

Probing Embryonic Stem Cell Autocrine and Paracrine Signaling Using Microfluidics

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Abstract

Although stem cell fate is traditionally manipulated by exogenously altering the cells' extracellular signaling environment, the endogenous autocrine and paracrine signals produced by the cells also contribute to their two essential processes: self-renewal and differentiation. Autocrine and/or paracrine signals are fundamental to both embryonic stem cell self-renewal and early embryonic development, but the nature and contributions of these signals are often difficult to fully define using conventional methods. Microfluidic techniques have been used to explore the effects of cell-secreted signals by controlling cell organization or by providing precise control over the spatial and temporal cellular microenvironment. Here we review how such techniques have begun to be adapted for use with embryonic stem cells, and we illustrate how many remaining questions in embryonic stem cell biology could be addressed using microfluidic technologies.

Autocrine: refers to diffusible signaling from one cell to itself or to another cell of the same cell type

Paracrine: refers to diffusible signaling from one cell type to a nearby, different cell type

Inner cell mass (ICM): the cells inside a blastocyst that give rise to all the cells in the adult organism

Blastocyst: an embryonic structure consisting of an ICM that forms the embryo and a trophoblast that forms the extraembryonic tissues

1. INTRODUCTION

Cellular phenotype is heavily influenced by the extracellular environment. Cells constantly create and respond to external signals, including soluble secreted factors, direct cell-cell communication through gap junctions and cadherins, and extracellular matrix-based signals. These external signals, in turn, drive signaling cascades that lead to maintenance or alteration of the cellular phenotype. Thus, technologies that aid in elucidating and controlling these external cues are critical to understanding cell biology and harnessing cells commercially or therapeutically.

Stem cells in particular are strongly influenced by their extracellular environment because of their pliability. Stem cells can create new copies of themselves indefinitely—a property known as self-renewal—while maintaining the ability to differentiate into specialized cell types (1). External cues strongly influence whether a stem cell decides to self-renew or differentiate, and one question in stem cell biology is how to determine the set of signals required to dictate any given stem cell decision.

One set of external cues that are particularly important to stem cell biology consists of the soluble signals that the stem cells themselves produce and respond to, known as autocrine signals, and the signals produced by other cell types in the stem cells' microenvironment, known as paracrine signals. These signals can act at short distances, down to the scale of a single cell (2), or can signal over many cell diameters (3). These autocrine and paracrine cues affect diverse processes in stem cells, including self-renewal (4, 5), growth (6, 7), and differentiation (8), and they may contribute to heterogeneity in stem cell cultures (5, 9). In short, they are incredibly important. However, their ubiquitous presence (i.e., they are difficult to remove) and intrinsic closed-loop nature (i.e., they may be difficult to detect externally) make them technically challenging to study in any cell system and in stem cells in particular.

Microfluidics provides opportunities to address these technical challenges. By allowing for control of cells and their environment with spatial resolution down to the scale of a single cell and timescales down to subseconds, microfluidic systems can play an important role in teasing apart the complex interactions involved in stem cell autocrine and paracrine signals. In this review, we describe how microfluidics can be applied to the study of autocrine and paracrine signaling in stem cells. We employ an expansive definition of microfluidics, including methods that involve manipulation of liquids as well as those that involve micrometer-scale control of cells (e.g., cell patterning). To keep focus, we concentrate on questions in embryonic stem cell (ESC) biology, although many of the technologies can be applied to related questions in other stem cell systems.

We first provide an introduction to ESC self-renewal and early differentiation, which leads to a set of open questions in the field. We then describe existing methods used to study autocrine and paracrine signaling in stem cells, providing context for the subsequent description of how microfluidics can aid in studying those questions. For the microfluidic technologies presented, we provide caveats and issues to consider when designing and implementing the techniques.

2. AUTOCRINE AND PARACRINE SIGNALS INVOLVED IN EMBRYONIC STEM CELL PROCESSES

2.1. Embryonic Stem Cells

ESCs are pluripotent cells—they can differentiate into all embryonic lineages—derived from the inner cell mass (ICM) of a blastocyst (10, 11). In addition to being pluripotent, they can also self-renew, by dividing while maintaining their pluripotent state, or differentiate, by dividing and exiting their pluripotent state to adopt other phenotypes. ESCs have been derived from several mammals (10–16), but mouse (10, 11) and human (12) ESCs (mESCs and hESCs, respectively)

are the most commonly studied. ESCs are readily available from cell banks and can be derived de novo, are relatively straightforward to culture, and can divide indefinitely in culture without losing their pluripotency. These cells' ability to differentiate into all tissues in the adult explains their clinical significance, as they have the potential to form cells or tissues for regenerative medicine, and clinical trials using hESC-derived cells are under way (17).

Endogenous:
emanating from a cell
or a population of cells

2.2. Autocrine and Paracrine Signaling

Autocrine signaling and paracrine signaling canonically refer to soluble signals produced by cells to which they respond (autocrine signals) or to which neighboring cells respond (paracrine signals) (18). In this review, we use the term autocrine signaling to refer to signals secreted by a cell that may bind to that cell or to a neighboring cell of similar phenotype, whereas paracrine signaling refers to signals produced by a cell to which that cell type cannot respond but other cell types can. Also, we differentiate between signals added to the culture media versus soluble signals released by the cells with the terms exogenous and endogenous, respectively.

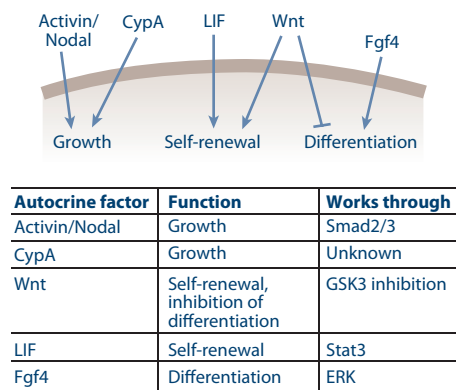
2.3. Self-Renewal

Self-renewal is the most fundamental process that ESCs undergo and is interesting both from a basic cell biological viewpoint (i.e., How do ESCs decide to stay ESCs?) and from a biotechnological viewpoint (i.e., How do we design bioprocesses that allow expansion of ESCs to therapeutic scales while maintaining pluripotency?). Thus, significant effort has been expended over the past ~30 years to understand ESC self-renewal, which has led to the identification of exogenous signals that are important for this process. hESCs and mESCs have differing self-renewal requirements that are thought to have arisen because of their different origins and/or species-specific differences. mESC pluripotency can be retained through the addition of leukemia inhibitory factor (LIF) with serum or in serum-free media with LIF and bone morphogenetic protein 4 (BMP4) (19, 20). hESCs do not self-renew in LIF media; instead, they require exogenous Activin and fibroblast growth factor 2 (FGF2) (21, 22). Although exogenously added soluble factors can induce self-renewal, are they sufficient? One cannot answer this question without knowing the contributions of endogenous factors.

