Review

High-throughput methods to define complex stem cell niches

Stefan Kobel and Matthias P. Lutolf
Laboratory of Stem Cell Bioengineering (LSCB) and Institute of Bioengineering (IBI), Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland

BioTechniques 48:ix-xxii (*Focus on Cell Culture Technology* April 2010) doi 10.2144/000113401
Keywords: stem cell; microenvironment; artificial niche; microfabrication; high-throughput; single-cell analysis

The potential of stem cells in clinics and as a diagnostic tool is still largely unmet, partially due to a lack of in vitro models that efficiently mimic the in vivo stem cell microenvironment—or niche—and thus would allow reproducible propagation of stem cells or their controlled differentiation in vitro. The current methodological challenges in studying and manipulating stem cells have spurred intense development and application of microfabrication and micropatterning technologies in stem cell biology. These approaches can be readily used to dissect the complex molecular interplay of stem cells and their niche and study single-cell behavior in high-throughput. Increased merging of microfabrication with advanced biomaterials technologies may ultimately result in functional artificial niches capable of recapitulating extrinsic stem cell regulation in vitro and on a single-cell level.

Introduction

Stem cells are defined by their unique capability to self-renew and produce differentiated progeny, which makes them extremely interesting cellular sources for clinical tissue engineering and for in vitro drug discovery applications. However, the clinical and pharmaceutical application of stem cells is still hampered by a lack of tractable stem cell culture techniques. In particular, many adult stem cell types cannot easily be maintained in culture without compromising multipotentiality, and the directed differentiation of embryonic or induced pluripotent stem (iPS) cells and their assembly into functional tissues is still highly challenging, if not impossible (1).

In vivo, stem cell self-renewal and differentiation are tightly controlled by a complex niche that physically hosts the stem cells in an anatomically well-defined location within a tissue (Figure 1A). The key function of the niche is the perpetual maintenance of a pool of slowly dividing stem cells. Stem cells in the niche are surrounded by support (or niche) cells and are exposed to additional extrinsic signaling cues originating from interactions with the extracellular matrix (ECM) as well as various soluble stimuli. The spatially and temporally controlled presentation of these stimuli is assumed to instruct stem cell behavior by balancing the number of quiescent and cycling stem cells. Cell divisions can result in two daughter cells with the same or disparate fates (Figure 1B). Asymmetric self-renewal division (asymmetric with respect to the function of the two daughter cells), resulting in homeostatic conditions, could either be induced by an asymmetric distribution of cell-intrinsic, fate-determining proteins or by exposing the two equal daughter cells to different local microenvironments (2). Symmetric self-renewal divisions would result in the expansion of the stem cell pool at the population level (2–4).

While classical biological methodologies—ranging from high-throughput gene expression analyses or fluorescence-activated cell sorting (FACS) to in vivo experiments—have significantly increased our understanding of the phenotypic stem cell makeup and the genetic mechanisms that control stem cell behavior, they are not ideally suited to elucidate the extrinsic mechanisms of stem cell regulation. In vivo experiments on stem cell niches are often hindered by low accessibility (e.g., niches in the bone marrow, brain, and muscle) and by the difficulties to specifically manipulate niches genetically. On the other hand, commonly used in vitro approaches lack the means to recapitulate the spatial and temporal niche signaling, and are built on materials with biophysical properties that do not mimic stem cell niches. Furthermore, many classical in vitro approaches are based on population-scale cell analyses, which neglect the fact that stem cell populations are not homogeneous, while FACS analyses miss the dynamics and genealogical relationships in behaviors of large numbers of single cells.

These shortcomings have spurred the development and application of new generations of cell culture platforms building on microfabrication technologies as well as advanced biomaterials approaches. Microfabrication is a generic term describing the construction of miniaturized structures—ranging from a few to hundreds of microns—that can be fabricated via numerous techniques such as photolithography, soft lithography, and microfluidics (5,6). The use of these techniques in stem cell biology is often motivated by the low reagent consumption, high throughput, and shorter analysis times (7). These technologies promise to enable specialized applications and processes not imaginable on a larger scale.

In this review, we discuss emerging applications of such miniaturized platforms in stem cell biology. Since the rapidly growing body of literature on micron-scale systems for cell culture applications is already too large to be discussed comprehensively, we will mainly focus on those methods that

