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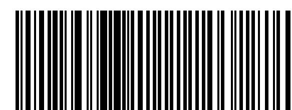

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Advancing stem cell research with microtechnologies: opportunities and challenges

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Stem cells provide unique opportunities for understanding basic biology, for developing tissue models for drug testing, and for clinical applications in regenerative medicine. Despite the promise, the field faces significant challenges in identifying stem cell populations, controlling their fate, and characterizing their phenotype. These challenges arise because stem cells are ultimately functionally defined, and thus can often be identified only retrospectively. New technologies are needed that can provide surrogate markers of stem cell identity, can maintain stem cell state *in vitro*, and can better direct differentiation. In this review, we discuss the opportunities that microtechnologies, in particular, can provide to the unique qualities of stem cell biology. Microtechnology, by allowing organization and manipulation of cells and molecules at biologically relevant length scales, enables control of the cellular environment and assessment of cell functions and phenotypes with cellular resolution. This provides opportunities to, for instance, create more realistic stem cell niches, perform multi-parameter profiling of single cells, and direct the extracellular signals that control cell fate. All these features take place in an environment whose small size naturally conserves reagent and allows for multiplexing of experiments. By appropriately applying micro-scale engineering principles to stem cell research, we believe that significant breakthroughs can be made in stem cell research.

1. Introduction

Stem cells have broad potential across bioscience, from clinical applications, such as creating neurons to treat spinal cord injury, to basic science, such as a model for understanding cell fate determination. While the end-point goals may differ, most stem cell applications involve three basic steps: identifying and expanding stem cells, differentiating them into a cell type or tissue of interest, and assessing the fate of the resulting cells (Fig. 1). Specific applications may require additional steps (*e.g.*, transplantation of tissues requires development of targeting and delivery methodologies) or fewer (embryonic stem cells are straightforward to generate and expand for basic research), but these three generic steps are ubiquitous across stem cell biology. Technical limitations in our ability to carry out these steps temper our ability to exploit the extensive potential of stem cells. For instance, improving the efficiency and completeness of somatic cell reprogramming back to their pluripotent state will circumvent obstacles associated with the use of human embryonic stem cells (hESCs) in clinical contexts, such as ethical issues regarding the use of human embryos and immune rejection after transplantation. It follows, then, that technological improvements in these steps will advance the field forward. In this review, we discuss how microtechnology, in particular, can help advance the field of stem cell research.

Microtechnology has already been applied to many biological problems, from microfluidics for dynamically *controlling* the extracellular environment[1] to technologies for making absolute *measurements* of intracellular molecules.[2] However, there are specific features of stem cells and stem cell biology that offer particular challenges for control and measurement, and at the same time present opportunities for the field. The phenotypic plasticity of stem cells is the reason that they are

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Insight, innovation, integration

Stem cell research is progressing at a rapid pace and has great potential to impact both clinical and basic biology. Yet, the field still faces many challenges, some of which can be addressed by integrating the capabilities of microtechnology into the experimentation and assay of stem cells. In this review, we change the conventional microtechnology-driven perspective to a stem cell biology-driven perspective to highlight research opportunities where microtechnology can help advance the stem cell field. We also provide insights into issues associated with the adaptation of existing micro-technologies to tailor to the unique characteristics of stem cells.

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interesting and useful, yet poses significant challenges in controlling that phenotype and in assessing it. However, we believe that microtechnology has two significant features that can be exploited for stem cell biology. First, microtechnology intrinsically enables control of the cellular environment at cellular resolution. This potentially enables researchers to replicate complex environments at a biologically appropriate resolution. Second, microtechnology facilitates high-throughput experimentation due to its ability to manipulate large numbers of small liquid volumes. We also emphasize that many current challenges in the field require biotechnological solutions that are *not* instrumentation, such as the development of small molecules to perturb cell fate or enhance reprogramming.

This review highlights the challenges in stem cell research that can particularly benefit from microtechnologies and approaches to adapt microtechnologies to the specific qualities of stem cell biology. Many of the issues are similar to those faced in other disciplines of bioengineering research, especially from a technological perspective. For instance, the ability to understand the microenvironment to better control cellular phenotype is an area that is being extensively studied for applications in tissue engineering,[3] and we will draw upon this large body of work when specific features or engineering principles can be translated to stem cell research. Therefore, instead of comprehensively reviewing the state-of-art in micro-technologies, most of the work that we highlight here is meant to demonstrate the application of engineering principles to cell biology that can be translated to address particular issues in stem cell research.

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2. Stem cells

Stem cells (SCs) are broadly classified by their developmental potential, with pluripotent stem cells (PSCs) having the broadest differentiation capability, able to form all the cell lineages of the adult organism. The canonical PSC is the embryonic stem cell (ESC) although recent advances in cell reprogramming have enabled the generation of PSCs from somatic cells.[4–6] Other stem cell classes (*e.g.*, adult stem cells such as mesenchymal stem cells), having a more limited differentiation potential, are referred to as being multipotent (able to generate multiple cell types in one lineage), or unipotent (able to form one cell type *e.g.*, spermatogonial stem cells).[5] In mammals only the zygote and the first cleavage blastomere are totipotent and can give rise to an entire organism.[5] In addition to differences in developmental potential, stem cell classes have differences in their ease of propagation *in vitro*. PSCs can be propagated indefinitely *in vitro* without change in their phenotype (aka self-renewal *in vitro*), enabling the generation of stem cell *lines*, while other stem cell classes typically have limited propagation ability *in vitro*. Their wide differentiation potential and ease of

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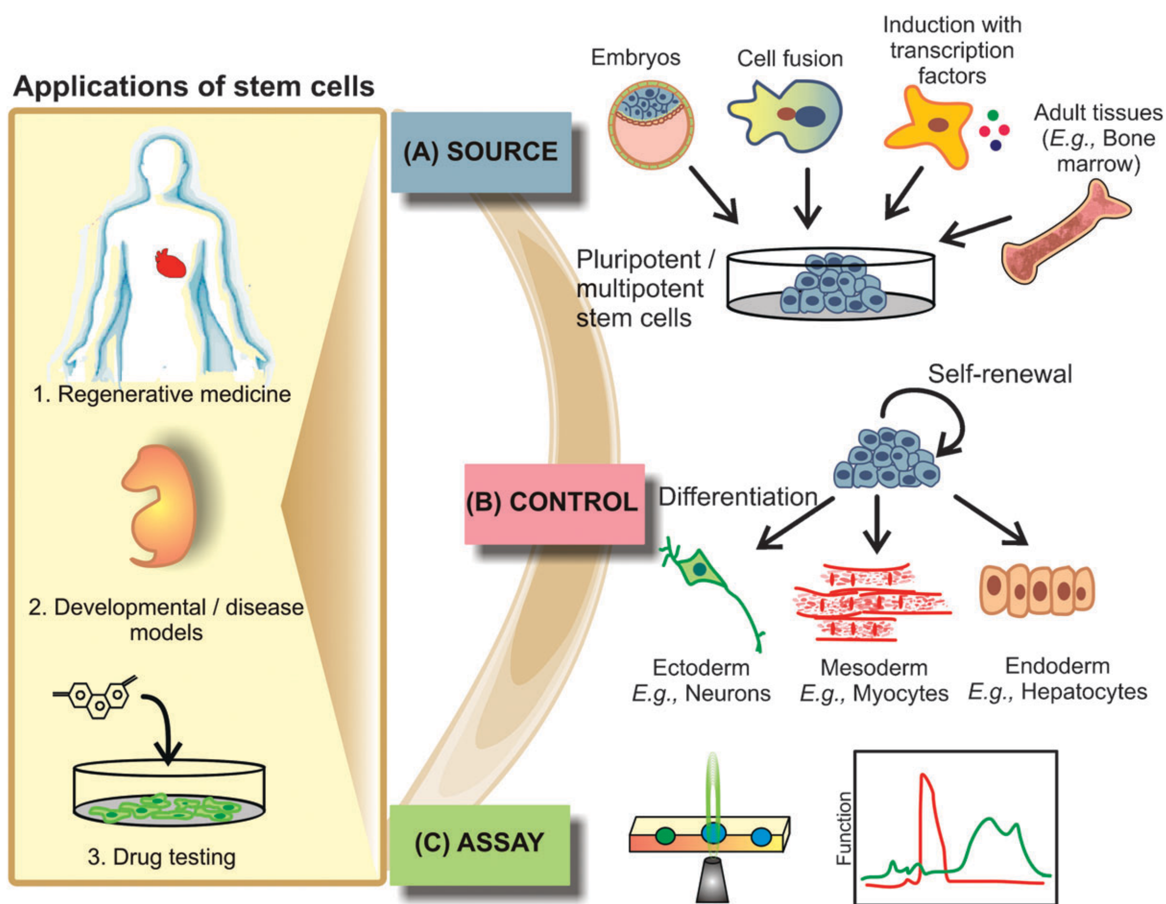

Fig. 1 Applications of stem cells range from regenerative medicine to developmental and disease models for basic biological studies or drug testing. Most applications involve three basic steps: (A) derivation of a stem cell source either from embryos, from fusing somatic cells with stem cells, *via* transfecting somatic cells with transcription factors, or from adult tissues, such as bone marrow; (B) controlling the stem cells to induce self-renewal or differentiation into a desired lineage; (C) assaying the resulting (stem) cells to determine their state or function.

propagation in the undifferentiated state make PSCs an especially interesting cell system, because if their fate could be controlled, they would have the potential to create any cell type needed for therapy, drug screening, or basic science. Here we focus on the developments and challenges facing the control of *pluripotent stem cell* fate, in particular maintenance and exit from the pluripotent state, although some of the issues are applicable to other stem cell sources.[7,8]

ESCs of murine and human origin are the most common form of PSCs and are derived from the inner cell mass (ICM) of preimplantation embryos.[9] The cells are routine to derive and since they can be propagated indefinitely in culture, are straightforward to store and distribute. Mouse ESCs (mESCs) grow as compact multilayer colonies *in vitro* with indistinct cell borders and high nuclear-to-cytoplasmic ratio. They divide quickly for mammalian cells ($\sim$12–18 h doubling time), can be propagated clonally ($\sim$30–50% single-cell plating efficiency), and because they are readily amenable to homologous recombination, a number of reporter lines are available for studying different aspects of their biology. Human ESCs (hESCs), in contrast, are typically derived from embryos discarded from *in vitro* fertilization (IVF) procedures. They grow as monolayer colonies *in vitro* with more distinct cell borders. They divide slower than mESCs ($\sim$24 h doubling time) and have extremely low single-cell plating efficiency ($<$1%),[10] which is successfully circumvented by the application of Rho-associated kinase (ROCK) inhibitor (efficiency increased up to 27%).[11] These phenotypic differences, when coupled to the transcriptional and epigenetic differences described below, have led to an emerging view that hESCs and mESCs do not represent analogous cells from mouse and human, but rather that hESCs may represent a slightly later developmental stage. The implications of these differences for downstream applications are as of yet unknown.

For clinical applications, hESCs derived from embryos obtained from IVF procedures will not be autologous, and hence any cells or tissues derived from these stem cells may be rejected by the recipient.[9] These obstacles could be circumvented by reprogramming terminally differentiated somatic cells from patients back into a pluripotent state. Early approaches to such reprogramming included somatic cell nuclear transplantation, where the nucleus of a somatic cell from patient is transferred into an enucleated donor oocyte,[12,13] and fusion of somatic cells with ESCs.[14,15] While these approaches in principle could provide autologous PSCs, ethical and technical issues related to the destruction of viable embryos, limitation of donor oocytes, and genetic stability of tetraploid fusion of cells render these methods unlikely to be clinically significant.[5,9]

A breakthrough in somatic cell reprogramming was first demonstrated by Takahashi and Yamanaka in 2006, who showed that viral transduction and forced expression of four transcription factors (Oct4, Sox2, c-Myc and Klf4) was sufficient to reprogram somatic embryonic or adult fibroblasts into PSCs,[16] a process whose mechanisms are now starting to be uncovered.[17] The resulting induced pluripotent stem cells (iPSCs) have been created in a number of animal systems,[18,19] and in murine models have met the most stringent pluripotency test by generating fertile live-born offspring *via* tetraploid complementation.[20,21] In most respects, iPSCs appear genetically and functionally similar to ESCs, yet their epigenetic state is demonstrably different[16,22] though the implications of this difference (if any) are unknown. Although the four so-called Yamanaka factors represent the most widely used set of reprogramming factors, not all are essential,[23–25] and intense effort is being undertaken to exchange these genetic factors for small molecules.[26] A critical feature of the reprogramming process is that reprogramming occurs at low efficiency. This raises fundamental questions about the origin of the inefficiencies and requires the development of methods to identify the reprogrammed pluripotent cells from partially reprogrammed and unaltered somatic cells.