Several mESC autocrine factors that influence proliferation have been identified (**Figure 1a**), including Activin, Nodal, and Cyclophilin A (6, 7). LIF also acts in an autocrine fashion (23–25), albeit not at levels sufficient to maintain self-renewal, whereas autocrine Wnt signaling is required for mESC self-renewal (4). Other factors probably contribute, but their activities are obscured in standard culture settings.

The known autocrine pathways in hESCs are mostly distinct from those found in mESCs (**Figure 1b**). Fgf2 is an autocrine factor (26, 27), a role that is reinforced in normal cultures by exogenous Fgf2 addition (28). Part of the mechanism may be due to a paracrine loop that forms in hESC cultures, whereby some fraction of the population differentiates to a more fibroblast-like state that then secretes insulin-like growth factor II in response to exogenous FGF2, which in turn serves to keep the hESCs in a self-renewing state (5). Transforming growth factor (TGF)- β /Activin signaling has also been proposed as an autocrine loop for maintenance of hESC self-renewal (29), and the addition of Activin may induce autocrine FGF2 production in hESCs (30). Growth differentiation factor 3 (GDF3), also in the TGF- β superfamily, is secreted from hESCs and acts to block BMP and thereby inhibit differentiation (31), whereas autocrine levels decrease during differentiation (32). Also, as in mESCs, endogenous Wnts help maintain hESCs in an uncommitted state, acting in hESCs by blocking neuronal differentiation (33).

a Mouse embryonic stem cell



b Human embryonic stem cell

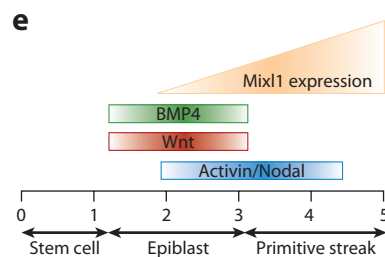
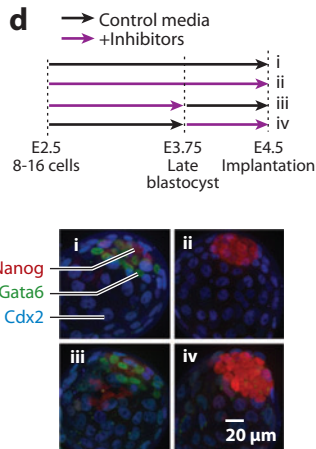
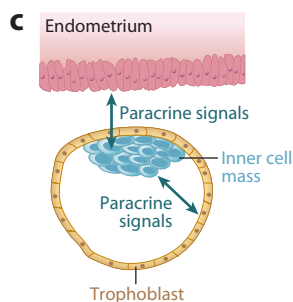
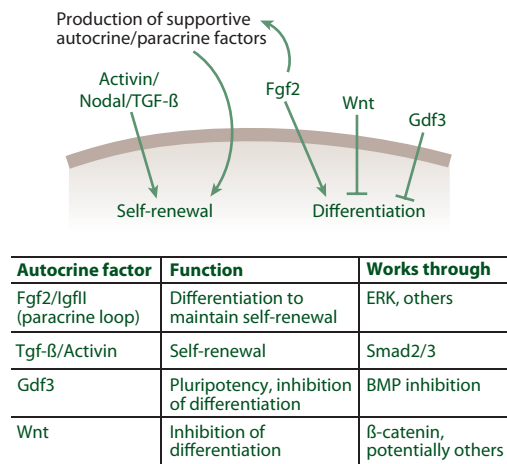


Figure 1

Embryonic stem cell (ESC) autocrine and paracrine signaling. (a,b) Functional autocrine-acting signals that have been identified in (a) mouse ESCs (mESCs) and (b) human ESCs (hESCs) and their roles. (c) Origin of paracrine signals involved in developmental specification in the early embryo. (d) Timing of autocrine fibroblast growth factor (FGF) signaling affects lineage specification. Exposure of embryos to control media (i) from embryonic day (E)2.5 to E4.5 resulted in normal development of trophoblast (Cdx2 expression, blue), primitive endoderm (Gata6 expression, green), and epiblast (Nanog expression, red), whereas continuous exposure to FGF-MAPK (mitogen-activated protein kinase) inhibitors (ii) affected primitive endoderm development. Treatment with inhibitors at (iii) early or (iv) late time points shows that embryos are sensitive to inhibition only at late time points (iv). Scale bar, 20 μ m. Reproduced from Reference 42 with permission from the Company of Biologists. (e) Depiction of time (in days) and differentiation stages during which ESCs are responsive to various stimuli (BMP4, Wnt, Activin/Nodal) for the induction of Mixl1. Abbreviations: BMP, bone morphogenetic protein; CypA, Cyclophilin A; ERK, extracellular signal-related kinase; GSK, glycogen synthase kinase; IGFL, insulin growth factor like; LIF, leukemia inhibitory factor; TGF, transforming growth factor. Panel e adapted from Reference 44.

Determination of the signals that affect pluripotency *in vitro* is related to our understanding of early embryonic development, given that the pluripotent state *in vivo* is transient and proper development requires an exit from this state as a result of maternal and embryonic autocrine and paracrine signaling (**Figure 1c**). Indeed, paracrine communication between cells that make up the ICM and the trophectoderm or between extraembryonic cells and adjacent epiblast cells is an important part of early mouse embryonic growth (34, 35), and blastocyst implantation in mice requires paracrine LIF expression in the uterus (36). Thus, numerous endogenous factors influence stem cell self-renewal and embryonic development, and complete elucidation of these signals is important for understanding ESC biology and for fully exploiting these cells therapeutically and commercially.

Epiblast:

a postimplantation blastocyst tissue that gives rise to all three germ layers that make up the adult organism

2.4. Exit from Self-Renewal

Although the constant addition of factors can induce the continual self-renewal of ESCs, the dynamics of factor additions play a crucial role in the exit from self-renewal and subsequent differentiation. Many differentiation protocols use timed exposure to various exogenous growth factors (37, 38) to drive cells to particular end points, often in an attempt to recapitulate timing of corresponding developmental stages. Timing of autocrine or paracrine factor presentation is also important. For example, signaling from the autocrine growth factor Fgf4 through extracellular signal-related kinase 1/2 (Erk1/2) is required for the adoption of an early neural fate in mESCs (39–41). The timing of Fgf4 presentation is important, and researchers have used pulsed addition of inhibitors to standard cultures to show that only a short period of Erk1/2 activation is required, although the exact temporal requirements have not been fully defined (39).