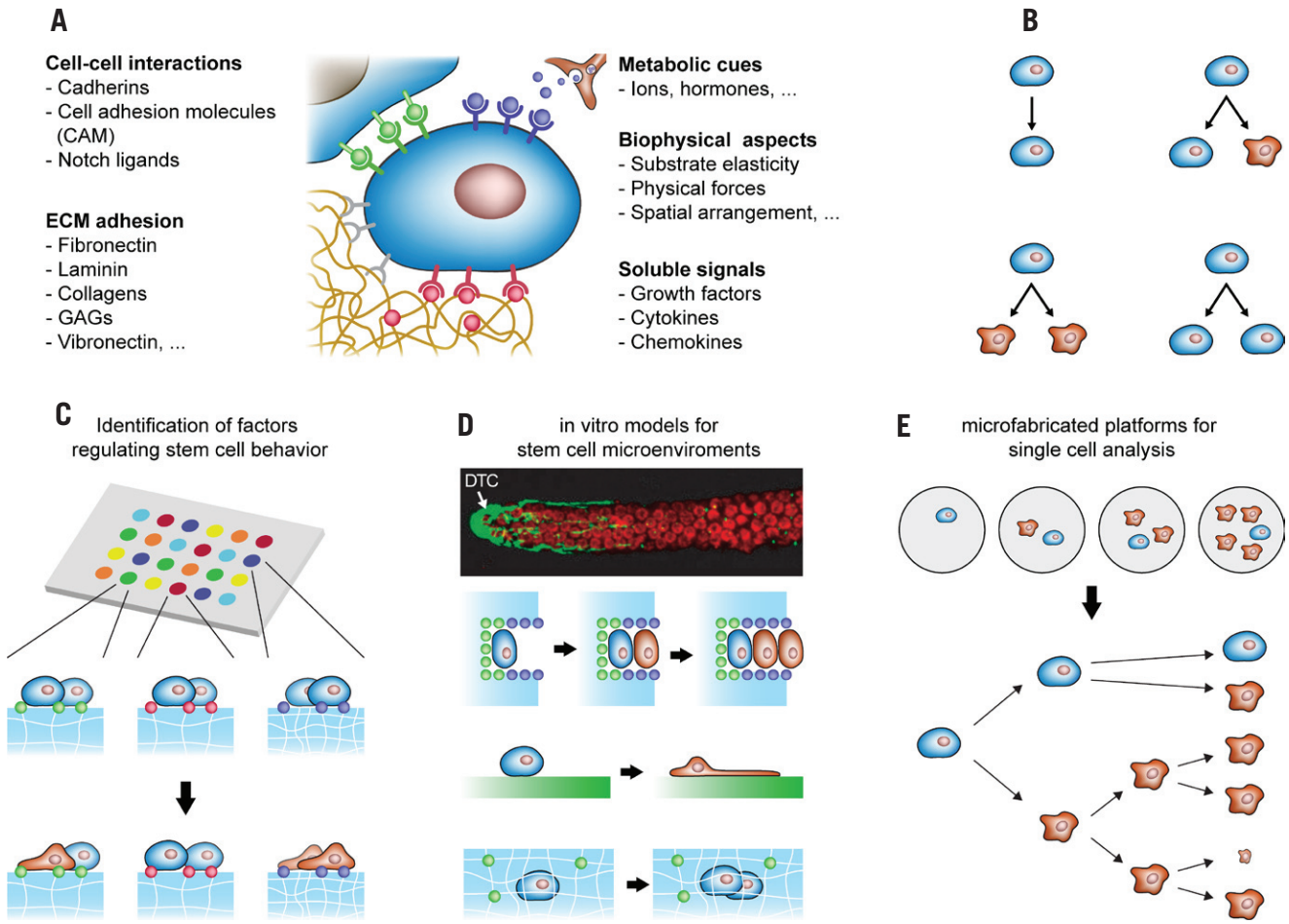

Figure 1. Microfabricated platforms to study the interaction of stem cells with their niche. (A) In the niche, stem cells are embedded within a 3-D structure composed of extracellular matrix proteins, proteoglycans, and other cells that provide a complex mixture of extrinsic cues. (B) The interaction and the physical distribution of these factors are believed to regulate stem cell fate, such as proliferation, survival, and quiescence. (C) Due to the complexity of niche signaling, much effort is dedicated to the identification of niche factors using miniaturized screening approaches. (D) Effort is also dedicated to in vitro models to recapitulate in vitro key aspects of the niche structure [here: adult gonad of *Caenorhabditis elegans* where the distal tip cells (DTC, in green) constitute the niche for the germline cells (in red). Adapted with permission from Reference 2. Copyright 2006 Nature Publishing Group]. Micro-scale artificial niches enable deconstruction of the effects of polar or graded distribution of signaling molecules, the nature of growth factor presentation, and the biophysical and 3-D architecture on stem cell proliferation and lineage commitment. (E) Because these events are difficult to detect in bulk culture, an increasing number of microfabricated platforms have been developed to track stem cell behavior at a single cell level in high-throughput.

have been applied in stem cell biology, as well as a few examples that have not yet been interfaced with stem cell biology but appear particularly promising in this context. We distinguish between three main types of applications: *(i)* high-throughput platforms to screen for many different niche factors and their combinations (Figure 1C); *(ii)* artificial niche models to recapitulate key aspects of natural niches in vitro, including spatially heterogeneous substrates, gradients, and 3-D microenvironments (Figure 1D); and *(iii)* high-throughput single-cell handling techniques to assess the heterogeneity and dynamics of stem cells and their progeny (Figure 1E). Notably, since many types of stem cells are sensitive to the biophysical characteristics of their niche, we emphasize approaches that incorporate advanced biomaterials into micron-scale platforms. We strongly believe that some of the discussed approaches will improve the validity of many in vitro stem cell experiments and will ultimately reveal novel biological insights not discoverable using conventional methodologies.

Micropatterning platforms to identify niche factors

Stem cells are exposed to a range of molecular cues in vivo, such as soluble growth factors and crosslinked ECM components, biophysical cues such as substrate elasticity, and metabolic cues (Figure 1A) (8). The complexity of these signaling networks has motivated the adaptation of microarray platforms to screen tens to hundreds of putative stem cell microenvironments. Such microarrays consist of robotically spotted combinations of cell adhesion molecules and growth factors on cell-repellent substrates, which eliminates the problem of migration of the seeded cells between spots. Due to the restricted cell movement, any observed change in cell behavior could be attributed to the unique microenvironmental combination to which cells were initially exposed (Figure 2). Pioneering work on protein microarrays was performed by Flaim et al., who fabricated a microarray consisting of 32 combinations of collagen I, III, and IV; fibronectin; and laminin

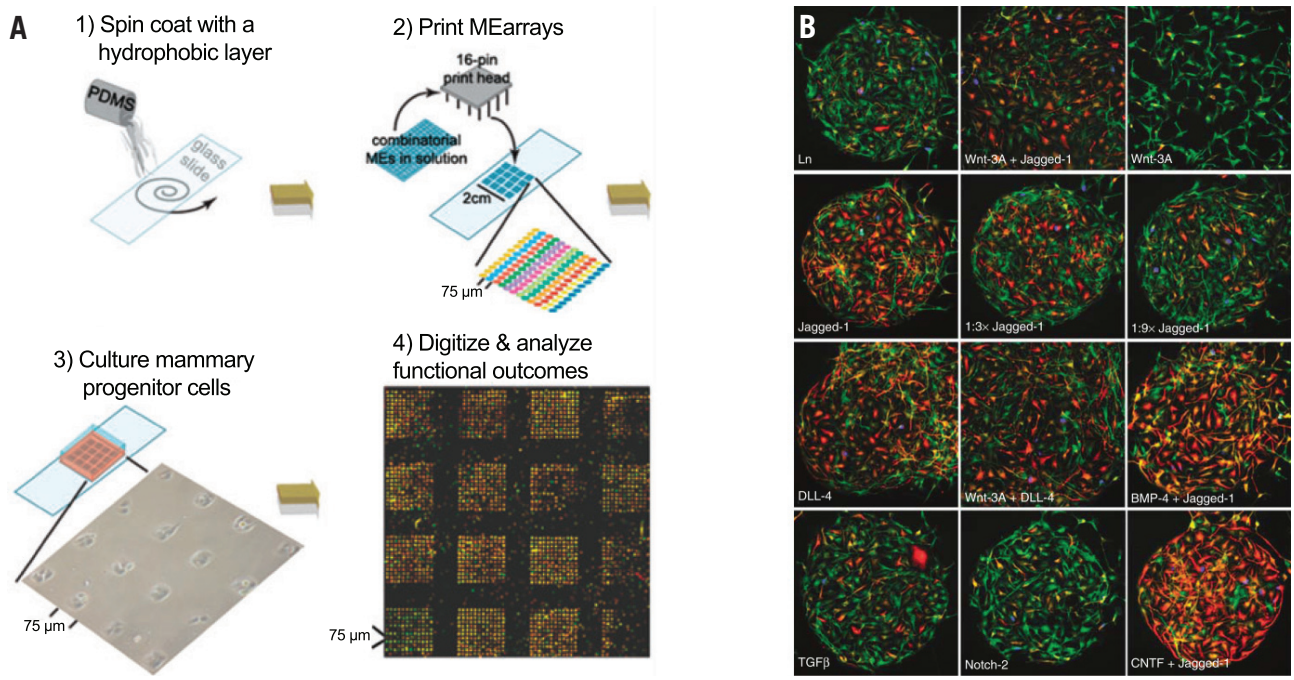

Figure 2. Protein microarrays for the high-throughput identification of niche factors. (A) Microarrays are typically fabricated on substrates such as poly-dimethylsiloxane (PDMS) or functionalized glass slides (step 1) to physically adsorb or covalently bind mixtures of growth factors and ECM components that are spotted using a robotic spotter or a printer from conventional multi-titer plate libraries (step 2). After passivation of the array to avoid nonspecific cell attachment, stem cells are cultured onto the arrays (step 3) and the effect of individual protein mixtures on proliferation or differentiation can be assessed using fluorescence stainings (step 4). Reprinted with permission from Reference 11. Copyright 2009 Royal society of Chemistry. (B) An example of primary human neural progenitors cultured on laminin (Ln) spots illustrating the strong influence of different growth factors combinations or concentrations on cell proliferation and the expression of neurogenic (TUJ1, in green) and glial (GFAP, in red) markers. Adapted with permission from Reference 10. Copyright 2005 Nature Publishing Group.