3. Controlling stem cell fate

Regardless of the potential application, it is of paramount importance to be able to reliably control stem cell fate. For instance, incompletely or incorrectly differentiated cells may become tumorigenic instead of therapeutic,[27] and the presence of such cells in a derived culture or tissue will lead to greater heterogeneity in results of screens and decrease the utility of *in vitro* models. In a typical use scenario, we want to expand stem cells in their pluripotent state and then direct them to differentiate into functional endpoint cells.[28] Due to the variety of endpoint cells that can be derived from PSCs, we will limit our discussion on differentiation to the onset of this process where stem cells exit from their pluripotent state. The specific mechanisms for differentiation into various lineages have been extensively reviewed.[27,29,30]

External factors in the stem cell environment alter the internal signals that ultimately regulate stem cell fate. Because PSCs can go from the same initial state to a range of diverse fates, the decisions that the cells make as to which fates to adopt must be provided at least in part by signals from outside the cells. In this context, it is useful to think about stem cells as existing in a niche: a local microenvironment that provides the external signals needed to drive stem cell self-renewal or differentiation. The idea of a niche is primarily based on germline and somatic (*e.g.*, adult) stem cell niches, most notably from the ovary and testis, bone marrow, hair follicle and intestinal villus.[31–34] Based on these studies, the different aspects of the niche controlling stem cell fate can be broadly classified into (i) soluble factors secreted by the stem cells and neighboring cells, (ii) oxygen, (iii) shear stress, (iv) direct cell contact with neighboring cells and (v) extracellular matrix (ECM).[35,36] PSCs exist only transiently during embryonic development (ESCs, which can self-renew indefinitely in culture, can be considered a culture artifact), and thus one cannot strictly conceive of an *in vivo* niche for these cells. However, maintenance or differentiation of PSCs *in vitro* still requires control of these five external environmental signals, which we identify with the concept of a niche. A variety of micro-technologies has been developed to control different aspects of the cell niche as covered by number of reviews.[37–40] Some of these technologies can be translated to control the stem cell niche albeit modification is necessary to tailor to the characteristics of stem cells which we shall discuss in the following sections.

The identification of the individual external environmental cues allows a bottom-up approach in reconstituting the stem cell niche to control the fate of PSCs. The external factors necessary for *maintaining* PSC pluripotency are relatively well defined as alluded by the fact that we can grow these cells *in vitro* in monoculture using standard culture techniques. In contrast, the direct *differentiation* of PSCs into specific end-point functional cells using a set of defined external factors in standard culture conditions varies in efficiency depending on a desired functional cell type as well as the applied protocol. Most differentiation protocols for PSCs involve first the formation of an embryoid body (EB), where cells are clumped together into a three-dimensional mass in non-adherent culture.[41] EBs recapitulate part of embryonic gestation where PSCs differentiate into precursors of the 3 germ layers. In this top-down two step approach, we first form an entity (the EB), whereafter a specific differentiation condition is applied to the EB to enrich for production of the desired end-point cell population. By controlling EB formation and its organization, we can indirectly modulate the differentiated fates of PSCs.

3.1 Soluble factors

Soluble factors secreted by PSCs or surrounding stromal cells regulate stem cell fate *via* autocrine and paracrine signaling (Fig. 2A). Soluble factors are the most extensively studied aspect of the niche since they can be easily supplemented in culture medium. mESCs have the best-defined extracellular requirements for self-renewal. Perhaps the most common culture environment is to co-culture mESCs with mouse embryonic fibroblast (MEF) feeder cells and serum. The fibroblasts secrete the cytokine leukemia inhibitory factor (LIF)[42] (and perhaps other molecules), which has been shown to be sufficient for self-renewal of mESCs in the presence of serum. The MEFs can be replaced by the addition of recombinant LIF in serum-containing medium,[43] the combination of LIF and bone morphogenetic factor 4 (BMP4) in serum-free medium,[44] or by knockout serum replacement (KSR) medium supplemented with adrenocorticotropic hormone (ACTH).[45] By avoiding serum, these latter media are fully defined and consistent, thus permitting more controlled experiments. However, various mESC lines perform differently in different culture environments; it is still common for biologists to culture cells in serum with LIF *and* with feeder cells (that secrete LIF). hESCs (and hPSCs) have different culture requirements than mESCs, now thought to reflect the fact that mouse and human ESCs represent different stages of development.[46] For instance, in contrast to mESCs, LIF is

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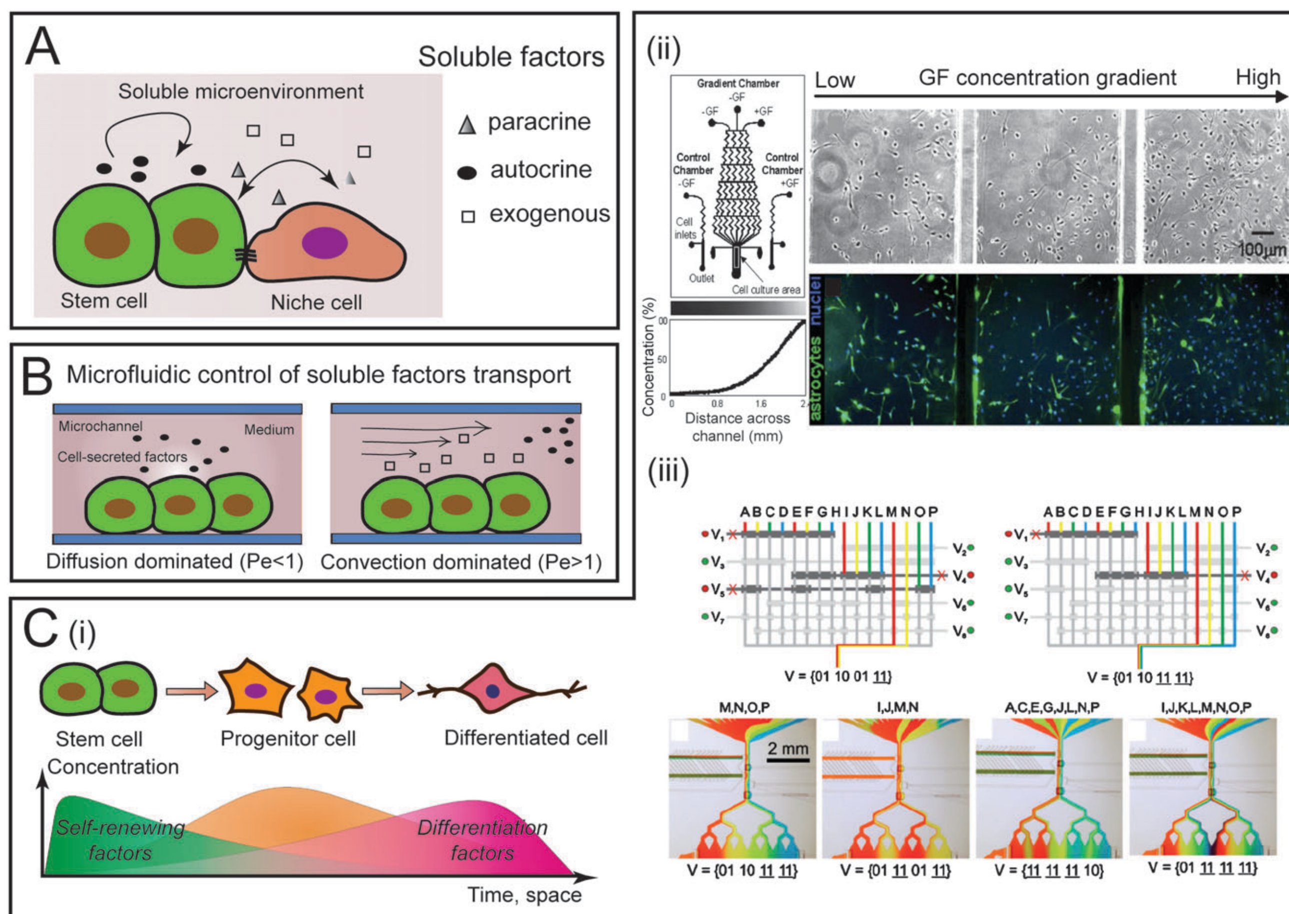

Fig. 2 Soluble factors for controlling stem cell fate. (A) Soluble factors in the stem cell microenvironment can be secreted by the stem cells themselves (autocrine factors) or supporting niche cells (paracrine factors) as well as exogenous factors supplemented in the culture medium. (B) The length scale of microfluidic systems are comparable to the diffusion constants of soluble factors, thus enabling one to control the mass transport regimes of soluble factors to be diffusion-(Peclet number (Pe $<$ 1)) or convection-dominated (Pe $>$ 1). (C) (i) The spatio-temporal distribution of various soluble factors change during the course of stem cell differentiation. (ii) To understand the role of a particular soluble factor, microfluidic gradient generators can be used to generate concentration gradients for investigating the dose-response of stem cells to that soluble factor. For instance, the "Christmas tree" network gradient generator creates a continuous concentration gradient of growth factors, which in turn control the proliferation of human neural cells.[52] (iii) Microfluidic multiplexers can also be employed for presenting multiple factors in a combinatorial fashion to screen for the most effective combination of factors specifying a desired stem cell fate. In one version of a binary multiplexer, where N fluidic inputs can be controlled by $X = \log_2 N$ pairs of pneumatic lines, Cooksey *et al.*, demonstrated the generation of 81 unique chemical combinations with 16 discrete fluidic inputs (A–P) that are controlled by 8 pneumatic lines (V_1–V_8).[64] Images are reproduced with permission from the Royal Society of Chemistry.

not required for maintenance of hESC pluripotency, but fibroblast growth factor 2 (FGF2) is indispensable for maintenance of pluripotency in hESCs for different media formulations,[47] including chemically defined medium.[48] Although hESCs can be adapted to serum-free and feeder-free culture, similar to mESCs not all cell lines adapt equally and adaptation may incur changes in developmental potential (*i.e.*, loss of pluripotency).

A limitation of studying fate-regulating soluble factors using conventional culture dishes is the accumulation of secreted factors by cells and depletion of oxygen and nutrients, which causes the soluble environment to change over time. In some cases this effect is desirable, as it arguably recapitulates autocrine/paracrine interactions that occur *in vivo* during development. Researchers have used static (*i.e.*, no flow) microfluidic devices to create controlled profiles of autocrine diffusible factors.[49] Here the advantage of microfluidics is that the small dimensions make convection less likely than in a macroscale open culture dish, although the use of microfluidics (or more precisely, enclosed environments) is not strictly necessary to study these effects, as autocrine/paracrine signaling gradients are commonly observed in stem cell systems cultured in conventional dishes.[50] Additionally, solute and solvent adsorption/absorption by commonly used polydimethylsiloxane (PDMS) can alter the soluble factor concentration profiles in complicated ways and must be considered.[51]

In other instances, where one wishes to study the effect of exogenously added soluble factors on development, these autocrine/paracrine signals can mask or exacerbate the effects of a particular soluble factor of interest. For these latter cases,

microfluidic perfusion devices have the potential to control the background soluble environment and make it more time-invariant and spatially uniform. For instance, one may desire a uniform baseline soluble environment; by continuously sweeping away cell-secreted factors, autocrine and paracrine signaling from extraneous sources can be minimized.[52] The spatial distribution of a soluble factor within a culture system can be defined by the mass transport regime that the system is operating at, which is characterized by the Peclet (*Pe*) number.[53] Because the length scales of the flow velocity and channel dimensions in microfluidic systems are comparable to the diffusion constants of soluble factors, it is possible to operate either at either diffusion-dominated ($Pe < 1$) or convection-dominated ($Pe > 1$) regimes (Fig. 2B). In comparison, macro-scale perfusion systems are almost always dominated by convective transport.[53]

Soluble factors are also key players in specifying differentiating stem cells, reflecting the importance of soluble factors in development. The effect of a particular soluble factor is concentration dependent,[54] time-dependent, and exhibits complex synergistic or antagonistic behavior when multiple factors are co-administered.[29] For instance, BMP4 when added to LIF in serum-free media promotes mESC self-renewal, but when added without LIF in serum-free media induces mesodermal differentiation.[55] These differences arise from the fact that the distribution of different soluble factors within the stem cell niche or during development is regulated in space and time (Fig. 2C, i). However, reproducing this spatio-temporal distribution in conventional culture systems such as culture dishes is difficult and is typically approximated by dosing independent stem cell cultures with discrete concentrations of soluble factors at different time points.[56] This presents an important opportunity for microfluidics, which can leverage laminar flow and small volumes to be able to recapitulate the spatio-temporal presentation of soluble factors to stem cells in a more sophisticated and cost-effective manner.

