell differentiation fate.

6 Molecular Mechanisms of Microwell Regulation of PSC Fate

Very little is yet known regarding the precise molecular mechanisms by which intercellular interactions drive the significant differences in cell fate observed between PSC culture in 3D microwells and on 2D substrates. Moreover, how the subtle differences in techniques for establishing 3D aggregates influence cell fate decisions have not been explored extensively.

While hESCs cultured within microwells and on 2D substrates both exhibited molecular signatures of pluripotent cells, including high expression of Oct4 and Nanog, quantitative differences in cell phenotypes were identified [58]. Within a few days after seeding, cell proliferation rates were lower in microwells than on 2D unconfined substrates and decreased as microwell size decreased. While the overall cell cycle duration in 3D and 2D culture were similar, the cells in microwells spent more time in G1 but less in G2/M than cells on 2D substrates. As early as 2 days after seeding, cells cultured in microwells were significantly smaller than cells on 2D substrates and cell size continued to decrease with longer microwell culture times whereas cell size on 2D substrates remained constant. Moreover, cell metabolism, represented by specific glucose consumption and lactate production rates, was lower in cells cultured in microwells, perhaps as a result of the lower proliferation rate. Since cell metabolism, growth and proliferation rates have been shown to be regulated by cell size [59], the smaller cell volumes in microwells may serve as signals to these changes. Though incompletely understood in mammalian cells, 3D confinement may mechanically alter the interactions between macromolecular complexes like the lipid membrane and ion channels, which are mainly responsible for cellular responses to volume perturbations [60, 61].

Furthermore, Azarin et al. recently examined how the increased cell-cell contact during hESC microwell culture modulates molecular mechanisms that govern lineage specification [62]. The surface density of E-cadherin, which mediates ESC cell-cell contact and is a key regulator of stem cell pluripotency and self-renewal [63], was found to be 5-fold higher in cells cultured in microwells as opposed to on 2D substrates. Also, though β -catenin was found to be colocalized with E-cadherin at the cellular membrane with both microwells and 2D culture, β -catenin predominantly localized to the cell membrane in cells cultured in microwells. In epithelial cells such as hESCs, β -catenin physically links E-cadherin to the actin cytoskeleton in adherens junction, but also acts as a transcription co-factor of the canonical Wnt signaling pathway [64]. In fact, hESCs cultured in microwells exhibited lower expression Wnt signaling pathway-associated genes when compared to hESCs cultured on 2D substrates [62].

3D culture of hESCs in microwells also affects developmental signaling during differentiation. Upon enzymatic removal of hESC colonies from microwells and EB differentiation, Azarin et al. also observed significantly higher canonical Wnt signaling in EBs made from microwell-cultured hESCs [62]. Moreover, cells that demonstrated higher levels of Wnt signaling activity exhibited greater expression of genes associated with the

primitive streak, mesoderm and cardiac lineage. These findings suggest that the morphology of undifferentiated ESC colonies regulates developmental signaling pathways which subsequently affect lineage commitment of the cells.

A different mechanism of colony morphology regulation of ESC signaling was described by Hwang et al. [65] Mouse ESCs were allowed to settle, aggregate, and differentiate within non-adhesive PEG microwells of controlled dimensions. The microwell size of 450 μm was reported to enhance cardiogenesis in EBs while smaller microwells of 150 μm increased endothelial cell differentiation. In the EB sizes that favored cardiogenesis over endothelial cell differentiation, non-canonical Wnt11 was expressed at a greater level than Wnt5a. When Wnt5a expression was knocked down using siRNA, endothelial vessel sprouting was ablated while cardiac markers became highly expressed. However, while exogenous Wnt5a addition did not significantly alter cardiac markers, the sprouting of endothelial vessel structures and expression of endothelial markers was increased.