Fgf signaling also plays an important role in development, and again, the timing of signal presentation plays a crucial role (42, 43). In one example, during ICM maturation, cells from the ICM segregate and become either primitive endoderm or epiblast. The mechanism behind this segregation was unknown until recently: The cells segregated due to either their position or external stimuli. Recent research has identified endogenous FGF signaling through mitogen-activated protein kinase (MAPK) as the determining factor and found a temporal requirement for FGF-MAPK signaling *in vivo* (**Figure 1d**) (42). Thus, for both neural precursor formation *in vitro* and the formation of epiblast *in vivo*, the timing of autocrine or paracrine signaling is important.

In another example, researchers sought to determine when specific additions were critical for specification of the mesoderm lineage, as assessed by *Mixl1* expression (44). The critical timing periods for BMP4, Wnt3a, and Activin A additions differed; if timing was off, *Mixl1* expression was not induced (**Figure 1e**). These signals also induced paracrine loops to propagate mesoderm induction. However, the temporal windows described were resolved only to days or half-days, which may not correspond to the true temporal resolution.

2.5. Areas of Further Study

Although significant progress has been made in understanding the involvement of cell-secreted signals in ESC processes, many questions remain. Two broad areas of study are described below.

The first involves determination of all the signaling components that are important in maintaining ESC self-renewal. Although many components are known to be important or sufficient, the inability to remove the contribution of autocrine signals means that we still do not know which signaling pathways are minimally required and which are important but dispensable. Is there one overarching pathway that is required, or are several required in conjunction? Are there separate

pathways that alone can provide the necessary signaling to maintain self-renewal? For us to fully understand the fundamental processes required for maintenance of pluripotency, we need to know precisely which signals are necessary versus expendable, and we should understand which signals contribute to the opposite phenomenon—exit from self-renewal.

The second question involves determining the dynamics of autocrine and paracrine signaling involved in the exit from the self-renewing state *in vitro* and the corresponding early embryonic state *in vivo*. These temporal requirements are important both in terms of exogenous additions that are frequently used to mimic paracrine signals sensed *in vivo* and in terms of endogenous signaling, as rapid cell-cell signaling may occur *in vivo* to trigger differentiation pathways. A thorough understanding of when the presence or absence of certain signals is required for a particular early cell-fate decision will also aid us in understanding the mechanisms that determine why cells decide to self-renew versus differentiate, and will allow for improved directed differentiation.

3. CONVENTIONAL METHODS FOR INVESTIGATING AUTOCRINE AND PARACRINE SIGNALING

Cell-secreted factors are the “dark matter” of soluble signaling: They are there in abundance but are challenging to study due to their intrinsic closed-loop nature. Below, we describe the approaches that have been undertaken to address questions of autocrine and paracrine signaling. These methods have been quite successful, but their remaining limitations present opportunities for microfluidic techniques.

3.1. Pathway Inhibition

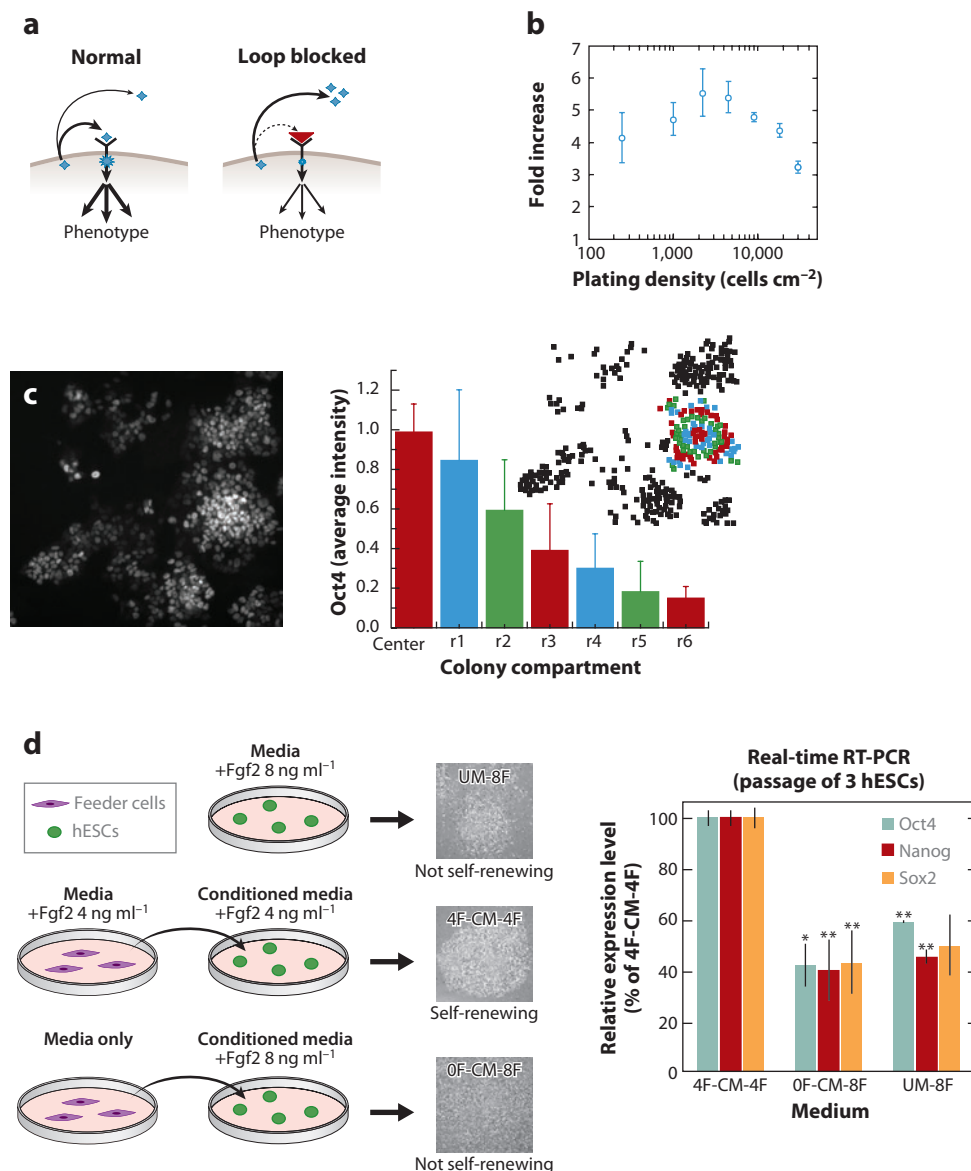
When the specific factors or receptors that are part of an autocrine or paracrine signaling pathway are known, the best way to investigate the contributions of the pathway is via inhibition with knockout cell lines or specific inhibitors. Many small-molecule inhibitors that are specific to receptors or downstream signaling molecules have been identified, and blocking antibodies can be developed to target known proteins or receptors. Cell lines can also be derived in which specific secreted proteins or receptors are knocked out. With these reagents, one can perform the definitive experiments to identify and characterize an autocrine loop (**Figure 2a**). By measuring the ligand