Laminar flow in microfluidics also allows for control of the soluble microenvironment. At multicellular length scales, gradient-generating devices as reviewed by Keenan and Folch are a popular approach to generate concentration gradients for investigating the dose response of soluble factor(s) of interest and thus optimizing culture conditions.[57] Although these devices were primarily developed to study phenomena where a gradient is intrinsic to the biological process (*e.g.*, chemotaxis), they can also be used to expose cells to many different concentrations at once. Noo Li Jeon's group used such a device to expose human neural stem cells (hNSCs) to gradients of growth factors, essentially performing a dose-response assay all at once (Fig. 2C, ii).[52] This approach complements the conventional tactic of applying discrete concentrations to multi-well plates. However, two challenges exist. First, differentiation is relatively slow process; the duration of which varies dramatically depending on the desired phenotype. For instance, mouse PSCs take $\sim$5 days to turn into neuroectodermal precursors and $\sim$11 days to differentiate into neuron-like cells. The developmental duration for other lineages are similar, which is thought to reflect the timescales of *in vivo* development (mouse gestation takes $\sim$20 days). Accordingly, differentiation of hESCs takes even longer (up to four weeks to generate neurons). Second, to see varying effects of molecules on differentiation, one must typically create a dose response curve with (at least) a few molecular doses spaced across a logarithmic scale, which are difficult to create within a single chamber with current gradient-generating devices. Thus, the long duration of PSC differentiation and large range of doses have to be addressed simultaneously in order to design a microfluidic system that will harness the full potential of microscale flow properties.

An appealing opportunity for microfluidic gradient generators is to create gradients that affect early development. It is known that gradients of soluble factors (known as morphogens) specify lineages (*e.g.*, bicoid gradients). While Ismagilov's group used microfluidics to spatially and temporally perturb embryonic patterning in *Drosophila*,[58] the application of multifactorial gradients to alter stem cell differentiation has not yet been demonstrated. For example, BMP4 and WNT-1 act antagonistically during development to direct cells into the mesodermal and ectodermal lineages respectively.[29,59] Using microfluidics to generate juxtaposed gradients of BMP4 and WNT-1 would allow study of how these two molecules interact to decide cell fate.

The unique properties of microfluidics allow us to control the concentration gradient of a soluble factor of interest, facilitating the understanding of its role in stem cell fate regulation. Conceivably, we can trace the trajectory of a stem cell's fate as we vary the spatial-temporal distribution of a particular soluble factor. However, soluble factors do not act independently and interact with each other synergistically or antagonistically. The ability of microfluidics to massively manipulate small volumes of solutions provides a practical mean of mapping the fate trajectories modulated by multiple soluble factors into a multi-dimensional space, which we can use to search for the most effective combination of soluble factors to achieve a desired stem cell fate. Using conventional macro-scale culture systems to establish a multi-dimensional soluble factor space is prohibitive due to the high cost of reagents and lack of widespread availability of automated liquid handling. Thus, a number of microfluidic cell culture arrays have been developed for combinatorial presentation of soluble microenvironments.[60–63] Microfluidics allows each unit of the cell culture array to be individually addressed,[60,63] and the incorporation of multiplexers,[64] microvalves,[65] gradient generators,[60] and mixers[66] greatly facilitate combinatorial input of multiple soluble factors (Fig. 2C, iii).

Microfluidics offers exciting opportunities to modulate the soluble stem cell microenvironment in a manner closer to *in vivo*, where multiple soluble factors vary simultaneously over space and time. However, a prerequisite to realizing this application is the robust culture of stem cells in perfused microfluidic systems. The technical issues related to performing perfusion cell culture in microfluidic systems as reviewed by Kim & Toh *et al.*[67] are generally applicable to stem cells. However, due to the plasticity of stem cells, one needs to pay special attention to how conditions imposed by the microfluidic system can influence the stem cell fate. For example, cells are dynamically seeded in most microfluidic systems,[67] which makes it difficult to control the seeding density. Variability in seeding density can change the proliferation and self-renewing

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capacities of stem cells *via* autocrine signaling. On this note, PSCs also need to be passaged (*i.e.*, dissociated and re-plated at a lower density) routinely since they spontaneously differentiate when grown to high cell density.[68] While it is possible to passage cells in microfluidic systems,[69] it adds considerable complexity to the design and operation of the system. Another factor to consider when culturing stem cells in microfluidic systems is the effect of fluid shear stress. For most cell types, the primary concern is to avoid operating under high shear stress, which is detrimental to cell viability.[67] As fluid flow mediates both mass transport of nutrients and shear stress, the perfusion flow rate needs to be balanced between supplying sufficient oxygen and nutrients for cell survival and minimizing shear stress damage. For stem cells, we also need to be cognizant of how shear stress can influence their fate since shear stress has been implicated to skew stem cells towards the mesoderm lineage,[70,71] a topic discussed in more detail in section 3.3.

3.2 Oxygen

Besides factors secreted by cells, soluble biochemical molecules also affect stem cell fate. Oxygen (O_2) is usually regarded as a nutritional requirement for normal cellular metabolic function and its concentration is fixed at the atmospheric partial pressure (21%) in most *in vitro* cell cultures. But for stem cells, the role of O_2 as a fate-determining external environmental factor must be considered because O_2 is known to affect embryonic development and stem cell phenotypes *in vitro*.[72] Tissues in the adult or embryo experience different O_2 levels under physiological conditions, many of which are hypoxic (low O_2 concentration ranging from 1–9%). Under hypoxic conditions, hypoxia induced transcription factors (HIFs) and related signaling pathways are activated, which modulate the development of blood, vasculature, placental, bone and other tissues.[72] For instance, hypoxia stimulates the secretion of vascular endothelial growth factor (VEGF) that controls angiogenesis.[73] O_2 concentration is also implicated in stem cell phenotypes *in vitro*. Low O_2 concentration (1–5%) promotes the undifferentiated state of several classes of stem cells, including hematopoietic stem cells (HSCs)[74] and ESCs,[75] perhaps because the corresponding *in vivo* niches (bone marrow and inner cell mass respectively) are at low O_2 tension. The molecular mediators involved include Oct4 and β-catenin, which are key regulators in maintaining stem cell pluripotency.[72] Changes in O_2 concentration alone can result in a switch in stem cell differentiation fates. As an example, trophoblast stem cells switch from a spongiotrophoblast to a giant cell fate when differentiated at 3% O_2 and 21% O_2 respectively.[76]

Conventional methods of studying O_2 effects typically involve setting the entire incubator at a particular oxygen concentration, making it difficult to investigate across a range of O_2 concentrations. Most studies only perform pair-wise comparisons between normoxic and hypoxic conditions. However, conditions defined as hypoxic cover over a wide range of O_2 concentrations (1–9%);[72] therefore it is difficult to compare results across different research groups since different O_2 concentrations may be selected to represent hypoxia. Because O_2 functions essentially as a soluble morphogen during development and exhibits concentration-dependent behavior, the characterization and control of O_2-dependent stem cell fate specification should be similar to those of soluble factors. Interrogating multiple O_2 concentrations to generate a dose-response curve for a given stem cell system provides a more reliable indication of the potency of O_2 as a mediator of stem cell fate. To this end, the principles for controlling mass transport regimes and generating gradients of soluble factors are generally applicable to O_2. However, we need to account for differences in solubility behavior because O_2 exists as a gas whereas soluble factors are solids at room temperature. O_2 solubility in water is dependent on its gaseous partial pressure. Its diffusivity in PDMS is also significant, rendering PDMS permeable to O_2,[67] which can subsequently alter the dissolved O_2 concentration in a PDMS microfluidic device.

There are generally two schemes for controlling O_2 concentration in microfluidic systems. The simpler approach relies on the inherent O_2 uptake by cells and convective transport by perfused culture medium to create a continuous gradient along the length of a microfluidic channel.[77,78] Due to laminar flow, the O_2 concentration at any point along the microfluidic channel length can be analytically or numerically determined by balancing the mass flux input (medium flow rate) and output terms (cellular uptake rate, permeability in PDMS), where many of the parameters in these terms have been previously determined.[78] Continuous axial O_2 gradients have been used to study liver zonation (*i.e.*, variation in liver cell phenotypes in response to an O_2 gradient).[78] While this approach is experimentally easy to implement for stem cells, we should note that the assumption of a constant cellular uptake rate in a typical mass balance equation might not be valid for differentiating PSCs. As the PSCs differentiate, their O_2 uptake rate may change correspondingly depending on the lineage of the cells (*e.g.*, O_2 consumption rate of liver cells is an order of magnitude higher than fibroblasts).[78] As a result, the cellular uptake rate becomes an O_2 concentration-dependent time-variable, which adds considerable complexity to the model. In another approach, arrays of microfluidic cell culture chambers are in close proximity with gas-filled microchannels, typically separated by a thin PDMS membrane.[61,79] Since PDMS is permeable to O_2, the dissolved O_2 in the microfluidic chambers is equilibrated with the O_2 partial pressure in the gas channels. Such double-layer microfluidic devices have been applied to shear-sensitive primary mammalian cell culture, where the gas microchannels serve as an oxygenator to decouple O_2 supply from medium flow rates (and therefore shear stress).[61,79] In a similar double-layer microfluidic device, Lam *et al.*, further demonstrated the generation of a step-function O_2 gradient by using solution gradient generators designs to mix O_2 and nitrogen in varying proportions.[80] Each gas channel at a discrete O_2 concentration is equilibrated with an underlying fluidic chamber, essentially creating an array of micro "incubators" maintained at different O_2 concentrations. These differential micro-oxygenators together with integrated O_2 sensors[77,80] provide opportunities to study stem cell fates under a range of normoxic to hypoxic conditions in a dose-response manner.

3.3 Fluid shear stress

The fluids used to introduce soluble factors in microfluidic cultures also convey shear stress to the stem cells in them. Fluid shear stress is in some cases developmentally relevant, such as during differentiation down cardiovascular lineages,[70,71,81] and sometimes a by-product of stem cell processing, such as during expansion of stem cells for clinical applications in bioreactors.[82] Thus, understanding how fluid shear affects phenotype can provide an additional fate input (enhanced cardiovascular differentiation) when desired and an understanding of how to avoid artifacts (adverse shear) when designing platforms for PSCs expansion. Macro-scale shear stress studies are typically performed with parallel-plate flow chambers[70] or a rotating cone apparatus,[71,83] which operate at a single shear stress magnitude. Therefore, most studies apply shear stress in a digital on-off manner, establishing a qualitative contribution of shear stress to changes in stem cell fate.[71,83] For instance, Fok & Zandstra developed two stirred-suspension culture systems for generation of ESC and ESC-derived cells—microcarrier and aggregate cultures, and characterized ESC developmental potential in shear-dependent conditions.[82] Agglomeration of ESC was formed under controlled shear conditions of approximately 9.86 dyn/cm^2 without adverse effects on ESC development potential.[82] In addition to PSC expansion and differentiation, shear stress/fluid flow plays a critical role during late embryogenesis and organogenesis. For instance, the growth and network rearrangement required for a vascular system will not occur if blood flow is not present, while directional flow of the extraembryonic fluid on the mouse embryonic node determines left-right asymmetry during development.[84] While quantitative studies across different magnitudes of shear stress are possible,[70,85] microfluidics provides for simultaneous application of shear stress spanning a defined range of magnitudes, and quantitative assessment of shear-dependent stem cell fate specification.