A study by Bauwens et al. also described the control cell colony size has over differentiation potential and a cellular mechanism by which this control may occur [66]. Formation of an outer epithelial layer of extra-embryonic endoderm (ExE) cells is one of the earliest events during EB differentiation. Since ExE and its derivatives have been shown to promote cardiomyocyte differentiation in cell populations residing in EBs, Bauwens et al. hypothesized a direct relationship between ExE cell concentration and cardiac differentiation efficiency. By varying expression of SOX7, a transcription factor required for ExE differentiation in hESCs, they demonstrated that cardiac differentiation is a function of endogenous ExE cell concentration. In addition, by producing spin EBs of different sizes, they established that initial aggregates of approximately 1000 cells were optimal for cardiomyocyte differentiation. Thus they showed that cell aggregate size, which specifies the outer surface area-to-volume ratio, can determine the amount of cardiac-promoting ExE in the EB, which in turn promotes cardiomyogenesis.

Much has yet to be identified regarding the milieu of molecular mechanisms by which morphology of PSC colonies regulates developmental signaling pathways and affects lineage commitment. Microwells and the morphology of 3D colonies and EBs likely modulate a host of biophysical factors including external mechanical forces, cell shape and geometry, and extracellular matrix topography and mechanics. The mechanotransduction processes responding to these physical differences in the local cellular microenvironment coalesce with other signaling pathways to influence and change PSC fate decisions [67].

7 Conclusions and Future Perspectives

The derivation of human ESCs at the turn of the century unleashed a torrent of interest and research in stem cell biology, which has greatly increased our understanding of cell and tissue development. At the same time, the importance of culturing cells in three dimensions was realized, in part catalyzed by Bissell and colleagues' seminal study [68]. Bissell demonstrated that antibodies against a particular cell-surface receptor completely changed the behavior of cancerous breast cells grown in 3D culture, but not in 2D culture [69]. Just by changing the way a cell interacts with its 3D environment, researchers can substantially alter its behavior. It is now well accepted that key events in the life cycle of a cell are regulated by cellular context, and 3D cell culture can establish physiologically relevant cell-cell and cell-ECM interactions [70].

For the past decade, stem cell scientists have sought to understand how the spatial organization of the 3D microenvironment plays a role in stem cell fate decisions. The 3D aggregation of PSCs into embryoid bodies has been integral in differentiating PSCs to many somatic cell types. Microwell systems have been a simple though effective method to

implement 3D cues in PSC culture. Though the use of microwells began as a method to generate EBs of predictable size, shape, cell number, and composition, microwells also permit researchers to probe the effects of culture dimensionality and endogenous, intercellular signaling on PSC fate. Through modulation of the biochemical and biomechanical microenvironment, microwell technology can help elucidate the molecular factors and pathways that lead to complex cellular responses.

In the future, much can be done to enhance the microwell platform to present growth and differentiation cues in a more physiologically-relevant microenvironment. For example, Gobaa et al. designed a microwell array with modular stiffness that could be functionalized with combinations of proteins spotted by robotic technology in order to delineate synergies in mechanical and chemical regulation of mesenchymal and neural stem cells in 3D [71]. Further development of similar microwell platform could be implemented to study cell behavior in a “deconstructed, systematically-controllable” microenvironment. The study of clonal PSC behavior can also be achieved using microwells, similar to the microwell array for hematopoietic stem cell culture designed by Lecault et al., which integrated microfluidics to enable non-perturbing, single cell capture [72]. This contrasts with the gravity settling or microfluidic seeding of many PSCs per well with the microwell technologies discussed within this review. As for reconstructing relevant microenvironments, there has been much progress in fabricating and implementing biomaterials into engineered microenvironments [9, 73]. Advances have been made in the incorporation of signaling factors via micro-/nano-particles [74] in microwells to more effectively direct cell fate decision. PSCs can be encapsulated in structural materials such as ECM [75] or in spatially organized, 3D hydrogels that incorporated signaling factors [76]. Microwells have even been developed in conjunction with microelectrode arrays that can be used to characterize electrical activity of cells [77]. Microwells have even been designed so that the bottom face is a fabricated electrospun network of nano/microfibers [78]. Depending on the fiber material, electrospinning technique and post-electrospinning functionalization, physiologically relevant scaffolds can be incorporated into microwells to potentially elicit specific cellular responses [79]. In efforts to facilitate better transport of media and nutrients for scale-up, self-loading, 3D microcontainers have also been designed for living cells [80]. Altogether, microwell platforms are a multifaceted tool for the study of PSC cell fate decisions and the underlying molecular mechanisms.