Figure 2

Conventional methods for investigating autocrine or paracrine signaling. (a) As autocrine factors are secreted from a cell, they bind to receptors to trigger a downstream response (*left*), unless the receptor is blocked by a receptor-blocking antibody, in which case autocrine signals accumulate in the surrounding media (*right*). (b) Varying cell density to study autocrine signaling. Growth of mouse embryonic stem cells (mESCs) on day 2 depends on initial cell-plating density, suggesting an autocrine loop. Reproduced from Reference 7 with permission from Elsevier. (c) Use of spatial information to study autocrine signaling. (*Left*) Image of Oct4 staining in a mESC colony along with (*right*) quantification of the intensity binned into concentric rings, showing that the intensity decreases with distance from the center of the colony. Reproduced with permission from Reference 24. Copyright 2006, Wiley. (d) Use of conditioned media to study paracrine signaling. Depiction of coculture experiment indicating that fibroblast growth factor 2 (FGF2) produces a paracrine-acting signal that is necessary for the maintenance of human embryonic stem cell (hESC) self-renewal. Conditioned or unconditioned media with the indicated additions were added to hESC colonies, and only feeder cells for which FGF2 was added to the media conditioned media that supported hESC self-renewal, as shown by morphology and expression of the self-renewal markers Oct4, Nanog, and Sox2. Abbreviation: RT-PCR, reverse transcription polymerase chain reaction. Data images reproduced with permission from Reference 27. Copyright 2007, Wiley.

in the media and characterizing the phenotype with and without a receptor-blocking antibody, one can determine that (a) the cells are secreting ligand, (b) the ligand binds to the receptor, and (c) ligand binding alters the phenotype (45, 46). Importantly, all these methods are limited to studies of known factors.

What is currently known about autocrine signaling in ESCs has been determined primarily with these methods. For example, when autocrine Wnt signals were identified as necessary for mESC self-renewal, the investigators found that addition of either a Wnt antagonist or an inhibitor of Wnt signal production could halt self-renewal, an effect that could be reversed with exogenous Wnt addition (4).



3.2. Using Global and Local Cell Density

In cases where the cell-secreted factors of interest have not been identified, one approach to assessing the contribution of autocrine signaling is to vary the global cell density and assess the density dependence of a particular phenotype, which then sets up a search for specific autocrine factors. This approach was used after observation of the density-dependent growth of mESCs (**Figure 2b**) to determine that this phenotype depended in part on the presence of the autocrine growth factor Cyclophilin A (7). However, varying density does not provide a complete picture because autocrine signals can be sufficient at clonal density (47), and both autocrine signaling and contact-mediated signaling typically vary with cell density.

Instead, one can study autocrine signaling by quantifying phenotypes on the basis of spatial position and local cell density. Quantitative single-cell immunocytochemistry, combined with computational modeling, was used with mESCs to determine that, in the absence of LIF, radial organization of self-renewal markers was observed, with higher marker expression in areas of higher local cell density. This phenomenon was attributed to an unknown autocrine non-LIF gp130 ligand (**Figure 2c**) (24). Spatial variation was also found in hESC colonies in terms of Oct4 levels and Smad1 signaling (9), and cells at the periphery of hESC colonies have distinct morphologies and different cell growth and death characteristics (48)—all phenotypes that were attributed to the presence of autocrine and paracrine cues.

Although varying global cell density or measuring local cell density in traditional cultures can be useful, it can be challenging to control for varying colony morphologies and to standardize immunofluorescent staining across experiments. A particular challenge with imaging mESCs is that the colonies grow as multilayered clumps, so studies measuring fluorescence intensity in two dimensions require either a way to functionalize the surface so that the colonies spread out (24) or a way to image cells in three dimensions.

3.3. Conditioned Media and Coculture Studies

An alternative to varying cell density is to use conditioned media assays. Experiments in which a medium that has been exposed to one population of cells for conditioning (i.e., to be loaded with cell-secreted factors) and then transferred to a separate cell population have been used in studies of both autocrine and paracrine signaling. In studies of autocrine signaling, conditioned media can be used as a surrogate for cell density because cells can be grown sparsely and conditioned media can then be added to simulate culture at high density. This approach has been used to find that autocrine factors are important for the maintenance of a short G₁ cell-cycle phase in hESCs (49).

Conditioned media studies are even more powerful in studies of paracrine signaling. Many *in vitro* protocols for differentiation of ESCs rely on conditioned media or on coculture with other cell types (50–52). One common method involves use of a transwell, an insert that allows paracrine signals to pass between cells cultured in a single well that are separated by a protein-permeable membrane. Conditioned media was used to determine the effect of exogenously added FGF2 on the maintenance of hESC cultures. Exogenous FGF2 is normally added in hESC cultures that consist of hESCs and feeder cells. However, FGF2 is not required for the maintenance of the hESCs; instead, it is required for signaling to their feeder cells, which results in upregulation and secretion of other proteins that are required for hESC self-renewal (**Figure 2d**) (27). Although useful, conditioned media assays may suffer from inconsistency because the complement of growth factors present may vary according to cell-seeding density, growth time, and preparation and storage of conditioned media.

4. MICROFLUIDIC APPROACHES TO THE STUDY OF CELL-SECRETED SIGNALING

Whereas conventional methods can be used to uncover the presence of autocrine and paracrine signaling pathways, reveal their importance, and identify the specific intercellular molecules that are or are not involved, there are issues that microfluidic approaches are better able to address. Specifically, microfluidic approaches can help provide a precise quantitative understanding of spatial and temporal parameters, thereby allowing for better-controlled studies of the cell-secreted signaling environment.

4.1. Physics and Scaling

To control cell-secreted signaling, the modes by which secreted molecules are transported in liquids need to be considered. In general, ligand that is produced by source cells at some rate (molecules per second) can bind back to cell-surface receptors (reaction sink), diffuse away, or be convected away (e.g., by fluid flow) (**Figure 3a**). Because it allows for small-scale manipulations, microfluidics can be used to arrange cells to define regions of ligand sources and sinks, and the predictability of microscale fluid flow allows for precise control over ligand transport.

Controlling cell arrangement at the microscale allows for control of local cell density. Density, in turn, determines the endogenous ligand sources and sinks, which can be varied in autocrine systems by altering the colony size or the spacing between colonies, whereas relative densities or distributions of different cell types can alter these parameters in paracrine systems. To directly control the transport of ligand in the media, one can use nondimensional numbers to compare different modes of transport and thereby determine the appropriate microfluidic operation regime. A diffusion velocity can be estimated by D/L , where D is the ligand diffusivity (for a ~ 20 -kD cytokine, $D \sim 10^{-6} \text{ cm}^2 \text{ s}^{-1}$) and L is a characteristic length (e.g., the chamber height). Similarly, a reaction velocity can be defined as $k_{\text{on}}R_s$, where k_{on} is the ligand binding on rate (in $\text{M}^{-1} \text{ s}^{-1}$) and R_s is the receptor density (in mol m^{-2}); the convection velocity is simply v , the characteristic fluid velocity in the system. Ratios of these values lead to the Peclet number (convection/diffusion, vL/D), the Damköhler group I (reaction/convection, $k_{\text{on}}R_s/v$), and the Damköhler group II (reaction/diffusion, $k_{\text{on}}R_sL/D$). By altering these transport phenomena, one can alter the balance among diffusion, convection, and reaction, and in turn modulate the activity of autocrine loops to discover their effects on phenotype.