Because flow in microfluidics is analytically well-defined (*e.g.*, Poiseuille flow), this allows precise control over the resulting shear stress.[38] For example, the wall shear stress in a rectangular microfluidic channel can be calculated as:[62]

$$\tau = \frac{6\mu Q}{h^2 W}$$

where τ is the shear stress, μ is the viscosity of culture medium, Q is the volumetric flow rate, h is the height of the channel and w is the width of the channel. It follows that shear stress can be defined geometrically (by changing the channel geometries) or controlled by varying the flow rate (*e.g.*, *via* syringe pumps). Kim *et al*. created a device that simultaneously varied the flow

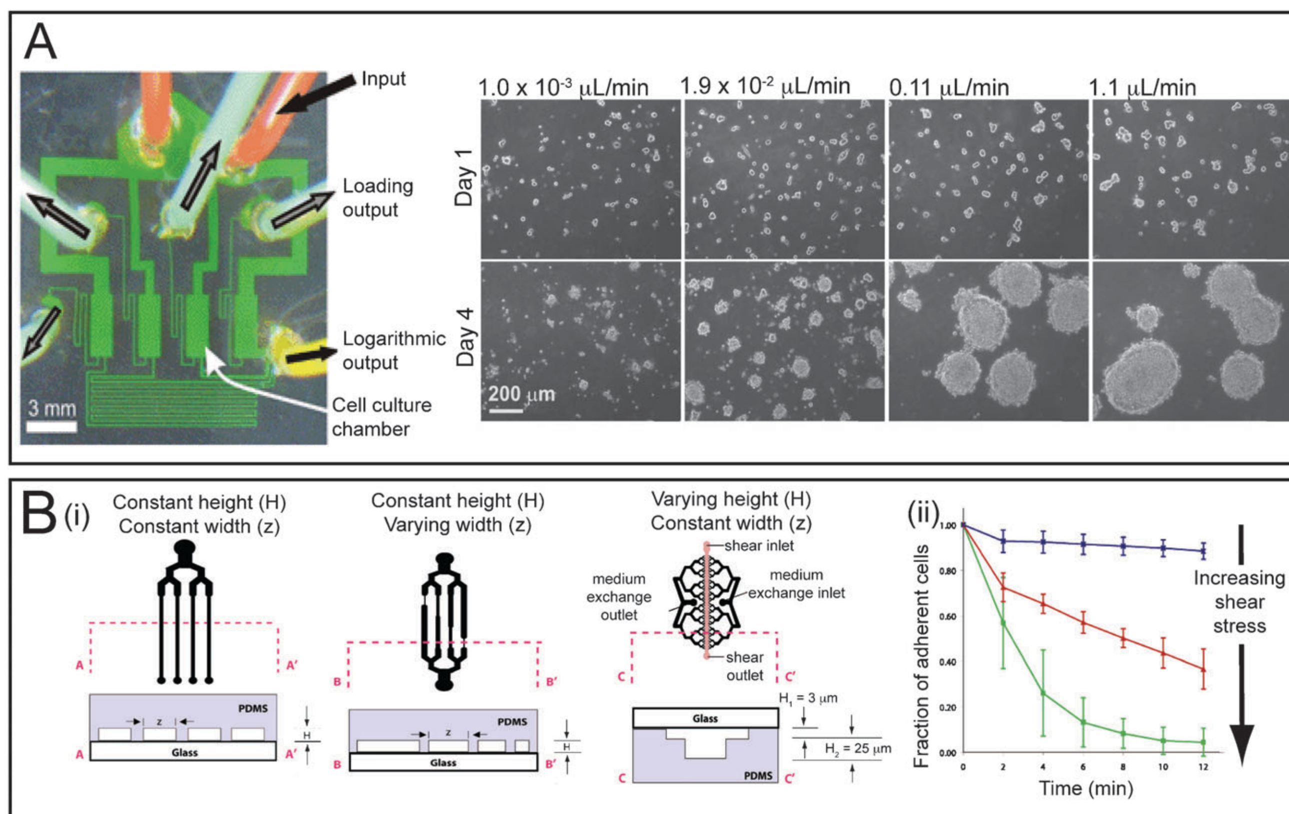

Fig. 3 Fluid shear stress for modulating stem cell fate can be parallelized and controlled precisely with microfluidic systems. (A) Shear stress within a microfluidic cell culture array can be controlled by varying flow rates with flow-setting resistor channels. This design allows simultaneous application of four different flow rates (hence shear stress) to modulate the proliferation rate of mESC cultures.[62] (B) Shear stress can also be controlled by changing the geometries of the cell culture chambers.[86] (ii) The different magnitudes of shear stress generated with different chamber geometries shown in (i) were used to investigate the dependence of cell attachment on shear stress. Images are reproduced with permission from The Royal Society of Chemistry and ACS Publications.

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rate in an array of perfusion chambers over a range of 256× by changing the geometries of flow-setting resistor channels.[62] mESCs exposed to this logarithmic varying flow-rates formed colonies of different sizes,[62] implying an effect of flow on mESC growth (Fig. 3A). Lu *et al.* developed a perfusion-shear device that varied shear stress by changing the geometries of the culture chamber, for quantitative analysis of shear effects on cellular adhesion of a fibroblast cell line (Fig. 3B).[86]

No microfluidic devices have been specifically designed to study shear in stem cell systems, which is an opportunity for the field. The effects of shear will be context-dependent, depending not only on the magnitude of the shear, but also on the soluble environment, and likely even on cell-ECM interactions, as those will alter focal adhesions and SC colony shape. It is important to discriminate between the two effects of flow in microfluidic systems: flow-rate (*i.e.*, shear)-dependent mass transport and flow as a mechanical force, and hence one must be cautious of possible misleading interpretations of results. One can envision microfluidic devices with culture chambers operating over a range of shear stresses simultaneously being used to study the effect of shear stress on the differentiation of mouse and human ESCs down specific lineages. In a similar manner, microfluidic devices could be used as scaled-down models for bioreactors to find a "safe" operating range of shear stresses in bioreactors where PSCs can be expanded in their pluripotent state without triggering spontaneous differentiations, which is of paramount importance for clinical applications.

3.4 Cell-ECM interactions

The interactions between stem cells and their surrounding ECM also influence their fate.[87] At a practical level, mESCs are typically cultured on gelatin, while hESCs are typically cultured on Matrigel™. Changing the ECM, even when in a self-renewing soluble environment, can change the phenotype of the cells, although the precise nature of how ECM signals affect self-renewal or differentiation in an inward manner is unclear. The extent to which cell-matrix interactions can skew the differentiation trajectory is nicely illustrated by the example where culturing mESCs in identical soluble micro-environment but varying ECMs directed their differentiation into mesoderm.[88] At a molecular level, ECM can influence stem cell fate specification in two ways. Mechanical traction forces felt by cells anchoring onto the ECM are transduced by force sensing elements, such as the cytoskeleton, to activate signaling pathways that affect cell fate (Fig. 4A).[89] The magnitude of these traction forces depends on the physical properties of the ECMs, such as their stiffness. Second, at the cell-ECM adhesion sites, different classes of surface receptors, most notably the integrins, interact with various ECM molecules to activate signaling pathways that mediate stem cell self-renewal or differentiation (Fig. 4A).[90] We shall treat these two aspects of cell-ECM interaction separately because they act through distinct mechanisms to modulate stem cell fates; therefore the avenues for controlling these two aspects will be different.

3.4.1 ECM-mediated mechanical forces. ECM-mediated mechanical forces, which act alongside shear, have been shown to be important mediators of stem cell fate. The differentiation of ESCs into hematopoietic or endothelial cells is augmented by cyclic strain,[91] while substrate stiffness can preferentially differentiate mesenchymal stem cells into osteoblasts instead of adipocytes.[92] A recent review by Guilak *et al.* also highlighted the importance of ECM physical properties, such as stiffness and dimensionality, in specifying stem cell fate *via* mechanotransduction signaling pathways, which are often manifested as changes in focal adhesions and cell shape.[89]

Mechanotransduction signaling mediated by the physical properties of ECMs is not as extensively studied as biochemical signaling triggered by the binding of different ECM proteins to cell surface receptors. This is primarily due to the fact that in conventional cell culture, ECMs are applied as a coating onto substrates. Therefore it is relatively straightforward to investigate the chemical composition of ECMs in this configuration by coating substrates with different ECM proteins but difficult to modulate their physical properties. Discher's group pioneered investigation of the effects of ECM stiffness on stem cell fate by culturing stem cells on polyacrylamide or collagen I gels, which can be cross-linked to various degrees to modulate their stiffness.[93] However, it is difficult to extend this strategy to other ECMs with less defined cross-linking properties, such as Matrigel™. Investigations on the three-dimensionality of ECM typically embed cells in 3D matrices before assessing changes in cell shape, focal adhesion complexes and cell function. Matrix encapsulation at the macro-scale often leads to mass transport limitations of nutrients and oxygen, which elicit changes in the cell physiology and thus potentially confound data obtained from such experimental set-ups.

The use of ECM micropatterning has emerged as an innovative approach to manipulating stem cells' interaction with ECMs for more in-depth investigation on how the physical properties of ECM influence stem cell fate. Various techniques, such as micro-contact printing (μCP),[94] ink-jet printing,[95] and mask spraying,[96] have been developed to pattern a wide range of ECMs at the micro-scale as reviewed by Falconnet *et al.*[97] Micropatterning can be employed to manipulate the cell shape by defining the footprint of the cell attachment area, thereby changing the differentiated lineage of stem cells. Using μCP to control the cell attachment area, Mcbeath *et al.* demonstrated that the switch in osteogenic/adipogenic lineage commitment in human mesenchymal stem cells (MSCs) could be regulated by cell shape (Fig. 4B, i).[92] ECM micropatterning can also modulate the traction forces experienced by cells through defining the geometry of the cell attachment area instead of changing the bulk substrate stiffness. Ruiz and Chen patterned fibronectin in various geometries such as squares, ellipses, annulus, sinusoidal bands, and used the deflection of microposts to determine the traction forces experienced by human MSCs on these fibronectin patterns (Fig. 4B, ii). They established that cells on concave surfaces experienced greater traction forces than those on convex surfaces, which preferentially directed them into osteogenic instead of adipogenic lineages.[98] These two-dimensional micropatterning techniques can be further complemented by three-dimensional approaches, where photolithography is used to cross-link cell-containing photo-polymerizable ECMs into defined patterns.[99]

It is conceivable that the ECM micropatterning techniques can be extended from multipotent SCs highlighted in the