(9). By screening for liver-specific differentiation in embryonic stem (ES) colonies seeded onto these ECM arrays, they found a ~140-fold difference between the least and the most efficient protein combinations. Other groups extended the microarray concept from a pure ECM protein screen to cell adhesion molecules (CAM) and various growth factors that were physically adsorbed to a spin-coated layer of poly-dimethylsiloxane (PDMS) or covalently bound to aldehyde-derivatized glass slides (10,11). Using this strategy, Soen et al. could, for example, demonstrate that Notch ligands only led to an effect on primary human neural progenitor cell growth when covalently immobilized onto the substrate; incubation with soluble protein did not induce differentiation. They could also begin to dissect the effects of single versus combinations of growth factors, showing that Wnt and Notch co-stimulation maintained a progenitor cell state, whereas bone morphogenetic protein-4 induced the expression of both gliogenic and neuronal markers, a previously uncharacterized phenotype (10). LaBarge and colleagues systematically screened pairs of proteins for the induction of human mammary gland progenitor cell differentiation (11). Because each constituent was present in at least five different microenvironments, dominant compounds could be identified based on trends in these unique, but related substrates. Quiescence of cells could, for example, be attributed to laminin-1, and differentiation of myoepithelial cells (MEP) to P-cadherin. Notably, microarrays were fabricated to screen for a variety of cell types and target signal libraries, including synthetic polymers and mediators of cell-intrinsic programs (12,13). More recently, the integration of microarrays into a 96-well footprint using a custom-build gasket and microarray holder was described to screen for combinations of ECM molecules and soluble factors (14). These groundbreaking studies illustrate the need to dissect complex niche signaling into its individual components that can then be reassembled in a controlled fashion.

Micropatterning technologies can also be used to dissect spatial or temporal effects in microenvironmental stem cell regulation. In several studies, stem cell colonies of various sizes or shapes were fabricated in high throughput by micro-contact printing (15–17). For example, the control over colony size and separation revealed that cell shape influences human mesenchymal stem cell differentiation via a tension-based mechanism (15), or that embryonic stem cell (ESC) colony size and distribution influence ESC differentiation (16), suggesting an underlying colony-size dependent paracrine signaling mechanism (17). To investigate these paracrine signaling effects on a single-cell level, an inverted micro-contact printing method termed FlipChip was developed to precisely pattern single ESCs (18). With this method, various spatial and neighboring effects on colony growth efficiency were tested, demonstrating that efficiency from single cells was very low (~40%) and could not be improved significantly by patterning multiple cells on one spot. However, when multiple cells were patterned close to each other but far enough to prevent cell-cell contact, colony forming efficiency reached nearly 100%. Thus micro-contact printing is a useful method for studying niche-related questions in stem cell biology.

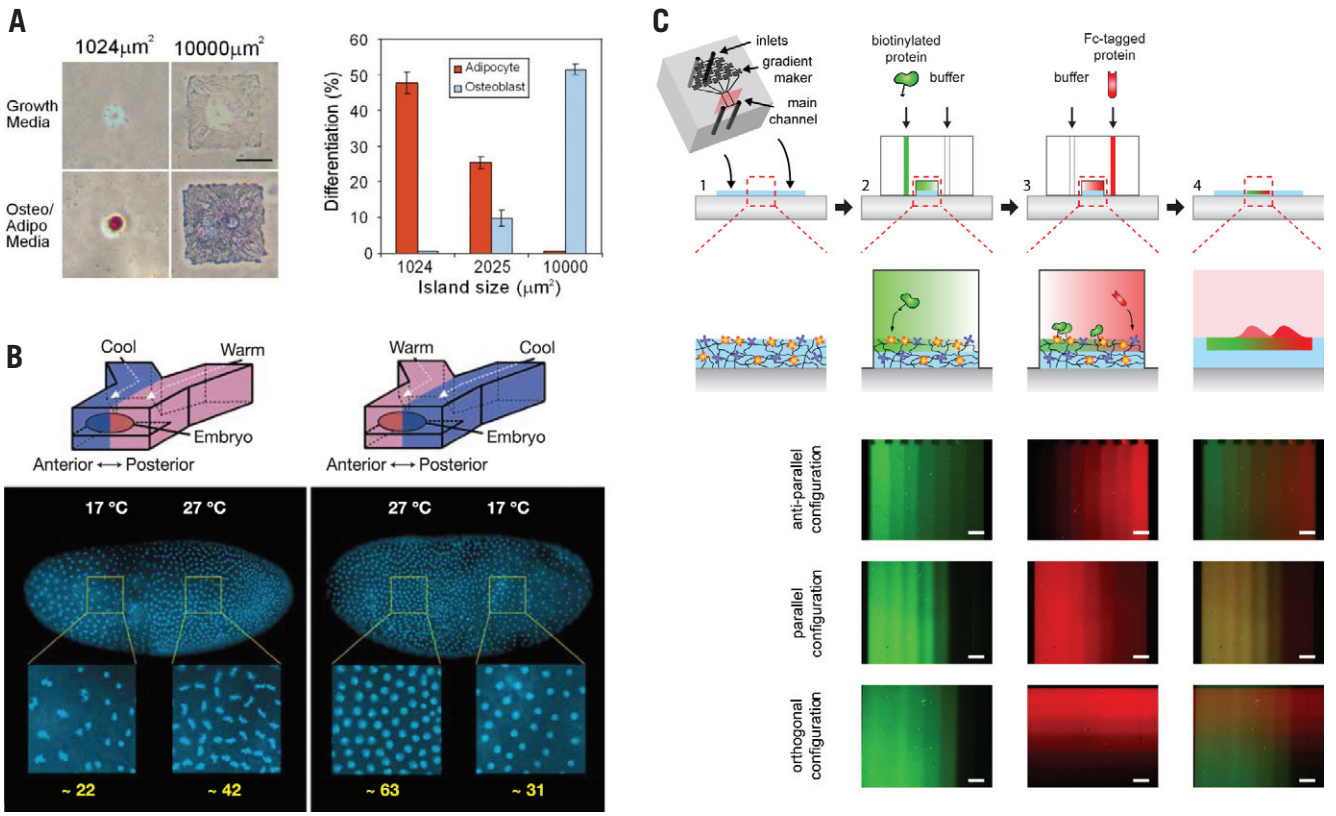

Figure 3. Micropatterning methods to probe the interaction of stem cells with their niche. (A) Adhesive micro-islands, typically generated by photolithography or micro-contact printing, confine colony size or even single cell spreading area to investigate spatial effects of stem cell regulation in a controlled and high-throughput manner. In the example shown, the hMSCs' spreading area was defined by fibronectin micro-patterns and, interestingly, regulated the stem cell fate. Reprinted with permission from Reference 33. Copyright 2004 Cell Press. (B) Whereas micropatterning is restricted to homogeneous patterns, hydrodynamic patterning can recapitulate the polarized niche architecture. Reprinted with permission from Reference 37. Copyright 2005 Nature Publishing Group. (C) Similar to the hydrodynamic patterning, microfluidics also allows the generation of continuous protein distributions by controlling the diffusive mixing of two streams. These gradients can then be used as soluble gradients or immobilized to a substrate, either via adsorption or via specific capturing proteins, such as NeutrAvidin or protein A, covalently incorporated in a hydrogel, allowing the generation of complex, overlapping gradients as found during development. Adapted with permission from Reference 55. Copyright 2009 John Wiley and Sons.

Microfluidic chips to identify niche factors

Microfluidic technology may also be utilized to optimize culture conditions for difficult-to-grow stem cells (e.g., References 19–21). At present, most research to identify niche factors is based on constant, static exposure of stem cells to extrinsic stimuli. Since stem cells can be regulated in a time-dependent manner [e.g., the circadian rhythm (22)], microfluidic platforms may be powerful tools to elucidate such time-dependent processes. Microfluidic systems, in contrast to static culture systems, allow rapid medium exchange and culture condition switching at desired time points during an experiment. An example of this was demonstrated by King and colleagues, who developed a microfluidic chip for the high-throughput variation of temporal stimuli (23). An implemented 'flow-encoded switch' enabled the simultaneous delivery of many different temporal profiles of a cellular stimulus. By controlling the pressure difference between the buffer and protein flow, variable pulse train widths, lengths, and frequencies were achieved nearly independently. With this device, the effect of tumor necrosis factor–alpha (TNFα)–induced apoptosis of hepatoma cells was investigated. By varying the recovery time after a heat shock treatment in a single experiment, the authors demonstrated that recovery was maximal after 4–7 h, supporting the dual role of TNFα in promoting both cell survival and apoptosis.