Microfluidics allows a decrease of L and the application of v , thereby allowing for tuning of both diffusion and convection. To increase endogenous signaling, one should operate in a convection-free environment ($v = 0$), where the reaction occurs at a comparable rate to or faster than diffusion. Although v is often assumed to be zero in macroscale static cultures, convection may still occur to some extent, so zero flow can be more accurately achieved by growing cells in enclosed chambers. To decrease soluble signaling, one should decrease the effect of reaction, which can be accomplished by increasing convection.

4.2. Organizing the Cells: Cell Patterning

Microtechnologies that enable cell patterning and organization have been adopted to investigate cell-cell signaling, either within colonies of cells or between colonies of the same or different cell types. Microscale ESC patterns can be created either by using substrates that include chemically modified regions to which cells can attach (53, 54) or by physically constraining cell location (**Figure 3b**) (55, 56). Regardless of the method, micropatterning can be used in many ways to

further understand how autocrine and paracrine signaling affects ESCs, for example, by uncovering endogenous signals involved in ESC processes, by determining the contributions of paracrine signals, or by recapitulating the *in vivo* microenvironment.

When assessing the contribution of a density-dependent signal such as an autocrine signal, the size of a colony of cells will affect ligand source and sink levels. Thus, controlling colony size can help to remove source and sink variations when quantifying the impacts of autocrine signaling. Likewise, modulating signaling by altering colony size can indicate whether cell fate is density dependent. For example, Peerani et al. (9) patterned hESCs into different-sized colonies using microcontact printing and assessed the ESCs' phenotype by using quantitative immunocytochemistry, ultimately implicating endogenous BMP2 and GDF3 as modulators of self-renewal (**Figure 3c**). Related studies with mESCs patterned at different colony sizes indicated the importance of endogenous Stat3 activation on self-renewal and showed that transcription downstream of Stat3 can be regulated by colony size (57).

To pattern cells without functionalizing the surface, stencil patterning is perhaps the simplest approach (**Figure 3b,e**) (58), and multiple cell types can be organized using patterning. Tuleuova et al. (59) used collagen and fibronectin micropatterns to pattern mESCs; they also used a silanized background surface to constrain the mESC patterns. Because the silane was only moderately nonpermissive, the patterned mESCs were backfilled with stellate cells for the study of paracrine interactions in hepatic differentiation (**Figure 3d**). Aqueous two-phase patterning is another substrate-independent patterning method that has been successfully applied to ESCs (60, 61). This method uses a pipette to deposit one aqueous phase (containing cells) into a solution of a second, immiscible aqueous phase; the cell-containing phase maintains its as-deposited pattern, thereby patterning the cells (**Figure 3f**). This method has been used to show that mESC neuronal differentiation increases with increasing colony size (61). A challenge posed by substrate-independent patterning is that, by its very nature of allowing for freedom of movement, this type of patterning makes long-term control and tracking of cells difficult, especially for motile cells such as ESCs.

Cell patterning can also help create experimentally convenient *in vitro* models of *in vivo* environments. Bio flip chip (BFC) cell patterning creates patterns by overturning a cell-loaded

Figure 3

Transport parameters and control over cell organization. (a) Transport modes for cell-secreted soluble signals, including diffusion, reaction, and convection of ligands from sources to sinks. (b) Depiction of cell patterning that relies on chemical modification (microcontact printing) or physical barriers (stencil patterning), including how coculture can be achieved with either method. (c) Microcontact printing of human embryonic stem cells onto MatrigelTM and immunofluorescence analysis. Images show that different markers vary from small to large colonies. Graph shows that larger colonies have higher levels of Oct4 and Nanog, probably because of the increased levels of secreted factors. Reprinted from Reference 9 with permission from Macmillan. (d) (Top) Microcontact printing of mouse embryonic stem cells (mESCs) without or with stellate cell coculture. Analysis of AFP (hepatocyte marker) in differentiated stem cells cultured alone or with stellate cells, showing (bottom) that stellate cells improve hepatic differentiation. AFP is shown in red, and DAPI (4',6-diamidino-2-phenylindole) is shown in blue. Reproduced from Reference 59 with permission from Elsevier. (e) (Top) Stencil patterning of mESCs alone or (bottom) cocultured with 3T3 cells. Cells were stained with PKH26 dye (mESCs) or CellTracker Blue (3T3s) for visualization. Reproduced from Reference 58 with permission from the Royal Society of Chemistry. (f) Polymeric aqueous two-phase noncontact printing. (Top) Schematic of the approach. (Bottom) Images of cells show that as cells are printed at increasing colony size, TuJ1 neuronal marker expression increases. Reproduced from Reference 61 with permission. Copyright 2010, Wiley. (g) Combined stencil and bio flip chip (BFC) patterning. Stencil patterning of trophoblast stem (TS) cells (green) and extraembryonic endoderm (XEN) cells (red), combined with BFC patterning of mESCs in a coculture resembling the organization of the late blastocyst, induces polarized differentiation (Wnt3 staining) similar to anteroposterior polarity. Reproduced from Reference 63 with permission from the Royal Society of Chemistry. (h) Use of microfabricated wells to control embryoid body (EB) size. (Top) Images of cells in wells of three different sizes and (bottom) EBs extracted from the wells (66).

EMBRYOID BODIES

When ESCs are removed from self-renewing cues and grown in suspension, they aggregate and form three-dimensional clusters of cells known as embryoid bodies (EBs). A remarkable feature of differentiating EBs is that they recapitulate some of the early steps of embryonic development, including the formation of a primitive streak-like region, anteroposterior polarity, and the ability to differentiate into all three germ layers (104, 105). These features make EBs a good model system for investigating cellular processes that occur early in development, especially for humans, in whom these processes are normally inaccessible. Developmental processes can occur to some extent in EBs that have no imposed architecture, but methods that can control EB organization or signaling could further expand the utility of these systems.

microwell array onto a recipient substrate, whereupon the cells fall out of the well and onto the recipient substrate while maintaining their arrangement (62). This technique has been combined with stenciling to pattern mESCs, along with other cell populations found in the early embryo, to create developmental models with which to study early embryonic patterning events *in vitro* (**Figure 3g**) (63). Other microscale approaches to control organization include flowing droplet arrays consisting of multiple cell types used to create controlled cocultures in small media volumes (64) and laminated microfluidic flows used to modulate cell-soluble signaling versus contact-mediated signaling (65), a technique that could be applied to stem cells. A more common way to control organization during ESC differentiation involves the use of three-dimensional aggregates of differentiating ESCs known as embryoid bodies (EBs; see the sidebar), whose size or shape can be controlled using microfluidics to bias differentiation, presumably by altering endogenous soluble or contact-mediated signaling (**Figure 3b**) (66, 67). By controlling EB size, investigators have shown that larger EBs contribute more toward the ectoderm lineage than do smaller EBs (66) and that differentiation can be tailored toward cardiac induction (67). One can also extrinsically control signaling in EBs by growing them in microfluidic traps that allow for the formation of gradients (68), by providing two separate culture conditions for a single EB (69), or by using microparticles to incorporate soluble factors within EBs (70). These approaches provide a platform for mimicking the microenvironment of the early embryo, in which morphogen gradients and signal polarities are important for early developmental decisions.