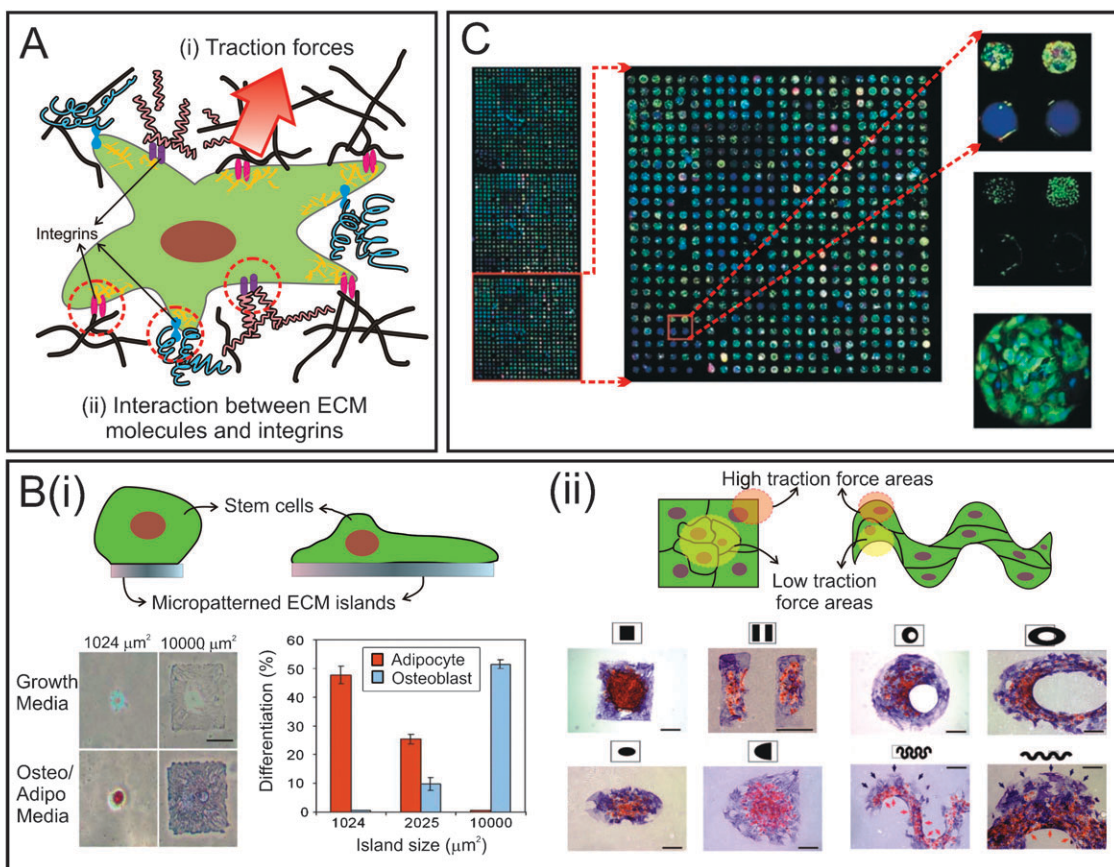

Fig. 4 Controlling stem cell fate *via* cell-extracellular matrix (ECM) interactions. (A) ECMs influence stem cells by (i) exerting mechanical traction forces that are dependent on the physical properties of the ECMs *e.g.*, stiffness and (ii) interaction between specific ECM molecules and cell surface receptors, such as integrins. (B) ECM micropatterning technologies provide innovative means of studying how physical properties of ECMs affect stem cell fates. (i) The sizes of ECM micropatterns can alter cell shape.[92] Constraining the cell attachment area with a small ECM island resulted in a rounded morphology that causes mesenchymal stem cells (MSCs) to preferentially differentiate into adipocytes (red); a stretched cell on a large ECM island favors osteogenic differentiation (blue). (ii) The geometrical shapes of ECM patterns can modulate traction forces experienced by cells. MSCs in patterned regions experiencing higher traction forces *i.e.*, at corners or concave surfaces preferentially differentiate into osteoblasts (blue) instead of adipocytes (red).[98] (C) ECM microarrays created by robotic spotting can be used to facilitate high throughput combinatorial screening (> 1000 combinations) of different ECM molecules to determine the optimal mixture of ECM molecules to specify a desired stem cell fate. Here, we show an ECM microarray employed to screen for synthetic biomaterials (blue spots) affecting the proliferation and differentiation of hESCs stained for the epithelial marker, cytokeratin 7 (green) and vimentin (red).[105] Images are reproduced with permission from Elsevier, AlphaMed Press and Nature Publishing Group.

above examples to PSCs. The increase in the number of possible lineages in differentiated PSCs poses a technical challenge since soluble factors are often applied in conjunction with the patterned ECMs to direct differentiation more efficiently, making it difficult to determine the contribution of changes in SC fate decisions to either of these cues only.[92,98] Nevertheless, it is conceivable to use a basal medium without LIF (used in EB formation), which does not bias differentiation toward any of the three germ layers. With a neutral differentiation medium, it is possible to investigate whether changes in cell shape or cell traction forces imposed by ECM micropatterning can bias the differentiation towards a specific lineage, and explore alternations in cell fate decisions contributed solely or mostly by ECMs.

3.4.2 ECM identity. The ECM contains a mixture of different protein molecules, whose exact composition is tissue-specific. As an example, the composition of basement membranes is rich in laminin while stromal ECMs in connective tissues comprise mainly of collagen.[90] Researchers have investigated the effect of various individual ECM protein molecules on stem cell fate specification. For example, laminin binds to $\alpha_6\beta_1$ integrin and is implicated in neuronal differentiation[100] while interaction with collagen II favors differentiation down the chondrogenic lineage.[101] Nevertheless, it is still not very clear how multiple ECM proteins signal collectively to modulate stem cell fate, a situation that mimics *in vivo* cell-ECM interactions more closely. Undertaking the endeavor of studying the combinatorial effects of ECMs will require the combination of multiple ECM molecules in varying proportions, which exponentially increases the number of experimental conditions. Performing these experiments using conventional well plates would be prohibitively expensive and impractical.

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Researchers are now leveraging on the ability of microtechnologies to massively handle small volume of reagents to address this issue. The most established format is the ECM microarray, where small volumes of ECM proteins and cells are distributed or immobilized onto a rigid substrate with a spatially addressable footprint using robotic spotting[102] or microfabricated wells.[103] Such cellular microarrays have been used to study stem cell responses to natural ECMs (*e.g.*, collagen, laminin and fibronectin),[102] peptides,[104] and synthetic biomaterials[105] in a combinatorial fashion (Fig. 4C). In a single experiment, typically > 1000 combinations of ECM molecules can be screened simultaneously,[102,105] which is particularly useful for determining the optimal ECM mixture for differentiating PSCs into a specific endpoint cell. For instance, Flaim *et al.* developed an ECM microarray that can combine five different ECM molecules in varying proportions and demonstrated that combinations of ECM were more effective than single ECM components for differentiating mESCs into liver cells.[102] Information obtained from such high throughout combinatorial screening using ECM microarrays can facilitate the formulation of more complex but chemically defined ECM mixtures for stem cell maintenance or differentiation. Such defined ECM blends will conceivably be more effective than single-component ECM in modulating stem cell fate while being less variable than animal or cell-derived complex ECMs such as Matrigel™.

3.4.3 Dynamic cell-ECM interactions. *In vivo*, stem cells differentiating to different endpoints are exposed to spatially and time-varying microenvironmental cues: ECMs, soluble factors, cellular interactions and mechanical forces, and thus the importance of ECM dynamics on cell fate *in vitro* is understandable. Dynamically altering ECM *in vitro* is much more challenging than dynamically altering soluble factors; thus the ability to dynamically alter ECM presentation to stem cells would put ECM on par with soluble factors and increase the overall complexity of microenvironmental cues presented to SC colonies. Whereas it is straightforward to change the soluble environment *via* wash steps at different time points of culture, changing cell-ECM interactions in conventional cultures would require passaging the cells onto new ECMs coated substrates, disrupting the microenvironment and stressing the cells. One possibility is to move from a temporal scheme (*i.e.*, changing ECM over time), typically used to dynamically alter soluble factors, to a spatial scheme (*i.e.*, changing ECM over space). This entails the ability to spatially organize ECMs on a culture substrate on which PSCs proliferate and differentiate. Further development of micropatterning technologies could create a spatial ECM gradient with varying physico-chemical properties corresponding to the temporal changes in ECMs presented to PSCs during the development. For instance, during vascular development and angiogenesis, immature capillaries proliferate in a fibronectin-rich ECM; as the capillaries matures, the composition of the ECM remodels to resemble that of the basement membrane, which is rich in laminin.[106] It follows that if one could spatially pattern fibronectin and laminin in a gradient to mimic the dynamic ECM environment during the maturation of vascular capillaries, it may facilitate more efficient differentiation of PSCs into vascular lineages.

3.5 Cell–cell interactions

In vivo, cell–cell interactions *via* membrane proteins or gap junctions also mediate cell fate. A classic example is in the *Drosophila* ovary germline stem cell (GSC) niche, where GSCs interact with somatic cells *via* cadherins and, upon division, one of the daughters migrates away from the somatic cells and begins to differentiate. In the bone marrow, hematopoietic SCs that lose contact with osteoblasts begin to differentiate.[107] Homotypic interactions between stem cells themselves are also important for their survival and self-renewal. For instance, hESCs have to be passaged as clumps to maintain their survival,[108] or alternatively supplementing media with a ROCK inhibitor allows for survival of dissociated cells,[11] suggesting the importance of cellular interactions in hESC maintenance.

Despite the importance of cell–cell interactions in modulating stem cell fate, it is difficult to precisely control these interactions using conventional biological techniques (Fig. 5A). A common method to control the extent of homotypic cell–cell interactions is to vary the cell seeding density; however it is difficult to locally control density and thus decouple the effects of paracrine signaling from actual physical contact between the cells in a culture dish. Gene knockdown/knockout approaches have been used to attenuate the function of specific cell adhesion proteins (*e.g.*, E-cadherin in mESCs[109]), but this approach is suitable only when there is prior knowledge of the identity of the molecules mediating the cell–cell interactions.

Additionally, the trend *in vitro* is generally to avoid introducing cell–cell interactions (especially heterotypic interactions) as a variable since they complicate the system and make the differentiation less defined; because the cell interactions are presumably mediated by molecules, researchers would rather replace the interacting cells with molecules to create a more-defined environment. The replacement of feeder cells for mESC culture by LIF is one example of this trend. However, the observation that ESCs need to be adapted when moved to feeder-free culture and that not all cell lines adapt as readily suggests that the feeders could be providing other signals (contact-mediated or even mechanical) to the mESCs. Additionally, because cells are dynamic systems, they can readily react to signals produced by the stem cells in ways that constant presentation of defined factors cannot (at least not currently).

Micropatterning of cell adhesion proteins (*e.g.*, collagen, laminin) can manipulate cell–cell interactions by stipulating the attachment sites of different cells on a substrate, and hence defining their spatial positions relative to each other. This approach of selectively patterning two different cell types could be potentially used to delineate whether the interaction of ESCs and feeder cells is mediated by soluble factors secreted by feeder cells only or whether cell–cell contact also plays a role. By micropatterning butterfly-shaped agarose patterns, Nelson & Chen were able to decouple cellular interactions from cell shape and found that direct cell–cell contact

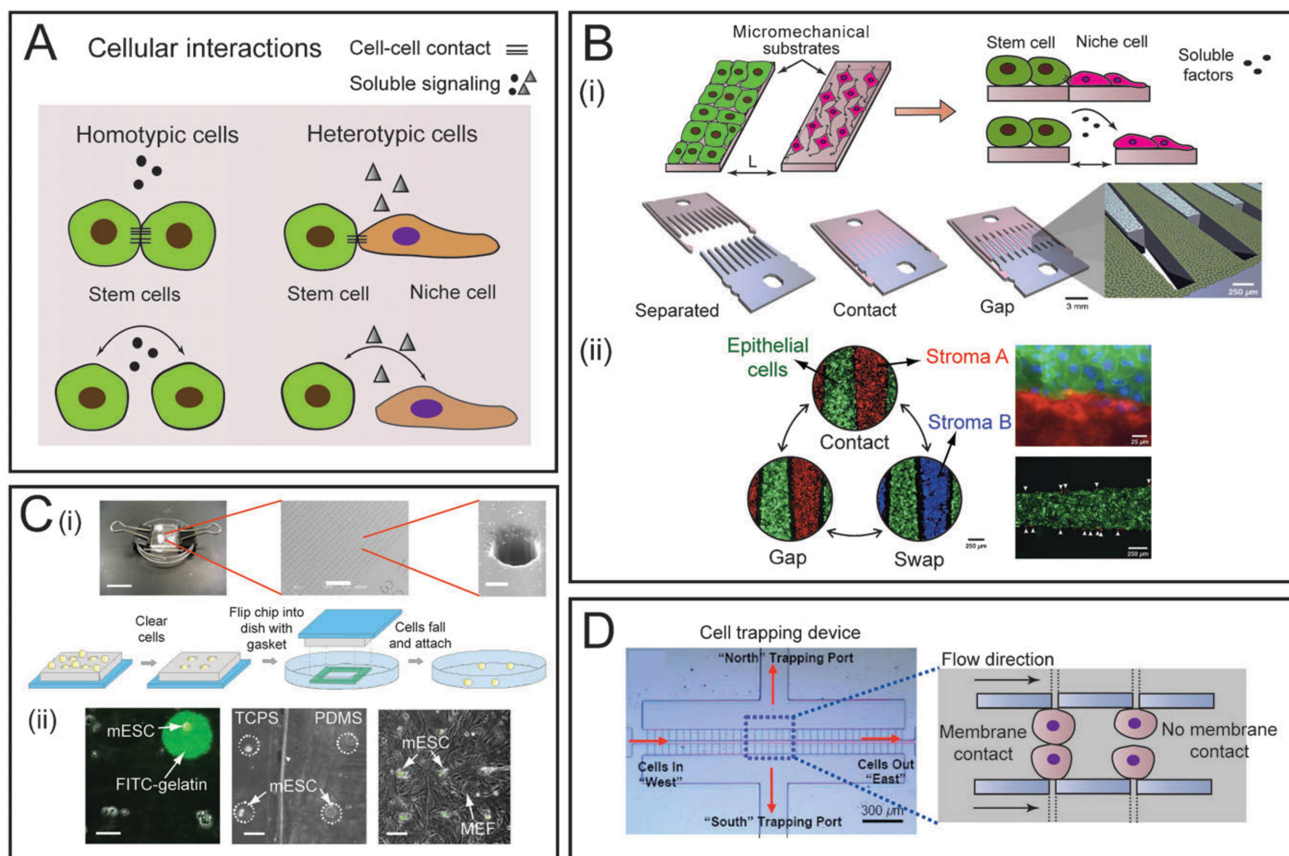

Fig. 5 Microtechnologies to manipulate cell–cell interaction for regulating stem cell fate. (A) A significant challenge in studying the role of homotypic or heterotypic cell–cell interactions is to decouple cell signaling resulting from direct physical contact and soluble signaling while not restricting the proliferation of stem cells. (B) Micromechanical substrates with two inter-digitated sections[111] which are pre-seeded with two different cell types can be used to manipulate the physical locality of the two cell populations without restricting their proliferation area as shown in (i). (ii) This device has been used to understand how different stromal cells support epithelial cell function in co-cultures.[111] (C) (i) Cell patterning with the Bio Flip Chip (BFC)[112] allows user to define the spatial location of single/group of cells with PDMS micro-wells. Cells within the micro-wells are then flipped onto a receiving substrate for subsequent culture. (ii) Since cell patterning is achieved independent of the culture (*i.e.*, receiving) substrate, a variety of substrates can be used, as demonstrated by patterning mESCs on a micropatterned gelatin island (left), tissue culture polystyrene (TCPS)/PDMS (middle) and mouse embryonic fibroblasts (MEFs) (right).[112] (D) Hydrodynamic localization of cells in microfluidic channel. Negative pressure generated by fluid flow in the two side channels flanking a central channel immobilize pairs of cells that are in direct contact or separated by a small gap.[113] Images are reproduced with permission from Elsevier, National Academy of Sciences and American Institute of Physics.