Cellular fate changes due to high shear stresses are potential confounding factors in microfluidic cultures. Conversely, perfusion is crucial for the delivery of fresh nutrients and growth factors. Consequently, microfluidic chips have been designed to optimize perfusion rates in stem cell cultures. Such chips typically consist of a network of parallel channels having different hydrodynamic resistances to vary the perfusion rate in each microchannel (24,25). Interestingly, such chip experiments have indicated that ESC culture can be improved at higher perfusion rates (21,25), one study showing a linear correlation of proliferation with perfusion rate (25). ESC growth reached a maximum at high flow rates, exchanging the chip medium in <5 min, and seemed not to depend on shear stress but rather limited by medium or cytokine supply.

Despite the apparent advantages of microfluidic technology and its successful application in many areas—including system biology (26), crystallography (27), and bioanalytics (28,29)—the impact of microfluidic technology in stem cell research thus far has been moderate. This may be explained by some unmet challenges regarding its use in a standard laboratory. One of the major limitations of microfluidic platforms is the so-called 'world-to-chip' problem: how to connect the picoliter microfluidic scale to our microliter

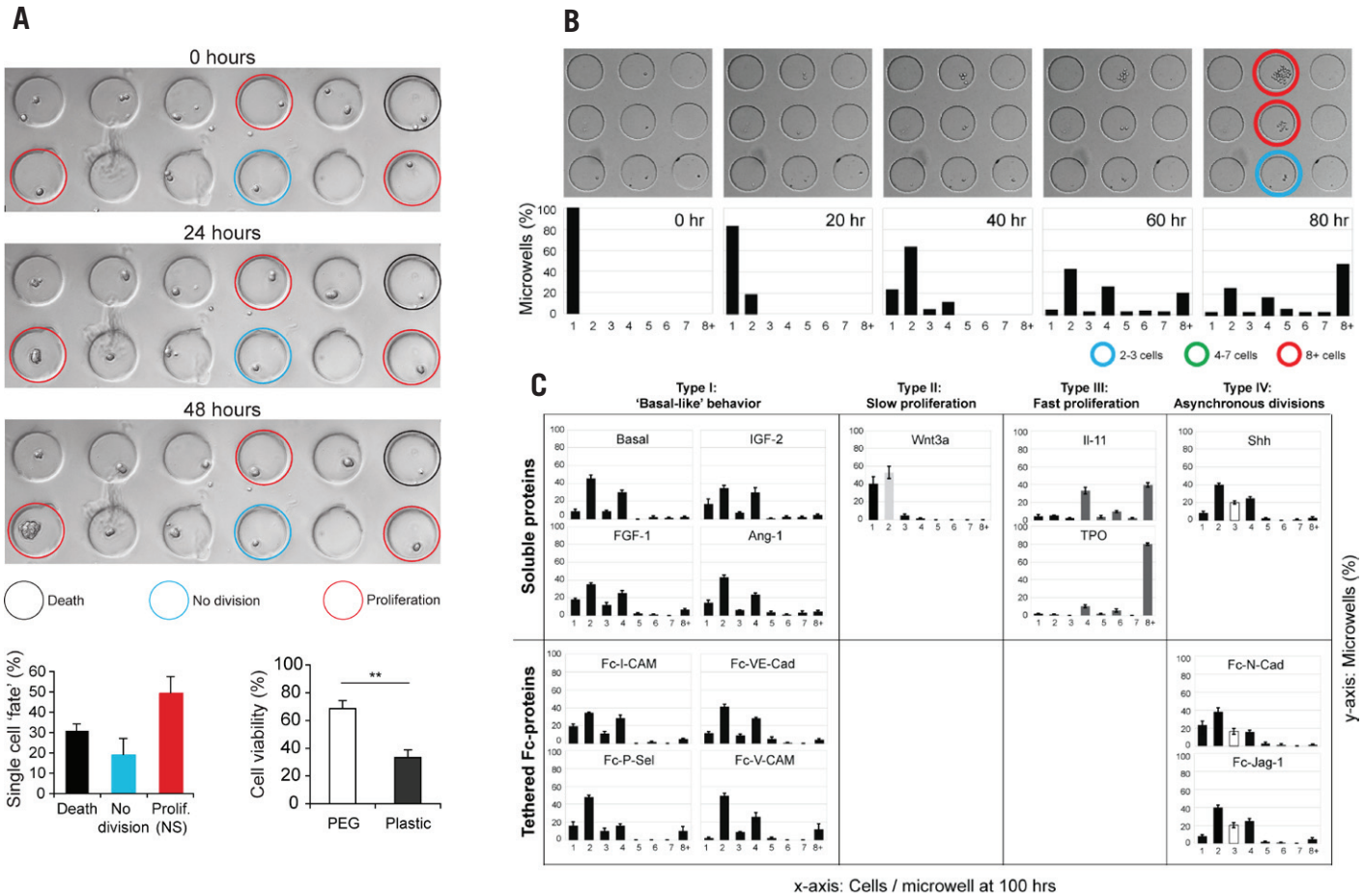

Figure 4. PEG Microwell arrays for high-throughput study of stem cell heterogeneity and fate. (A) PEG microwell arrays facilitate single-cell observation by restricting cell migration, thereby allowing the efficient imaging of the clonal growth of single stem cells. In addition, PEG microwell arrays provide a hydrated and soft substrate for cell culture and can significantly increase cell survival compared with normal tissue culture plastics. Adapted with permission from Reference 81. Copyright 2008 AlphaMed Press. (B) The physical confinement of cells to a microwell enables the study of clonal growth in high-throughput, and can reveal population heterogeneity (e.g., as found in hematopoietic stem cells). (C) This capability can also be used to perform screens on the single-cell level, as shown, revealing unexpected effects on stem cell fate. Adapted with permission from Reference 83. Copyright 2009 Royal Society of Chemistry.

world. Efforts to facilitate this down-scaling (or up-scaling) of over six orders of magnitude has involved automated on-chip valves and pumps that could be actuated using pressure and external solenoid valves (20,21) or via a Braille display incorporated into a microfluidic chip (30). Although these external valves can be controlled digitally and multiplexing allows the fabrication of arrays of thousands of individually addressable chambers with a limited number of on-chip valves (31), the reliable delivery of hundreds or thousands of different microenvironments to microfluidic devices remains a bottleneck (32). Parallelization of inputs can significantly increase throughput, as Maerkl and Quake demonstrated by combining spotting technology and microfluidics (26), and can possibly alleviate current limitations of microfluidic technology for stem cell applications.

Mimicking the spatially controlled display of niche signals

The stem cell niche is not a homogeneous microenvironment as emulated by microarrays spotted on rigid cell culture substrates, but rather a spatially well-defined 3-D heterogeneous structure (Figure 1D). Thus, the impact of the biophysical niche properties on stem cell fate (including its mechanical properties and its 3-D architecture) should not be underappreciated (2–4). Cellular- or subcellular-scale approaches to simplify this spatially complex system and to study structural aspects of the niche can be implemented using microfabrication or microfluidics. For example, McBeath et al. observed that the differentiation of human mesenchymal stem cells (hMSCs) cultured on plastic depended on cell shape (33). By confining single hMSCs to small or large fibronectin islands under mixed osteogenic and adipogenic culture conditions, they proved that restriction of cell spreading area induced adipogenesis whereas cells that could spread widely preferentially differentiated into osteoblasts (Figure 3A). This fate switch was mediated by RhoA, a small GTPase involved in regulation of the actin cytoskeleton, and its downstream effector ROCK. The role of RhoA in lineage commitment was so central that cells transfected with constitutively active or dominant negative RhoA differentiated into osteoblasts or adipocytes, respectively, independent of soluble factors.