Although controlling cell organization is useful in that it allows for the assessment of cell-cell signaling in a controlled setting, these techniques are most commonly used in a static culture environment in which autocrine and paracrine signals are continually present. Another set of methods is required to alter the transport of ligand to augment or block the signaling entirely.

4.3. Controlling Transport: Microscale Culture in Enclosed Environments

Methods to culture cells in enclosed microfluidic environments are fairly mature and have been applied to systems ranging from neurobiology (71) to pulmonary biology (72). In the context of studying endogenous soluble signaling, these methods can be divided into those that try to maximize ligand concentration and those that aim to minimize it.

One can use microfluidics to confine cells in small volumes to increase the concentration of their secreted soluble signals by utilizing small polydimethylsiloxane (PDMS) chambers or microdroplets loaded with cells (**Figure 4a**) (73, 74). These methods have been used for high-throughput antigen screening (75) and to capture and lyse single cells to determine the presence of specific signaling events (76). However, the use of small-scale culture environments

Polydimethylsiloxane (PDMS):

a biocompatible polymer used for many cell-based microdevices

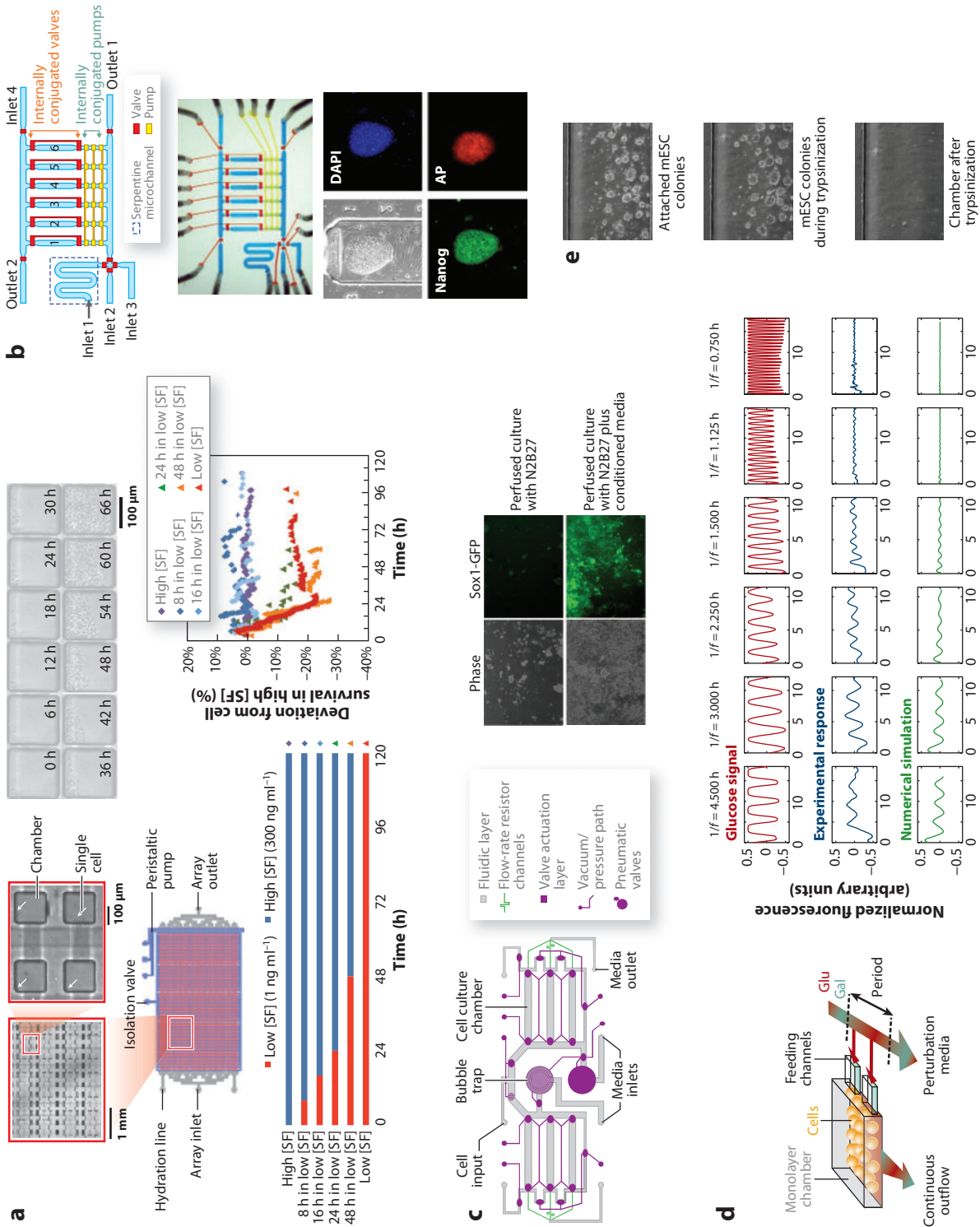
to increase endogenous signaling in ESCs is not common, probably because of concerns about nutrient limitations and because convection is not a fundamental problem with traditional cultures (i.e., one can observe gradients in autocrine factors within colonies, which would be obscured if convection always dominated). The added difficulty of working in an enclosed microenvironment must be balanced against the new information that could conceivably be obtained. Alternatively, one can use microfluidic systems to remove soluble signals from stem cell cultures. The fundamental requirement for these systems is that they have some mechanism for exchanging the medium in the culture chamber. Thus, these systems are typically composed of PDMS microfluidic chambers with inlets and outlets, and they often have valves (77, 78) and debubblers (79) to provide additional functionality.

Several microfluidic platforms have been described for the culture of ESCs, primarily to minimize reagent volumes for screens. For example, Kamei et al. (80) developed a valved PDMS microfluidic platform that allowed for hESC culture in chemically defined conditions for screening and quantitative imaging (**Figure 4b**), and another study used a simple microfluidic channel to deliver stimuli to different parts of single hESC colonies (81). Flow has also been used in microscale cultures to periodically replace the media in cell cultures to minimize nutrient depletion while allowing for periodic accumulation of secreted factors, as has been shown for hESCs grown on a feeder layer that required a short pulse of media every 2 to 4 h (82).