positively regulates proliferation in endothelial and smooth muscle cells *via* a PI3K-dependent pathway.[110] A similar approach could be used to study the effects of cell–cell interactions in hESC proliferation.[11] However, any type of static patterning will pose a challenge as PSC colonies proliferate, and thus restrictive patterns may introduce artifacts. Micromechanical devices have also been employed to manipulate cell–cell interactions. For instance, micromechanical substrates consisting of two inter-digitated sections have been designed to position hepatocytes and stromal cells reversibly in contact or at specific distance apart, thus enabling the study of contact-dependent intercellular communication while controlling the extent of soluble signaling (Fig. 5B).[111] Such cell-patterning approaches leave more room for the PSCs to proliferate, and could be utilized to delineate the role of cell–cell contact from soluble signaling. Rosenthal & Voldman developed a substrate-independent cell patterning technique by preloading ESCs into PDMS microwells and flipping them onto a substrate, thus achieving precise control over the spatial positions of different cells on any substrates, including feeder cell layers (Fig. 5C).[112] This simple technique allows patterning of layers of cells on top of cells, which could be potentially useful to arrange different cell types as they occur during development.[59] Microfluidics can also position pairs of cells in direct contact or in close proximity to study gap junction communications (Fig. 5D), but given the constraints of PSC doubling time, this would only permit study for relatively short times.[113]

The adaptation of such microtechnologies to stem cell research will enable researchers to better decipher the role of cell–cell interactions in determining stem cell fate. For example, Parekkadan *et al.* adapted Chris Chen's butterfly-shaped microwells to physically confine an undifferentiated mESC with an mESC-derived neuronal precursor in the presence or absence of direct contact.[114] Preliminary results indicated that contact with differentiated mESCs could induce the neuronal specification of undifferentiated mESCs in a dynamic manner

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mediated by the connexin-43.[114] However, stem cells present a challenge to interpret results from such experiments. The non-zero motility of mESCs means that the spatial organization imposed by a particular microtechnology may be disrupted (*i.e.*, cells originally in contact may cease contact, and non-contacting cells may move into contact). Additionally the 12-hour cycle time of the cells is much shorter than the typical time-scale over which we observe significant changes in SC state, making it difficult to directly attribute any observable phenotypic changes to the physical organization of cells (*i.e.*, whether they are in direct contact with each other). One possible solution is to pattern the cells in a manner that allow ESC proliferation without disrupting the plane of contact between two single cells or two populations of cells. Another solution is to select for a phenotypic marker that responds within the time frame before cell–cell contact is grossly disrupted by cell proliferation, although this option may not always be applicable.

3.6 Control *via* embryoid body formation and organization

Embryoid body (EB) formation recapitulates early embryonic development into the three germ layers (ectoderm, endoderm, mesoderm) and is commonly used as an *in vitro* pluripotency test for PSCs and an inductive step for directed differentiation of ESCs into various lineages. The size and shape of EBs dictate soluble factor gradients and the organizational architecture of cells within the EB, which in turn control the fate of the differentiating stem cells. Hence, control over the size and shape of EBs becomes a key approach to achieving reproducible differentiation of stem cells within the EB. Indeed, Hwang *et al*. found EB size-dependent differentiation propensity towards either endothelial lineage (for smaller EBs) or cardiomyogenic lineage (for larger EBs).[115] Although some of the conventional techniques used for EB generation permit some control over EB size, they are generally not suitable to scale-up.[116] Instead, a few research groups have used micro-well-type high-throughput array systems that allow creation of homogenous and easily retrievable mouse and human EBs (Fig. 6A, i).[117–120] An inventive way of forming uniform sized EBs in a format compatible to further on chip manipulation was demonstrated by Torisawa *et al*., where formation of uniform sized EBs is aided by non-adhesive semi porous polycarbonate membrane in between two PDMS channels and a microfluidic channel width (Fig. 6A, ii).[121] Such simple yet flexible systems allow for *in situ* analysis of EBs, accessibility for sequential off-chip experimentation, and can be scalable, which is important in clinical contexts.

Controlling the size and shape of EBs is an indirect way of controlling the cellular organization within the EB. This approach relies on signaling gradients that are established for a given EB geometry to direct the self-organization of the differentiating cells. We believe that established micro-technologies could be combined with EB formation devices to provide an active mean of controlling EB organization and architecture. Conceptually, gradients of soluble factors or ECMs established with microfluidics or micropatterning,

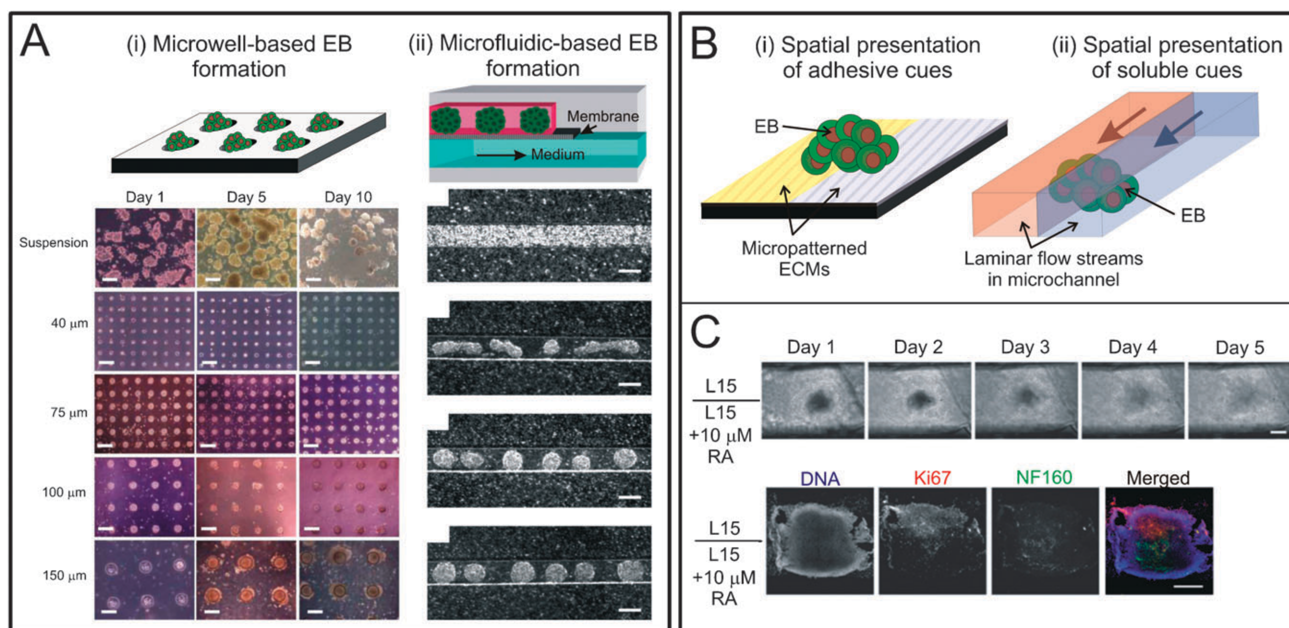

Fig. 6 Microtechnology-aided embryoid body (EB) formation and organization for more reproducible and controlled *in vitro* differentiation. (A) High throughput, uniform formation of EBs with (i) micro-wells[117] or (ii) microfluidic channels.[121] EBs of uniform shape and size develop similar cellular organization and soluble factor gradients, therefore providing a consistent baseline for different differentiation protocols. (B) A conceptual schematic on how established microtechnologies, such as ECM micropatterning and laminar flow patterning, can be combined with EB formation devices to directly control EB organization and differentiation by spatially presenting differential (i) adhesive or (ii) soluble environmental cues to a single EB. (C) A proof-of-concept device for using laminar flow patterning with self-renewing and neuronal differentiating media containing retinoic acid (RA) to control the differentiation of a single EB. The localization of the mitotic (Ki67) and neuronal markers (NF160) corresponded with the laminar streams of self renewing (L15) and differentiation (L15 + RA) media.[122] Images are reproduced with permission from The Royal Society of Chemistry.

respectively can be applied to EBs to direct the spatial organization of the three germ layers (Fig. 6B). For example, uniform-sized EBs can be formed within microwells in a microfluidic channel with a gradient generator to apply gradients of endoderm, mesoderm and ectoderm inducing factors. Fung *et al.*, demonstrated a proof-of-concept device for active control over EB organization by using laminar flow to present half of an EB with differentiation factors and the other half with self-renewal factors (Fig. 6C).[122] The spatial organization of self-renewing and differentiated cells within the EB corresponded with the localization of the laminar streams of soluble factors.[122]

4. Assaying stem cells and their differentiated progeny

Alongside control of stem cell fate, one must measure the state of the resulting cells. The phenotype of a stem cell (and almost any cell) is ultimately defined functionally, and in the case of stem cells, specifically defined by their function *in vivo*. How does one determine whether a cell is a PSC? Self-renewal and developmental potential of PSCs are assessed by different functional standards. Using mESCs as an example, a common functional test is the ability of ESCs injected into immuno-compromised mice to form mixed-cell tumors known as teratomas. Formation of tumors comprising ectoderm, endoderm and mesoderm lineages is one indicator of ESC pluripotency.[5,9] A more stringent *in vivo* assay is chimera formation – the introduction of mESCs into recipient blastocysts to determine whether they can incorporate into the embryo and give rise to all cell lineages in the body, including germline cells. The latter method, for obvious ethical and practical reasons, is not feasible in human PSC systems. Instead, pluripotency of hESCs *in vivo* is commonly tested by their ability to form teratomas in mice.[46]

While *in vivo* functional assays of PSC developmental potential remain the gold standard for probing their "stemness", they are slow (weeks to months) and, more importantly, necessitate the destruction of the cells whose function one wishes to test. Researchers have thus developed *in vitro* assays that balance stringency for practicality. *In vitro* differentiation of PSCs into all three germ layers can be attained through EB formation (see section 3.6).[123] The resulting cells can be analyzed for mRNA (*via* RT-PCR) or protein (*via* immunofluorescence or flow cytometry) expression representative of the three germ layers. PSCs can also be differentiated *in vitro* in adherent monoculture, and protocols have been established for ectoderm,[124] mesoderm,[88] and endoderm[125] lineages, though in general, monoculture differentiation is not as well established as EB-based approaches. As with *in vivo* differentiation assays, *in vitro* differentiation destroys the cells being tested and is thus a retrospective assay. Functional assays are also commonly used to assess the phenotype of endpoint differentiated cells, including beating for cardiomyocytes, action potential generation for neurons, and albumin synthesis for liver cells.[126] In contrast to assays testing the functionality of SCs, assays to test the function of differentiated cells are not necessarily destructive.