Individual stem cells in the niche may be exposed to different local microenvironments. Spatially controlled patterning of microenvironments in vitro at the subcellular scale may be achieved using hydrodynamic patterning, whereby cells or multicellular aggregates can be asymmetrically stimulated by flowing two different fluid streams over the cell

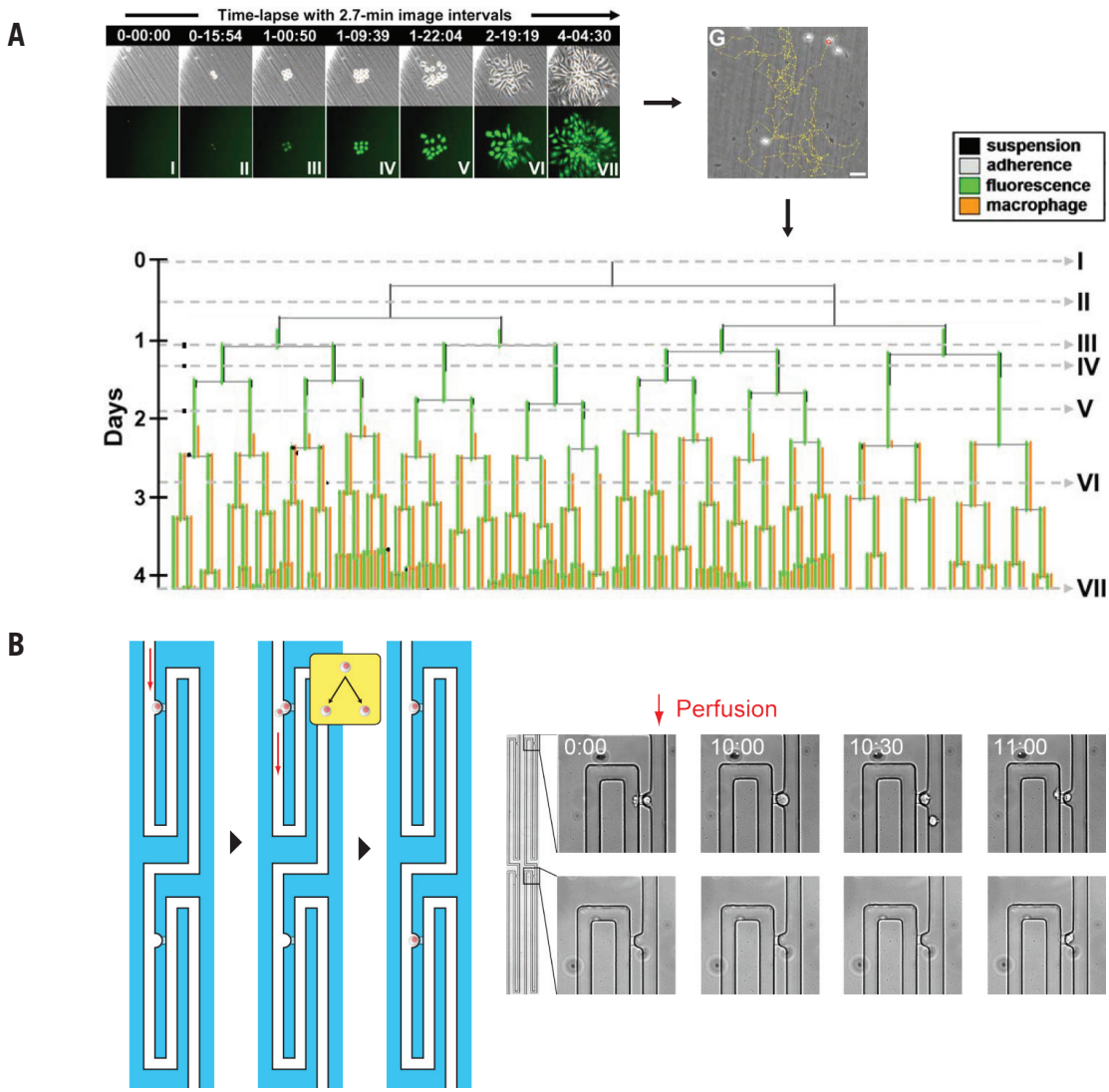

Figure 5. Tracking the genealogies of single stem cells. (A) Due to the lack of software algorithms that can reliably track cell divisions in time-lapse movies, the state-of-the-art in the construction of single-cell genealogies is manual cell tracking. The dynamics of certain stem cells can require the continuous observation of cells, therefore requiring continuous imaging of the cells in the bright-field spanning over multiple days at intervals of several minutes (top left panel). Manual tracking (top right panel) and the use of fluorescence markers allow the generation of pedigree trees, revealing important facets of single-cell behavior. Adapted with permission from Reference 96. Copyright 2009 AAAS. (B) Microfluidics devices, typically consisting of hydrodynamic traps to handle single cells, have recently been introduced to simplify single-cell fate analyses and dissect genealogical relationships of dividing stem cells. Adapted with permission from Reference 102. Copyright 2010 Royal Society of Chemistry.

in a microfluidic chip (34). The possibilities of this method have been explored to stimulate single cells (34), stimulate entire ESC colonies (35), produce asymmetrically patterned co-cultures (36), or even stimulate entire organisms. For example, Lucchetta et al. utilized hydrodynamic patterning to stimulate single *Drosophila* embryos with different temperatures (37). The temperature step across the embryo affected the local proliferation of nuclei, resulting in a density step of nuclei across the entire embryo (Figure 3B). Interestingly, a normal expression profile of the homeobox transcription factor 'even-skipped' established independently of the temperature step. ES colonies placed at the interface of two different streams (normal versus differentiation-inducing media) were observed to asymmetrically respond to the medium composition (35). Others have used a similar technique to asymmetrically pattern co-cultures of mouse ESCs and HepG2 cells, a hepatocarcinoma cell line. The latter seemed to induce an asymmetric differentiation pattern in stem cells (36), indicating the potential of hydrodynamic focusing to study mechanisms of stem cell regulation.

The above examples demonstrate some of the exciting possibilities of hydrodynamic patterning. However, this technique may not be well suited to asymmetrically pattern microenvironments of individual cells. Notably, Théry and colleagues showed that the geometry of micropatterns on a substrate can define the orientation of the mitotic spindle (38). Asymmetric micropatterns of immobilized ECM molecules and proteins mimicking cell-cell interactions such as cadherins or Notch ligands would therefore provide an excellent niche model system to study these phenomena in vitro with sufficient spatial resolution. Fabrication of asymmetric micropatterns can be achieved using microfluidics-controlled

deposition (39–41) or, with higher spatial resolution, using photolithographic techniques. For example, Derda et al. described the high-throughput photolithographic patterning of adhesion peptides for the cultivation of ESC colonies (42). The accurate alignment of photo-lithographical masks may enable the multi-step generation of well-aligned patterns of different proteins. Such an approach, possibly in combination with photo-patterning of capture molecules, such as NeutrAvidin (43), should enable the precise patterning of surfaces with asymmetrically distributed proteins to assess extrinsic mechanisms of cell divisions.

Gradients to assess stem cell behavior

Above we discussed the opportunities and potential limitations of microfluidic technology to fabricate biomolecule patterns with discrete, step-wise concentration profiles. However, microfluidics also allows the generation of continuous concentration gradients of biomolecules. Such gradients play a key role in many biological processes, including embryonic development, tissue regeneration, or tumor metastasis. During development, a multitude of overlapping gradients of signaling molecules, termed morphogens, govern the transformation of an initially homogeneous cell mass into an 'organized' arrangement of differentiated cells that, when fully developed, make up a tissue or an organ (44,45). In vivo, the establishment of morphogen gradients is tightly regulated by their binding to ECM components, including heparan sulfates. ECM binding not only limits the diffusion of morphogens, but can also increase signaling activity by clustering morphogens such as fibroblast growth factors (FGFs) or bone morphogenic proteins (BMPs) at the cell surface (46).