Although these studies provide optimized platforms for the growth or screening of stem cells, determination of the cell-secreted signals that are sufficient and necessary to maintain ESC self-renewal can also be aided by the precise control afforded by microfluidics. The use of microfluidics to control soluble-factor mass transport has been demonstrated for both hESCs (83) and mESCs (84–86). For hESCs, a system was developed that could be tuned to operate in either a convection- or diffusion-dominated regime, resulting in different percentages of differentiated cells (83). This effect was attributed primarily to the effects of shear in the convection-dominated regime but also to a decrease in soluble signaling because the relative amount of differentiation was density dependent. To control for the effects of shear and microscale culture, mESCs have been grown under microfluidic perfusion with or without added cell-secreted factors (**Figure 4c**) (85). In this study, mESCs were differentiated toward a neuroectodermal fate under perfusion, and cell-secreted factors were required for cell viability during this process because their removal resulted in very little cell growth. In another study, researchers used microfluidic perfusion to remove cell-secreted factors during self-renewal and found that without them, cells could not self-renew even in the presence of the canonical LIF and BMP4 self-renewal cocktail, which implicated autocrine factors in self-renewal (86). In this way, these studies show how the necessity of cell-secreted factors for a specific mESC process can be verified, which has led to the possibility of testing ESC autocrine signal sufficiency and necessity in various self-renewal and differentiation contexts.

4.4. Temporal Analysis

In addition to allowing for spatial control, microfluidics can also provide control over the timing of signal addition or removal to address unanswered questions in ESC biology. As stem cells transition out of their self-renewing state and differentiate to particular cell types, the timing of factor addition is critical, as it is in the developing embryo (**Figure 1e**). The timing of additions to ESCs *in vitro* is therefore used to mimic endogenous autocrine or paracrine signaling processes that occur *in vivo*. Identifying those temporal requirements is necessary to understand and achieve certain developmental end points, both *in vitro* and *in vivo*, and provides an intriguing opportunity for microfluidics.



Although the timing of signal presentation is clearly important throughout stem cell and developmental biology, controlling timing *in vitro* is more difficult than controlling exogenous factor identity and concentration. Multiwell plates and robotics make it feasible to screen different combinations and concentrations of factors, but these devices are more limited in their ability to test different temporal profiles of factor addition. Because typical microfluidic chamber volumes for mammalian cell culture are $\sim 1\ \mu\text{l}$ or less, media can be exchanged very rapidly and in an automated fashion, allowing precise temporal requirements to be addressed.

Several microfluidic devices have been developed that allow for precise exogenous factor additions at specific times, but these have focused primarily on the study of yeast signaling and gene-regulation dynamics (**Figure 4d**) (87–89). These studies revealed the presence of important signaling events, including negative feedback loops that acted at different timescales (87), but such devices have rarely been used to study timing in stem cells. However, the ability of microfluidics to exert temporal signal control could be combined with our increasing understanding of the exogenous signals involved in development to further study these processes *in vitro* and thereby to better control stem cell differentiation. A potential application of these systems is to study the temporal cues required to exit from self-renewal. The timing of Fgf4 signaling is critical to mESC specification and blastocyst development (39, 40, 42), and microfluidics could provide further resolution as to the temporal windows that are required or sufficient for this addition so that we may better understand how this autocrine or paracrine signal works *in vivo*.

Paracrine signals during development act both spatially and temporally, and both parameters can be controlled with microfluidics. Space can be controlled as described above, whereas exogenous inputs can be switched to stimulate and/or inhibit pathways, as has been shown by quantifying the differentiation and motility of human primary mesenchymal stem cells after transient stimulation in a 96-chamber device (90). Another example involved the testing of different timescales of Steel factor stimulation in a microfluidic device to define when this protein is required to exit quiescence in adult hematopoietic stem cells *in vitro* (**Figure 4a**) (73). Paracrine interactions can also be temporally controlled with microfluidics, as has been demonstrated with a valved two-chamber device in which signals from one chamber were directed to the other chamber; this device showed that cancer-secreted cytokines stimulate the transformation of fibroblasts into myoblasts (91).

Figure 4

Microscale stem cell culture. (*a*) Device for culturing cells in a system with small media volumes and minimal convection. The white arrows point to single cells. Device layout and chambers (*top left*) and images of ND13 mouse preleukemic cells (*top right*) growing in chambers for several days. At bottom is a time-course schematic of Steel factor (SF) application to hematopoietic stem cells grown in chambers, and the graph shows cell survival at the indicated conditions. Cells appear not to tolerate more than 16 h in low-SF concentrations to remain viable. Reprinted from Reference 73 with permission from Macmillan. (*b*) Microfluidic device for culture of human embryonic stem cells (hESCs). Schematic (*top*), optical micrograph (*middle*), and immunofluorescence staining of self-renewal markers (*bottom*) in an hESC colony grown on chip for six days. Reproduced from Reference 80 with permission from the Royal Society of Chemistry. (*c*) Microfluidic perfusion culture for modulating diffusible signaling. (*Left*) Schematic of microfluidic device for multiday culture of mouse embryonic stem cells (mESCs) under continuous perfusion. (*Right*) Images showing that only cells perfused with serum-free N2B27 media with added conditioned media (containing cell-secreted factors) grew and differentiated toward neuronal precursors (Sox1–green fluorescent protein expression) (85). (*d*) Microfluidic control of timing. (*Left*) Schematic of a device for rapidly controlling inputs to yeast cells, showing that sinusoidal perturbations of added signals can be controlled through the feeding channels. (*Right*) Graphs show experimental and computational results of fluorescence of a yeast fusion gene as it responds to glucose waves at varying oscillation periods. Reprinted from Reference 88 with permission from Macmillan. (*e*) Embryonic stem cell (ESC) removal. mESC colonies growing in an individual culture chamber are trypsinized on chip and collected for further analysis. Abbreviations: AP, alkaline phosphatase; DAPI, 4',6-diamidino-2-phenylindole; GFP, green fluorescent protein.

The use of microfluidics to mimic the *in vivo* environment can be extended to optimize protocols for directed differentiation and to better understand the interplay between exogenous factors and endogenous loops that operate during differentiation by altering timing and concentration of factor addition. Such studies are performed at moderate scales by use of traditional culture conditions (92), but they could be performed more easily and at lower cost with microfluidics.

5. EXPERIMENTAL CONSIDERATIONS

Microfluidics provides powerful tools with which to investigate unresolved questions in cell biology. However, with any use of microfluidic systems, it is important to ensure that the advantages outweigh the significant effort involved in developing and optimizing such systems.