In general, a key distinguishing characteristic is whether assays are performed *in vitro* (typically easier but less stringent) *versus in vivo* (more stringent, but more laborious). As mentioned, the latter are (often) tedious to perform and destructive, therefore researchers use surrogate assays comprising a cohort of molecular factors to define cell state. These molecular factors include gene expression (*i.e.*, mRNA), epigenetic modifications, regulatory micro-RNAs (miRNA),[5,127] and the resulting protein levels, which ultimately define function at the molecular level. The compromise is always between comprehensiveness (*i.e.*, measuring many factors) and ease of assay.

4.1 Transcription factors and miRNAs

Transcriptional networks formed by transcription factors keep stem cells in their pluripotent state by repressing the expression of differentiation genes.[5,128] These networks are relatively conserved across species, and most notably Oct4, Sox2, and Nanog have emerged as the essential trio of factors in the transcriptional network that specifies pluripotent stem cell identity.[5,129] These three factors bind together at each of their promoters forming a positive-feedback motif, co-occupy their target genes, and work together to target two groups of genes, one actively expressed and one repressed/silenced but poised for upregulation during differentiation.[5,130] As a result, molecular assignment of pluripotency often involves measurement of these factors at the mRNA or protein level.

Another set of factors regulating fate-determining gene expression are microRNAs (miRNA). miRNAs are small endogenous non-coding RNAs that silence gene expression by limiting the number of mRNA transcripts available for translation either by pairing with the mRNA or directing the degradation of the target mRNA.[131] Similar to efforts in obtaining gene expression profiles, mapping the expression profiles of miRNAs provides information on the stem cell state and serves as potential "markers" for pluripotency or differentiation.[131,132] Although exact miRNA profiles differ across ESC lines, researchers have identified a set of ESC-specific miRNAs.[133]

Stem cell cultures (and their differentiated progeny) are heterogeneous. Researchers have observed heterogeneous expression of the core transcription factor Nanog across cells and over time, as well as other pluripotency-associated factors such as Rex1.[134] There is increasing evidence that heterogeneity in stem cells is a fundamental feature, and that stem cell differentiation may be a consequence of switching between bi-stable states rather than a gradual transition from undifferentiated to differentiated states.[135] Additionally, even factors involved in the core transcriptional network are not unique indicators of the state if pulled out alone and not put in context with other transcription factors. For instance, Oct4 is expressed not just in ESCs but in certain blood cells and cancer cells.[136] Heterogeneity of the population also means that molecular definition of the pluripotent state requires not just positive expression of Oct4, Sox2, and Nanog, but suppressed expression of lineage-specific markers. Researchers commonly measure markers of early mesoderm (Brachyury, Flk1), early ectoderm (Sox1, Pax6), or early endoderm (Gata4, AFP).[30,70] One sees that the number of markers that one wishes to measure quickly rises and that measuring these markers at

single cell resolution is often tedious or impossible with conventional approaches (microplate qPCR, DNA sequencing, *etc.*). Moreover, conventional bulk assays measure population-averaged expression of molecular markers and will be unable to detect heterogeneity in states within a stem cell population. Together, the potentially inherent heterogeneity of the cultures and the requirement for multiple molecular markers to assess stem cell state strongly motivate the need for highly sensitive assays to measure multiple markers at single-cell resolution.

The development of microfluidic tools for sensitive, high-throughput analysis of nucleic acids has advanced quickly (reviewed by Liu & Mathies),[137] which makes it possible to assess SC phenotype at the single-cell level, potentially allowing insights into how heterogeneity within a population contributes to SC fate specification. Microfluidic RT-PCR systems are proficient at performing rapid and parallel reactions in picoliter volumes and require minute amount of starting material (*i.e.*, nucleic acids extracted from single cells).[137] For example, gene profiling of a single hESC has been performed in a microfluidic device with highly scalable valved architecture, which made it possible to extract mRNA from an individual cell and synthesize cDNA on the same device with high mRNA-to-cDNA efficiency.[138] Mathies's group developed an integrated microfluidic bioprocessor for single-cell gene expression analysis, which completes an entire assay, from cell capture to quantitative detection of genes of interest, in less than 75 min.[139] The capability of microfluidic RT-PCR systems to determine gene expression at single cell resolution within a short time makes it practical to measure the expression of multiple molecular markers for a large (statistically significant) number of cells in a stem cell population. It is conceivable to apply these tools in the study of stem cell heterogeneity to better understand how *in vitro* stochastic fluctuations in gene expression during development can influence cell fate decisions.

4.2 Epigenetic modifications

During differentiation, stem cells and their progeny transfer their transcription state to daughter cells epigenetically. Thus, examining epigenetic modifications to the genome is a critical aspect of their phenotype. Epigenetic modifications control gene expression by making DNA more or less accessible by transcription factors and transcriptional machinery.[140] This can occur *via* DNA CpG methylation (associated with transcriptional silencing) or by modification of histones, the proteins associated with DNA packaging.[128] For instance, methylation of Lysine 4 of histone H3 (H3K3me) promotes stem cell transcriptional activity while trimethylation of Lysine 27 of histone H3 (H3K27me3) represses transcriptional activity.[128,141] In general, pluripotent stem cells are transcriptionally promiscuous and, as they differentiate, their epigenetic profile changes to a transcriptionally repressive state.[142] This helps explain why under normal conditions, differentiation is a unidirectional process. Reprogramming of cells to an induced pluripotent state thus requires removal of epigenetic modifications, and indeed this reprogramming can be stabilized by treatment with an inhibitor of DNA methylation, 5-aza-cytidine.[128,143]

The epigenetic state of a stem cell is primarily measured by bisulfite DNA sequencing and chromatin immunoprecipitation (ChIP).[107] Bisulfite DNA sequencing provides binary (yes/no) information as to whether specific DNA sequences are methylated. Bisulfite treatment changes the nucleotide cytosine to uridine but leaves methylated cytosine unchanged. The change in DNA sequences is detected by regular Sanger DNA sequencing, reflecting the methylation state of a coding sequence. ChIP interrogates protein-DNA interactions and is therefore used to detect histone-associated epigenetic modification. In ChIP, an antibody against a particular modified histone is used to purify the histone together with fragments of DNA that are associated with it. The DNA fragment can then be identified either by RT-PCR, DNA sequencing or microarray. Both of these core technologies have been miniaturized to a microfluidic format. Key advantages demonstrated by proof-of-concept prototypes over conventional techniques are similar to those of microfluidic RT-PCR. Blazej *et al.*, developed a nano-liter scale Sanger DNA sequencing chip that reduces the amount of DNA template required by 1000-fold to $\sim$1 ng.[144] Microfluidic ChIP devices can significantly reduce assay time primarily because small reaction volumes and integrated mixers shorten the time to equilibrate antibody-protein binding, which is limiting in bulk ChIP assays.[145,146] In one version of microfluidic ChIP, Quake *et al.*, performed four parallel ChIP assays with 2000 cells (as compared to 10^6–10^7 cells used in a conventional ChIP assay) automatically with integrated valves and mixers.[146] These miniaturized assays have demonstrated comparable accuracy to conventional bulk techniques for standard PCR amplicons[144] or cultured cell lines[145,146] although they have yet to be validated for epigenetic characterization of stem cells.

Microfluidic DNA sequencing and ChIP open up opportunities to measure the epigenetic state of stem cells cultured or manipulated in microsystems since they require a smaller number of cells. Currently, an equivalent of 500–2000 cells is needed;[144,146] more development is needed to reach a similar level of sensitivity as microfluidic PCR devices where gene expression of single cell can be measured. The routine adoption of microfluidic DNA sequencing or ChIP to probe the epigenetic state of stem cells in microsystems allows for better characterizing of stem cell responses to engineered microenvironments. This comprehensive data on the stem cell genetic state (*i.e.*, mRNA, miRNA and epigenetic expression) can help researchers to iterate the design of a given microsystem for better control of the stem cell niche, and relate information obtained from microsystems to current literature based on conventional biological techniques.

4.3 Protein expression

Proteins define cellular functions at the molecular level; hence assessment of stem cell state involves measurement of the protein expression level for key factors regulating stem cell fate (*e.g.*, Nanog, Oct4). For the convenience of measurements, these proteins are commonly labeled by fluorescent antibodies. While live-cell staining is possible for cell surface proteins, immunofluorescent labeling of intracellular proteins, such as

transcription factors, is destructive because of membrane permeabilization procedures. To avoid endpoint assays, one can use fluorescent reporter cell lines, which have been created for a number of stem cell transcription factors as well as differentiated progeny or surface immuno-phenotyping (*e.g.*, SSEA1 for mESCs and SSEA3/4 for hESCs). Reporter assays typically report on only one marker, making them particularly prone to incorrect assessment of phenotype since SC states cannot be accurately determined with a single marker. However, fluorescent reporter cell lines are still commonly used because they are experimentally convenient in measuring changes in protein expression level due to the external stimuli.

In addition to measuring the molecules in cell lysate, microfluidic tools have emerged for measuring molecules (proteins, essentially) in intact cells. A number of groups have attempted to scale down fluorescence-activated cell sorting (FACS), which can be used to measure fluorescent protein levels in cells and then sort cells based upon that expression. Translating conventional FACS to microfluidic-based systems enables sorting small numbers of cells (one cell in a population of 10^2–10^3 cells) as compared to sorting one out of 10^5 cells, typically considered as a small population in conventional FACS.[147] In particular, Wang *et al.*, demonstrated microfluidic sorting of cells expressing fluorescent proteins by optical force switching to maintain high cell viability after sorting.[148] By efficiently combining optical forces with a simple Y-shaped device layout, they attained not just high purity and recovery rates ($\sim$90% for both), but performed experiments with as few as 1000 cells. Apart from demonstrating a microfluidic FACS for a small number of cells, their all-optical switch for live cells could be implemented in a high-throughput system where cells would be exposed to further experiments, or it could conceivably be scaled up to a high-throughput fluorescence activated microfluidic sorter.

Another approach to measure and sort stem cells for their protein expression at the microscale is to use cell microarrays, where single cells are loaded into an addressable array, imaged with microscopy and then sorted based on arbitrary phenotypes. Cell microarrays can complement FACS, since they enable sorting based on morphological criteria in addition to whole-cell fluorescence. Such microarrays for assaying and sorting cells have utilized active cell trapping with dielectrophoresis (DEP),[149] or passive hydrodynamic trapping with microwells[150] or extruded micro-structures.[151] Once loaded, selection by visual criteria using microscopy is straightforward because most cell microarrays are optically transparent. Retrieval of small quantities of viable cells following visual inspection has also been demonstrated in cell microarrays with a micromanipulator[152,153] or a combination of optical and hydrodynamic forces.[154]

Although quantitative assessment of SC state at the protein level currently typically employs flow cytometry,[54,88,155] one can argue that image-based microsystems are powerful tools for quantifying protein expression level *in situ*. While both techniques allow protein measurement at single-cell resolution, imaging approaches enable dynamic SC state assessment over multiple time points as opposed to a single time-point assessment with flow cytometry analysis. Moreover, imaging-based assessment of SC states and heterogenity can be based on other visual criteria, such as morphology or spatial localization of markers, besides fluorescence signal. In an ideal scenario, one can envision loading a population of stem cells into cell microarrays, visually monitoring and selecting for fluorescent-tagged cell surface markers or unique morphologies, such as the compact colonies formed by self-renewing ESC or rosettes formed by neuronal cells, in real time and then retrieving the desired colony or part of a colony for further enrichment or characterization.

5. Combining control and assay

Although we are becoming more knowledgeable about the internal and external determinants of SC fate, it is still unclear how signaling pathways activated by external factors eventually integrate with the internal regulators. Pioneering work by Chen *et al.* provides one framework for how to integrate experimental investigation of external and internal factors that regulate SC fate (Fig. 7A).[156] By using high-throughput ChIP-sequencing, they identified DNA sequences that bind to both transcription factors activated by LIF and BMP4 signaling pathways (*i.e.*, STAT3 and Smad1) and pluripotency transcription factors, Oct4, Nanog and Sox 2.[156] The identification of such sequences may serve as points of convergence between signaling pathways activated by external factors and internal transcriptional regulatory circuitry.