Classical methods to generate gradients in vitro, such as micropipets or the 'Boyden chamber,' afford a limited control over gradient formation or do not allow direct visualization of the single-cell response to a gradient. Microfluidically generated gradients can overcome these limitations (47,48). Since turbulent mixing is normally absent in microfluidics devices, the degree of mixing between two fluid streams can be manipulated by controlling diffusion between the streams to generate virtually any gradient shape. Li Jeon's group used a microfluidic gradient generator based on a pyramidal network of interconnected microchannels (47) to study the effect of a cocktail of three growth factors on the differentiation of neural stem cells. They could demonstrate a concentration-dependent proliferation and differentiation response (49). However, the combination of growth factors made it difficult to attribute these effects to individual growth factors. To overcome these effects, an osmosis-driven, long-term culture, and gradient-generating system was developed and utilized to assess the differentiation of ESC-derived neuronal progenitors (50). Park and colleagues produced overlapping anti-parallel gradients of Sonic Hedgehog (Shh) and BMP4 (or FGF8), morphogens described to specify neuronal identities during early development of the vertebrate nervous system. Increased proliferation in antagonistic Shh-FGF8 gradients and increased neurite outgrowth in Shh-BMP4 gradients was observed, exemplifying that microfluidic gradients can serve as powerful tools to deconstruct some of the complexity of in vivo morphogen interactions.

Arguably, some limitations of existing biomolecule gradient systems lay in the nonphysiological makeup of the substrate on which the gradient-exposed cells are cultured, as well as the fact that many gradients are soluble rather than substrate-tethered. Improvements of the physiological relevance of micron-scale gradient systems have resulted in approaches to tether gradients, for example via micro-contact printing of discrete cell-guidance patches (51), photo-polymerization of hydrogels containing a microfluidically generated gradient (52,53), or adsorption of laminin gradients onto cell culture plastic (54). However, the generation of in vitro model systems composed of independent overlapping gradients has been challenging. To address this technology gap, we have recently developed a versatile poly(ethylene glycol) (PEG) hydrogel system to capture from solution microfluidically generated gradients of biotinylated and/or Fc-tagged fusion proteins via NeutrAvidin or protein A, respectively, displayed on the gel surface (Figure 3C). The selectivity and orthogonality of the chosen protein binding schemes enabled the independent formation of parallel and orthogonal overlapping gradients of multiple proteins (55). The presentation of overlapping gradients on biomimetic substrates should expand the possibilities for studying a wealth of in vitro biological questions, and potentially enable high-throughput investigation of combinatorial effects of biomolecules on cell fate.

Patterned 3-D niche models

So far, we have focused on 2-D stem cell culture systems. Most stem cell niches, however, are 3-D microenvironments composed of hydrated, crosslinked networks of ECM proteins and sugars (Figure 1A). The three-dimensionality can change cell behaviors and induce striking differences in cell shape, proliferation, migration, and differentiation (4,56,57). In typical 3-D cultures, cells are randomly embedded in scaffolds either formed from naturally derived ECM components such as collagen I, Matrigel [i.e., a gel formed from a complex mixture of laminin, collagen IV, heparan sulfate proteoglycans, and growth factors (58)], or synthetic polymers. However, efforts are underway to recapitulate the heterogeneous 3-D architecture of stem cell niches using microfluidics and micromolding approaches. Micromolding typically involves the replication of gel surfaces against a 'positive' microstructured stamp, and the subsequent layer-by-layer assembly of hydrogels and cells (59,60). Whereas this method can be used to create well-defined but not interconnected cellular structures in one focal plane, compared with microfluidics it is less versatile to generate spatially modular microenvironments. Gillette et al. fabricated microfluidic scaffolds from interpenetrating networks (IPN) of collagen I and either alginate, Matrigel, or fibronectin to pattern cells and collagen I matrices in 3-D (61). 'Doping' the IPN with collagen induced the nucleation of the microfluidic collagen I phase at the interface to the IPN, which ensured the proper crosslinking of the two ECM phases and eliminated the risk that contractile forces of seeded cells could destroy the intended geometry. Others have fabricated multilayered hydrogel structures in an array of hexagonal posts that provide support and contain the gels during the injection process by balancing capillary forces and surface tension (62) or using microfluidic devices (63). Notably, microfluidically generated or micromolded 3-D gel structures enable the fabrication of multicellular, tissue-like structures exhibiting important physiological behaviors such as epithelial-to-mesenchymal transitions (60,61) and can be combined with microfluidic devices to generate 3-D gradients (64,65). These are impressive advances, but the spatial resolution of microfluidic patterning is currently limited and this technique does not allow the dynamic modification of the 3-D matrix properties.

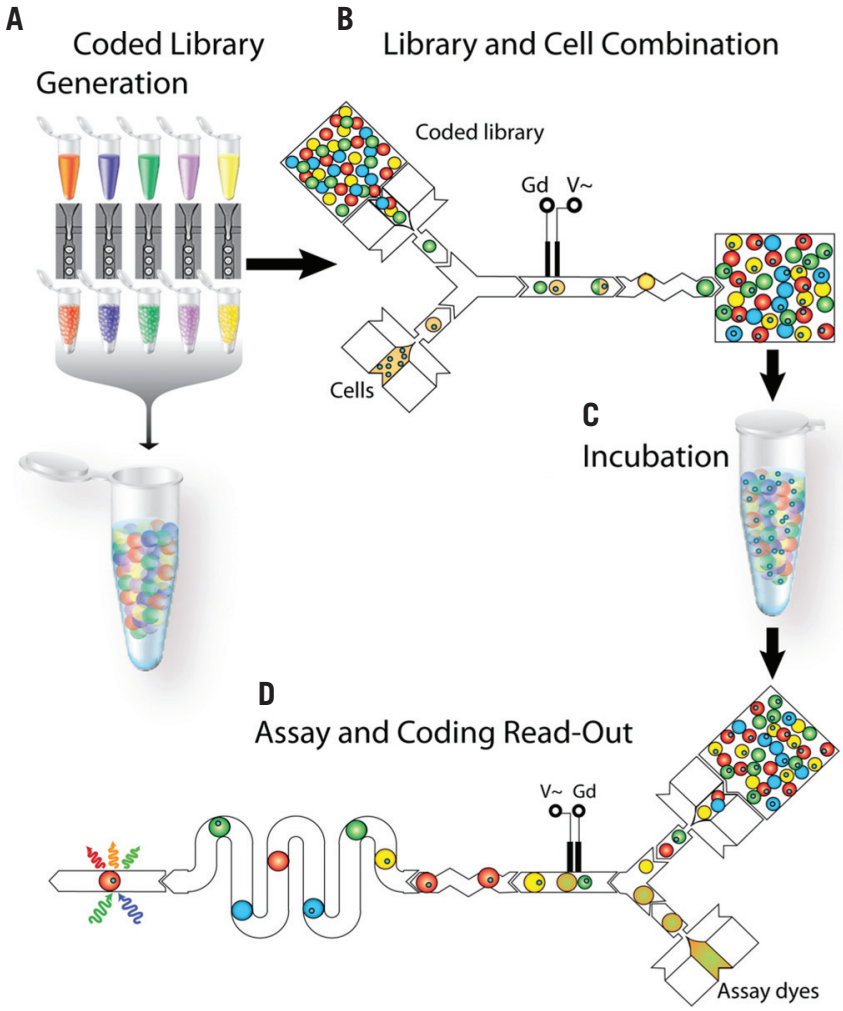

Figure 6. High-throughput screening using microdroplets. The monodisperse fabrication of microdroplets is an emerging application of microfluidics. Advances in on-chip microdroplet handling open the door for elegant screening methods at picoliter volumes and at a single-cell resolution. Stable surfactants enable the fabrication and storage of coded droplet libraries (panel A) that can be merged on chip with cell-containing droplets (panel B). The linearity of microfluidic devices thereby ensures a strict 1:1 ratio of sample and cell droplets, yielding droplets with well-defined and coded compositions. Microdroplets can be removed from the chip to incubate (panel C) and reintroduced into an analysis chip where they can be stained and analyzed (panel D). Reprinted with permission from Reference 107. Copyright 2009 National Academy of Sciences.