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Fig. 1.

(a) Phase contrast images of human embryonic stem cells cultured in microwells of different shapes. Approximately 6 days after seeding cells into the wells and expanding cells in MEF conditioned medium, the wells were confluent and the colonies were removed by enzymatic (Dispase) treatment (b) The resulting EBs possessed the shape of the microwell. (c) Confocal fluorescent image of a hESC colony stained with DAPI (blue) and phalloidin (red) in a microwell is shown. (d) Colonies were removed from microwells shown in c and cultured in suspension for one day to form EBs. Cells were stained with DAPI (blue), anti-E-cadherin antibody (red) and anti- β -catenin antibody (green). After 1 day in suspension culture the cuboidal colony became a spherical EB, and spatial gradients in protein expression were detected.

Fig. 2.

Summary of methods for generating 3D aggregates of human pluripotent stem cells. References are provided for examples of these methods.

Table 1

Summary list of examples of 3D microwell cell culture platforms discussed within this review. miPSCs: mouse induced pluripotent stem cells, HepG2: human hepatoblastoma cells, mNPS: mouse Neural Progenitor/Stem cells, mEC: mouse embryonic carcinoma cells, PDMS: poly(dimethylsiloxane), PEG: poly(ethylene glycol), PEGDA: poly(ethylene glycol) diacrylate, PMMA: polymethylmethacrylate, PNIPAAm: poly(N-isopropyl-acrylamide)

Authors	Cell types	Application	Materials	Fabrication & Key Features
<i>3D microwell cell culture platforms utilizing photolithography</i>				
Mohr JC et al. [36]	hESCs	culture, EB formation	Polyurethane	soft lithography, protein resistance applied outside of wells
Khademhosseini A et al. [37]	hESCs, MEFs	culture, EB formation	PDMS	soft lithography
Karp JM et al. [38]	mESCs	EB formation	PEG	soft lithography
Moeller HC et al. [39]	mESCs	EB formation	PEG	protein resistant PEG
Park J et al. [40]	mESCs	culture, EB formation	PDMS, glass	stencil with “micro-holes”
Ungrin MD et al. [41]	hESCs	EB formation	PDMS	soft lithography, pyramidal wells
Yukawa H et al. [42]	miPSCs	EB formation	PDMS	tapered soft stencil
<i>3D microwell cell culture platforms bypassing photolithography</i>				
Kang L et al. [43]	mESCs	culture	PEGDA	photomask when UV-cross linking PEG
Nguyen D et al. [44]	mESCs	EB formation	PDMS	laser-jet printing on heat shrinkable polystyrene
Choi YY et al. [45]	mESCs	EB formation	PDMS	SU-8 cast by vacuum deflected PDMS convex microstructures
Sakai Y et al. [46]	mESCs, rat hepatocytes, HepG2, mNPS	EB formation	PMMA	programmable micromilling technology
Selomovi S et al. [47]	mESCs, HepG2	culture, EB formation	Polyester film, glass	laser ablation
Tekin H et al. [48]	HepG2	EB formation	PEG, PNIPAAm	temperature responsive (swells at <32°C)
<i>3D microwell cell culture platforms incorporating microfluidics (requiring photo- & soft lithography)</i>				
Torisawa Y et al. [49]	mESCs	EB formation	PDMS	microchannel constrains EB diameters
Kim C et al. [50]	mEC	EB formation differentiation	PDMS	microwells trap cells from laminar flow
Khoury M et al. [51]	hESCs	EB formation differentiation	PDMS	micro-cups trap cells and allow diffusion of nutrients through micro-cup design
Kang E et al. [52]	mESCs	EB formation differentiation	PDMS	two-tiered device for formation and then attachment of EBs
Jeong GS et al. [53]	mESCs	EB formation	PDMS	deep, concave microwells with slanted back wall, easy harvesting