5.1. Cell Growth

Whether microfluidics is used to alter cell organization or ligand transport, there are growth requirements that need to be considered. First, the alteration of soluble signaling via microfluidics implies an alteration of bulk media volumes. At low media volumes, a lack of media nutrients and/or a buildup of waste products can become an issue. This issue makes it difficult to interpret experimental results; is a change in phenotype of cells cultured in a no-flow environment due to soluble signaling or due to nutrient issues? As long as the media are exchanged periodically in accordance with volumes in typical bulk culture conditions ($\sim 50\text{--}500\text{ pl h}^{-1}\text{ cell}^{-1}$ for an ESC), cells should not experience nutrient limitation.

5.2. Shear Stress

Any device that uses convection to alter soluble signaling also introduces shear stress. Shear stress at the walls of a parallel-plate flow chamber is described by the following equation:

$$T = (6\mu Q)/(b^2 w),$$

where μ is the viscosity; Q is the volumetric flow rate; and b and w represent the height and width of the chamber, respectively. Shear stresses could alter cell physiology or even remove cells from the substrate entirely. In terms of cell removal, the detachment shear stresses for fibroblasts are $\sim 30\text{--}50\text{ dyn cm}^{-2}$ (93), and shear stresses of 5 dyn cm^{-2} have been applied to an endothelial monolayer for a week without noticeable cell detachment (94), indicating that the shear required to detach adherent cells from substrates is typically $\gg 1\text{ dyn cm}^{-2}$. For mESCs in particular, removal shear stresses are $> 6.5\text{ dyn cm}^{-2}$ (95).

Shear stresses that alter the signaling pathways of cells are lower than those that remove cells from a substrate. For ESC-derived endothelial cells to begin expression of endothelial and tight junction markers, a shear of 5 dyn cm^{-2} has been used (96), and induction of endothelial cell-specific genes in mouse embryonic endothelial cells to study the activation of downstream pathways has been performed with $5\text{ to }25\text{ dyn cm}^{-2}$ (97). The effect of shear stress is likely to be context dependent, given that mechanical stresses affect self-renewing cells to a greater extent than they affect differentiated cells (98). However, whereas mESCs sense shear stress and respond to it dose dependently at stresses from $0.016\text{ to }16\text{ dyn cm}^{-2}$ (99), ESCs can grow indefinitely without any effects on self-renewal properties at shear stresses up to 6.1 dyn cm^{-2} (100). Thus, operation at shear stresses $\ll 1\text{ dyn cm}^{-2}$ is not likely to affect the ESC phenotype.

5.3. Adsorption and Absorption

Another issue in microscale devices, which are molded primarily out of PDMS, is the permeability of the chamber walls and surface adsorption. The gas permeability of PDMS is often regarded as a beneficial feature, as it allows for oxygen–carbon dioxide gas exchange to cells grown within PDMS chambers. However, PDMS also allows for the transport of water (101), which can lead to dehydration and thus convection and changes in media composition. Absorption can also be an issue for small molecules, especially hydrophobic ones (102), but is not significant for the >10-kD proteins typically found as signaling molecules. However, the adsorption of molecules to PDMS surfaces is enhanced in high surface area–to–volume microfluidic devices. Such adsorption tends to lower the fraction of secreted molecules that are active (the walls act as a sink), which may bias results obtained with static enclosed cultures or in measurements of secreted molecules collected from perfused cultures. These issues have been addressed with many solutions, including growing the cells in adequately humidified devices (73), prepriming devices with equilibrated cell media before loading the cells (84), and precoating PDMS devices with BSA (bovine serum albumin) to minimize the adsorption of cell-secreted proteins (103).

5.4. Cell Loading, Recovery, and Assay

Successful loading of ESCs into microscale devices can be challenging because ESCs tend to require long attachment periods and, in the case of hESCs, growth in clumps. Typically, cells are loaded through a cell-input chamber via syringe, with the benefit that only a small number of cells are needed for microscale chambers. Then, media manipulation (e.g., application of convection or addition of exogenous stimuli) should not begin until an adequate amount of time has elapsed to allow cells to fully attach, which takes between 12 and 18 h after loading for mESCs. To remove cells, trypsin can be applied in the same input into which cells or media are added; then the cells can be replated or used for downstream assays (**Figure 4e**).

The assays that can be performed on cells grown in microfluidic systems are often limited by the small cell numbers involved. This limitation is a challenge for experiments involving stem cells because they are, by definition, functionally defined. Thus, the phenotype that is assayed in a self-renewal or differentiation experiment must always be carefully chosen. Most experiments that have been performed in microsystems use cell growth or fluorescence as a readout; these assays do not require recovery or off-chip analysis of cells. However, they provide information about only a handful of parameters (80). Obtaining more complete phenotypic information is an open challenge in the field, but doing so will be necessary for more sophisticated questions to be asked.

6. CONCLUSION

The endogenous signals sensed by ESCs significantly influence the future state of the cells. Although much is known about how endogenous signaling affects ESC fate, many questions remain. These outstanding questions require techniques that provide precise control of cell organization, control of ligand transport in the media, and control of temporal cues. These factors, in turn, can be employed to better mimic the *in vivo* environment or to optimize differentiation and self-renewal *in vitro*. Whether microfluidics is used to manipulate endogenous or exogenous signals or to create artificial microenvironments, its ability to operate on scales closer to the scale of the cell itself allows this technology to emulate the fundamental biological lengths and timescales. With the appropriate design and experimental considerations, microfluidic methods can significantly improve our understanding of ESC biology.

SUMMARY POINTS

1. Cell-secreted signals are important for the maintenance of ESC self-renewal, but the full repertoire of signals and their functionalities remains to be determined.
2. Autocrine and paracrine cell-secreted signals are critical for patterning the early embryo, and their function depends on their proper spatiotemporal presentation.
3. Cell-micropatterning techniques provide the ability to control cell-secreted signals by organizing the cells that produce the signals. Both autocrine and paracrine systems can be modulated.
4. The use of static and perfused microchambers allows for control over endogenous signals that have been secreted into the media.
5. By allowing for control over timing of factor addition, microfluidics provides an opportunity to study the temporal requirements of endogenous and exogenous signals during differentiation.
6. The application of microfluidic techniques to ESC biology requires consideration of issues such as cell growth, fluid shear stress, molecular adsorption and solvent absorption, and cell handling.

FUTURE ISSUES

1. Robust and accessible microfluidic systems for long-term culture and manipulation of ESCs must be developed before these systems can be widely adopted.
2. Because of the small cell numbers inherent to microfluidic studies, researchers need to determine how to interface these devices with downstream assays, such as reverse transcription polymerase chain reaction and immunoblotting, to fully exploit the power of microfluidics.
3. The operation of microfluidics in a convection-dominated regime will allow for investigations into the cell-secreted signals required for ESC self-renewal.
4. The timing and placement of signals required during differentiation can be further defined and resolved with microfluidics.

DISCLOSURE STATEMENT

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