An alternative approach addressed these limitations by incorporating photolabile building blocks into a PEG hydrogel (66). The resulting hydrogel could be cleaved partially or completely by exposure to UV light, allowing the rapid prototyping of 3-D patterns at micron resolution using a two-photon laser-scanning microscope. hMSCs responded to locally induced changes in stiffness and availability of photolabile cell adhesion ligands. Natively, hMSCs produce the adhesion protein fibronectin and corresponding integrins. In differentiating cells, the secreted ECM is extensively remodeled by a gradual upregulation of collagen II and glycosaminoglycan (GAG) synthesis. By removing the photolabile fibronectin-derived peptide RGDS after 10 days of culture, this dynamic change in ECM composition could be efficiently mimicked and led to a significant increase of chondrogenic differentiation.

The approaches discussed above represent important advances in the fabrication of patterned 3-D cell culture systems. In combination with advanced, cell-instructive biomaterials (58), these technologies are anticipated to make exciting contributions to the field of stem cell biology. However, 3-D cell culture remains challenging because the sparse and homogeneous distribution of cells within a gel matrix may involve demanding imaging requirements. Another drawback of current

3-D patterning methods is the inefficient alignment of micropatterned protein structures to cells at a single-cell level. Several groups have explored dielectrophoresis (DEP) to handle and pattern cells in 3-D (67–69). DEP relies on the migration of cells in an externally applied heterogeneous electrical field and hence allows immobilizing cells into a hydrogel after cell patterning. However, the toxicity of some buffers and heat generation remain challenges of DEP for 3-D cell patterning (18,70). Other techniques such as acoustic traps or optical tweezers (71), in combination with appropriate microfluidic systems and biomaterials may be used alternatively to align cells and protein patterns in 3-D.

Microwell arrays as pseudo–3-D niche models

Because of the above challenges in precisely controlling cell and protein distribution in 3-D matrices, as well as the laborious imaging of cells in 3-D, the use of microwell arrays as engineered pseudo–3-D microenvironments has emerged as an alternative strategy to study single stem cells or multicellular colonies (72). Microwell arrays are topographically structured surfaces with hundreds to thousands of miniature cavities, arrayed into a regular grid. A wealth of methodologies has been developed to fabricate microwell arrays with microwell diameters ranging from tens to hundreds of micrometers. Cells are trapped by gravitational sedimentation and hence the number of cells per microwell can be controlled by the cell seeding density and microwell diameter. Due to the stochastic capturing process, the number of single cells per trap follows a Poisson distribution. The maximal frequency of microwells containing only one single cell is ~30–40%. However, microwells containing more than one cell at the onset of an experiment can be efficiently eliminated by retrospective image analysis.

Just as micropatterned ECM adhesion sites allow the control over cell spreading in 2-D (33), the capture of single cells in sufficiently small microwells can allow a quasi–3-D control over cell shape and spreading (73). For example, Kurth et al. recently probed the influence of microwell size on human hematopoietic stem cell (HSC) fate (74). Single HSCs were seeded on fibronectin-coated microwell arrays. Small microwells that nearly encircled single cells led to decreased proliferation and differentiation, quantified by the expression of the HSC marker CD34. Increasing the microwell diameter resulted in faster proliferation and lower expression of CD34, indicating that the adhesive cell-matrix contact area—and consequently the number of engaged integrins—could be involved in regulation of HSC quiescence.

Production of homogeneous stem cell colonies

Microwell arrays also allow the clonal growth of stem cells (such as ESCs) into larger 3-D cell colonies, termed embryoid bodies (EBs). These multicellular spheroids mimic some of the early stages of embryonic development, including an initial formation of the three germ layers. The traditional production of EBs by scrapping ESC monolayer culture results in heterogeneous mixtures of different shapes and diameters of EBs, which negatively affects experimental data and reproducibility (75). The alternative technique, the hanging drop culture, is based on the aggregation of cells at the tip of a single drop hanging on a lid of a Petri dish. Due to a tight control over the initial cell number per drop, this technique produces very homogeneous EBs. However, hanging drop cultures are laborious and practically preclude automation and high-throughput handling. Microwell arrays, due to their defined diameter, allow a simple choice over the initial cell number in a single spheroid and hence afford control over EB size. Microwell arrays fabricated from non-adhesive culture substrates—such as those made of PEG (76,77), agarose (78), or poly-dimethylsiloxane (PDMS) (73,75)—completely restrict cell migration, eliminating merging of multiple spheroids. These reproducibly fabricated and size-controlled EBs exhibit a more homogeneous differentiation pattern than ESC aggregates produced by traditional methods (75). Indeed, the size-controlled EB formation in microwell arrays led to insights on how EB diameter can affect differentiation. For example, it was found that cardiac—but not endothelial—differentiation was primarily induced in larger EBs. In contrast, small EBs resulted in significantly higher endothelial cell differentiation with reduced cardiogenesis (79).

The need to homogenize cell spheroid size is not only crucial in ESC biology, but also for various other stem cells such as neural or mesenchymal stem cells (80,81), as well as in cancer cells (82). Multicellular tumor spheroids, for example, reflect some of the physiological properties of metastasis and can acquire radio- and chemoresistance to apoptosis-inducing drugs, mimicking the resistance found in solid tumors. The ongoing integration of microwell arrays into classical cell culture platforms [such as multi-titer plates (83–85)] should make microwell arrays a useful tool for many fundamental studies in stem cell biology as well as applications in drug screening.

Analyses of single stem cell fates

Stem cells are inherently heterogeneous cell populations. For example, although HSCs can be isolated with relatively high purity, they show distinct reconstitution patterns in single-cell transplantation assays (86), the gold standard to prove stem cell function. A similar heterogeneity can be found in ESCs with regard to the expression levels of the pluripotency marker Nanog. Five to twenty percent of cells in a typical ESC population express low levels of Nanog even under self-renewing, leukemia inhibitory factor (LIF)–containing conditions (87). Recent experiments indicate that this heterogeneity is caused by an oscillating expression of a set of synergistically and antagonistically acting genes and could therefore be considered an integral part of pluri- and multipotency for both embryonic and adult stem cells (87–89).

To investigate these often low-frequency events, single stem cell fates have been traditionally analyzed in standard multititer cell culture plates, such as the 96-well plate (90). These unicellular cultures allow cells to be analyzed at the single-cell level and followed over time as single cell–derived clones, but they require disproportionately high amounts of cell medium and are highly inefficient. Microwell arrays are well-suited to analyze large populations of single cells at the single-cell level. Of course, similar single-cell analyses can also be conducted on micro-contact printed substrates (11) but the topography of microwells extends single-cell analyses to non-adherent cells with clinical relevance such as neurospheres or hematopoetic stem cells (81,84).

A pioneering high-throughput single-cell microwell study was conducted by Chin et al. on rat adult hypocampal progenitor. Using time-lapse microscopy, a large number of quiescent or slowly dividing cells were identified alongside a small fraction (3–4%) of highly proliferating cells. As a consequence of differences in proliferation capacities, 62%

of all the cells at day 4 of culture were derived from only 23% of the initial population (91). Our group expanded on these studies to investigate the clonal growth of single mouse neural stem cells into multicellular neurospheres on microwell arrays fabricated from PEG hydrogels. The hydrogel proved to be a superior cell culture substrate in terms of cell survival compared with tissue culture plastic, presumably due to its biomimetic properties. The microwell-confined culture revealed a subpopulation of slowly dividing cells that would be masked by the merging of spheroids in conventional cultures (81) (Figure 4A).