Aside from the work of Chen *et al.*, a number of issues arise that make these types of studies challenging. First, there is a mismatch in the scales of experimentation employed to study internal and external regulatory networks. Internal networks can be studied comprehensively (*i.e.*, genome-wide), albeit among populations of cells. Comprehensively presenting different external landscapes to stem cells, however, is prohibitive both technically and financially. The extent to which we can manipulate multiple external environmental factors simultaneously in conventional cell culture is still too small to investigate their collective function in a combinatorial fashion. Given the high inter-connectivity between regulatory factors,[156] modulating a small subset of external factors (*e.g.*, LIF and BMP4, in the case of Chen *et al.*) is unlikely to be sufficient to establish clear correlations to genome-wide changes in transcriptional and epigenetic profiles. Thus, increasing the range of the modulation space of external factors to meet the global analysis available to internal phenotypic indicators is needed.

Micro-scale cell culture arrays developed for screening drugs, ECMs, biomaterials, and proteins (reviewed by Fernandes *et al.*)[157] have tremendous potential to define the stem cell microenvironment in a parallel, high-throughput format, thereby increasing the modulation space of external factors. As discussed in section 3, combinatorial effects of ECM molecules and soluble factors can be investigated with the use of ECM and microfluidic cell culture arrays respectively (Fig. 7B). However, since these platforms are designed to separately modulate immobilized (*i.e.*, cells and ECMs) and fluid-dependent (*i.e.*, soluble factors, oxygen and shear stress) external factors, the current configurations of static and microfluidic cellular microarrays are not compatible

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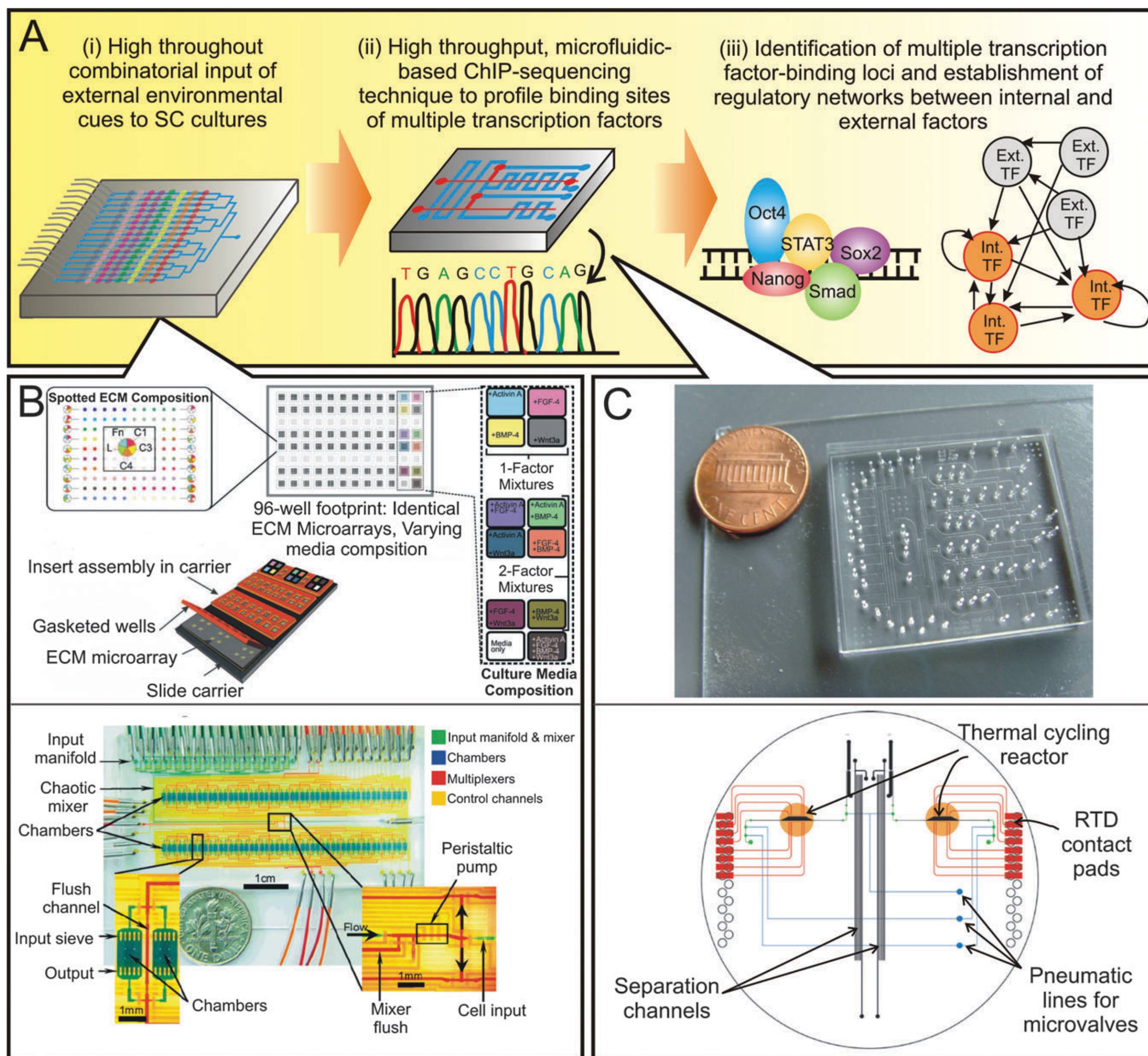

Fig. 7 Integrating experimental investigation of internal and external regulatory factors that control stem cell fate. (A) A framework for how we can integrate experimental investigation of external and internal regulatory networks that regulate stem cell fate. (i) Multiple external environmental cues are presented to stem cell cultures in a combinatorial fashion using micro-scale cell culture arrays. (ii) High content and throughput microfluidic chromatin immunoprecipitation (ChIP)-sequencing techniques are then used to profile for binding sites of various transcription factors associated with both the core stem cell regulatory networks *e.g.*, Nanog, Oct4, Sox2 and signaling pathways activated by external factors. (iii) Common TF binding sites can be identified as converging points for external and internal regulatory networks. (B) Comprehensive combinatorial presentation of both immobilized (*e.g.*, ECMs) and fluidic (*e.g.*, soluble factors) external environmental cues will require integration of existing cell/ECM microarrays (top panel)[159] with microfluidic cell culture arrays that are independently addressed from fluidic inputs controlled by microfluidic components such as mixers and multiplexers (bottom panel).[138] (C) Cells can then be analyzed with microfluidic ChIP (top panel)[146] followed by microfluidic Sanger DNA sequencing (bottom panel)[144] to determine changes in the internal genetic landscape correlating to the presented combination of external factors. Images are reproduced with permission from Mary Ann Liebert Inc., ACS Publications, The Royal Society of Chemistry, and National Academy of Sciences.

operationally; it is difficult to pattern ECMs and cells in an enclosed microfluidic channel. Although ECM patterning in microfluidic channels has been demonstrated by selectively altering substrate surface properties to modulate protein absorption,[158] the technique is not feasible for patterning large numbers of ECMs in a highly combinatorial fashion. Conversely, the standard approach for assembling a PDMS microfluidic channel (*i.e.*, plasma oxidation and mechanical clamping) will damage a statically pre-patterrned cell/ECM microarray. The challenge is to improve upon these micro-technologies to enable us to seamlessly manipulate both immobilized and fluidic external factors in a high-throughput manner, thereby achieving a comprehensive modulation space of external factors that we can search through.

One strategy is to adapt current ECM microarrays into a micro-well format so that the micro-wells can isolate the fluidic (specifically soluble) environment. Flaim *et al.* demonstrated a proof-of-concept in this approach by modifying their cellular microarray to robotically spot ECMs into 96-wells.[159] Using this system, they were able to interrogate the combined effects of various ECMs and soluble factors in 240 discrete microenvironments on stem cell cardiac differentiation.[159] To further increase the number of soluble environments will entail the use of higher density formats, such as 384 or 1024 micro-well plates, which will require expensive robotics to precisely spot ECMs into smaller wells and handle fluid mixing and dispensing.

In another approach, we can incorporate microfluidics into ECM/cell microarrays. This is now possible with the development of reversibly-sealed microfluidic systems where PDMS microchannels can be sealed onto wet substrates containing cells and ECMs.[160] Although this approach may involve substantially more development, it has two advantages over spotting ECMs and cells into micro-wells. Microfluidic systems are more efficient and cost-effective to handle the combinatorial presentation of the soluble and other fluidic-dependent external factors *e.g.*, shear stress. Another strong motivation to incorporate a microfluidic component is its compatibility with many of the downstream high-throughput techniques used to profile the internal genetic landscapes of stem cells, thus potentially realizing the aim of *probing and correlating the external and internal* stem cell landscapes. For instance, miniaturized versions of qRT-PCR,[138,139] ChIP,[145,146] and DNA sequencing,[144] have all been developed on microfluidic platforms (Fig. 7C). Therefore, microfluidic cell culture arrays can potentially be applied in tandem with microfluidic qRT-PCR or ChIP sequencing to study how signaling pathways activated by distinct collections of immobilized and fluidic external factors change the internal genetic landscape, which eventually determines the stem cell fate.

6. Outlook and opportunities

Researchers are capitalizing on two features of microtechnologies to advance the stem cell field. Microtechnology can manipulate external environmental cues at cellular resolution, therefore enabling aspects of the stem cell niche to be reconstructed. On the other hand, we are trying to scale the number of parallel experiments that one can perform since microtechnologies, because of their small feature size, consume minute amounts of reagents and can fit into a small footprint thus making high-throughput experimentation practical. So where are we in terms of engineering *in vivo*-like stem cell niches or achieving high-throughput experimentation and assays? As highlighted in this review, microtechnologies have been developed to manipulate a myriad of external factors in the stem cell niche, which determine stem cell fate at least in some context of self-renewal or differentiation. To eventually recapitulate a rudimentary stem cell niche *in vitro* requires figuring out how to assemble all these factors together in the right proportion. This endeavor is not trivial technically because different platforms are designed to manipulate specific environmental factors. Currently, the engineering designs and operations to implement control over a single factor are substantially complex such that other environmental factors are usually incorporated in the simplest possible configuration and are likely to be sub-optimal. For example, microfluidic systems that apply shear stress employ mono-cell culture with single component ECM coating (*e.g.*, gelatin). Thus, a major technical hurdle that must be overcome is the ability to build micro-scale control over various external environmental factors on a common platform. In our view, one key is to reconcile the segregation between static micropatterning technologies (for controlling cell–cell and cell-matrix interactions) and microfluidic perfusion systems (for controlling fluid-mediated factors *e.g.*, soluble factors, shear stress). Once we can control multiple factors at the micro-scale, we can start to address their spatial and temporal relationships (how the different factors are spatially located relative to one another over time), which define the stem cell niche.

Microtechnologies for high-throughput experimentations include technologies that increase the experimental modulation space (screen more experimental conditions) and those that allow more comprehensive measurements of stem cell fate (measure more read-outs). While it is intuitive that microtechnologies possess features, such as their small scales and integrated components (*e.g.*, valves, pumps) that render them amenable to multiplexing for parallel experimentation, the practical implementation is somewhat challenging. Most microfluidic systems for combinatorial input of soluble factors are still grappling with the creation of an efficient multiplexer that can generate large numbers of output solutions with a limited number of fluidic inputs (since it is impractical to have large number of inputs with dedicated valves and tubing). While individual external factors (ECMs and soluble factors) have been presented to stem cells in a high-throughtput manner (>1000 conditions), research efforts to develop microtechnologies that can perform combinatorial presentation of external environmental factors are generally limited. In comparison, the development of microassays is a lot more established. Many microassays have already demonstrated significant improvement in sensitivity and reduction in assay time. To move forward, we must validate these microassays with actual biological samples (instead of mock samples) and compare them to conventional bulk assays. We also need to demonstrate another purported advantage of microassays: multiplexing for performing multiple assays simultaneously, which is mainly limited by our ability to build highly parallelized microfluidic systems.

For the most part, microtechnologies for either fine-control of the cellular environment or high-throughput experimentation/assay are coming to the end of the proof-of-concept phase. Utilizing the unique features of microtechnologies to advance stem cell research requires addressing general issues necessary to achieve practical and routine application in biological research (*e.g.*, manufacturing consistency, standardization) as well as adapting the technology to meet the specific requirements of stem cells culture. Moving beyond the proof-of-concept phase towards applying the technologies to address specific issues in stem cell research will also help to guide and refine the design of future generations of biological microtechnologies.

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