Another stem cell population increasingly investigated using microwell arrays are HSCs (Figure 4B) (74,83). Confirming work by others (92), Lutolf and Blau et al. demonstrated that cell cycle times of individual long-term repopulating stem cells were significantly prolonged and showed a wider distribution compared with multipotent progenitor cells. Expectedly, the cultured cells did not show any repopulation potential when transplanted into irradiated mice (83). To further elucidate if the addition of exogenous factors can induce self-renewal divisions in culture, 14 putative niche factors—either provided as soluble proteins or tethered to the bottom of individual microwells—were screened for their effect on single-cell proliferation in vitro using microwell arrays cast in 96-well plates. To probe gel-immobilized stem cell regulatory proteins, micro-molding and micro-contact printing were combined and specific protein immobilization was achieved by functionalizing the regulatory proteins with a heterofunctional PEG linker covalently bound to the hydrogel. Site-specific immobilization of protein A extended the method to pattern any Fc-tagged transmembrane proteins. Strikingly, 40% of the tested 14 growth factors yielded significant changes in single-cell proliferation kinetics compared with the basal medium. For example, Wnt3a significantly slowed down proliferation, whereas some factors, such as thrombopoietin, led to accelerated cell division rates. A third class of growth factors, including Shh or immobilized N-cadherin, yielded a significantly higher frequency of single cells producing only three progeny within a week, suggesting that the founder cell had undergone an asymmetric division (Figure 4C). Importantly, the slow or asynchronous in vitro proliferation behavior correlated with the in vivo blood repopulation capacity (83).

Microwell arrays provide a powerful tool for the culture of cell spheroids, to assess the spatial and dimensional effects of geometric patterns at a single-cell level, as well as the tracking of clonal growth of populations of single cells. However, it should be mentioned that microwell arrays are passive structures, which restricts the possibilities to manipulate trapped cells. Therefore, integration of microwell arrays into microfluidic devices (93) or the development of releasable microwells (94) may provide new opportunities to handle and analyze cells cultured in microwells.

Microfluidic approaches to track single stem cell development in vitro

Results of time-lapse experiments on single stem cells cultured in microwells (e.g., Reference 83) suggest that HSCs can undergo self-renewal divisions in vitro, and that cell fate choices are under the control of niche factors. However, the distinction between symmetric and asymmetric divisions (Figure 1B) cannot be made a priori using microwell cultures alone. The understanding of these particular fates in stem cells is of utmost importance for clinical applications of many stem cell types (e.g., HSC expansion for transplantations to treat blood cancers). Indeed, in many mammalian tissues it is not yet known whether homeostasis is maintained by asymmetric divisions or by a 'population-based' strategy that uses symmetric divisions to balance stem cells and differentiated progeny (2). Because rare adult stem cell divisions can hardly be imaged in vivo (95) (e.g., HSCs that are buried in a poorly accessible bone marrow), there is a considerable demand for in vitro platforms to address this question in live cells.

State-of-the-art in vitro approaches to assess single-cell fate changes—in particular, the symmetry of division—include the manual tracking and analyses of dividing cells in conventional cultures. Although this approach is technically highly demanding, Schroeder and colleagues succeeded in visualizing blood generation from hemogenic epithelium and in tracking the progeny of single hematopoietic progenitor cells (Figure 5A) (96,97). To achieve this goal, these researchers imaged single cells seeded in standard culture plates at a frequency of 2–3 min and developed an advanced cell tracking software to cope with the tremendous data volumes. Although it should be possible to increase the efficiency of data acquisition in these studies using, for example, microwell arrays, the rate-limiting step of this approach is the downstream semi-manual image analysis. Notably, there are a few ongoing efforts addressing the image analysis challenge in microwells. For example, Kachouie et al. developed an algorithm to detect cells and PEG microwells in fluorescence images, taking advantage of the autofluorescence of the PEG hydrogel (98). A software that can recognize PDMS microwells in bright-field images, and that detects and clusters cells based on fluorescence images was also reported (99). However, because both algorithms depend on fluorescent images for cell recognition, and because frequent fluorescent imaging can lead to phototoxic effects in the imaged cells, fluorescence-based cell recognition may be problematic to reliably track cell divisions requiring continuous, long-term observation of primary stem cells (96).

Rowat et al. reported on a microfluidic device overcoming the difficulties associated with continuous imaging. Their chip allowed the tracking of multiple yeast lineages in parallel by trapping single cells and constraining them to grow in channels for as many as seven generations (100). Aligning all of the single-cell progeny in lines facilitates image analysis because it reduces the complexity of cell recognition from a 3-D to a 1-D problem. Whereas cell tracking on normal culture substrates requires finding a cell in both space and time, cell recognition in microfluidic devices can be reduced to detecting the time-dependent occupancy of predefined microfluidic traps; it should therefore be more amenable to complete automation.

Despite these exciting advances, the question of divisional symmetry remains poorly addressed. We believe that microfluidic technology has the potential to aid in revealing the molecular mechanisms that govern the fate of individual stem cells—in particular, the symmetry of stem cell divisions. Importantly, any imaging-based analysis of single-cell behaviors would need to be complemented by further assays to characterize cell fate, such as by differential phenotype or gene expression pattern. This could be done by physically separating daughter cells in a predictable manner and removing them

from their microfluidic environment after separation. Along those lines, Faley et al. designed a microfluidic device to study the behavior of human HSCs and chronic myeloid leukemia cells (101). Their microfluidic device consisted of an array of hydrodynamic traps that allowed the anecdotal observation of cell divisions using stained mitochondria. However, the parallel arrangement and the size of the hydrodynamic trap were not designed to reliably separate stem cells upon division. To accomplish this more effectively, we adapted a microfluidic perfusion chip having a consecutive number of serial hydrodynamic traps (102,103), small enough to host only one cell and reliable enough to capture daughter cells after division (102) (Figure 5B). By optimizing the hydrodynamic conditions of this trap and the perfusion of the chip, we reached a trapping efficiency of up to 97% and showed viability of nearly 95% of the trapped cells in long-term in vitro experiments. These prerequisites enabled us to visualize cell divisions of non-adherent leukemia cells under perfusion. Because each single cell trap was small enough to host only one single cell, one of the daughter cells generated upon division remained in the trap whereas the other one was transported into the next free trap by perfusion (Figure 5C). The automated physical separation of daughter cells via microfluidics, combined with additional (on-chip) fate analyses including PCR (28), could provide the foundation to rationally assess the regulatory mechanisms that govern single stem cell proliferation and lineage commitment.

Enhancing existing stem cell culture platforms

To date, microscale technologies for stem cell biology have primarily been based on classical cell culture substrates, in particular, tissue culture plastic and glass. However, it has been increasingly acknowledged that the biophysical characteristics of a cell culture substrate can significantly influence stem cell behavior. A seminal study by Engler et al. demonstrated the influence of substrate stiffness on mesenchymal stem cell differentiation (104). Soft hydrogels mimicking the elastic properties of the brain led to the preferred differentiation of hMSCs into neurons; stiffer matrices were myogenic and relatively rigid substrates (as found in the collagenous bone) induced osteogenesis. We expect that the awareness of cell sensitivity to biophysical factors will result in the increased use of soft biomaterials as cell culture substrates in microfabricated platforms (9,81,83), and will also initiate the increased development of approaches to micropattern such materials using micro-contact printing (83), photolithography (66) or microfluidics (53,55). This convergence of existing technologies could generate truly unique microenvironments for cell fate manipulation. To this end, the integration of advanced hydrogel chemistries, micromolding, and micro-contact printing into a single platform has been demonstrated (83). This engineering approach yielded PEG microwell arrays displaying tethered proteins on a soft, tissue-like substrate. This enabled the effect of tethered factors on single non-adherent hematopoietic stem cells to be investigated, which would not have been possible on unstructured substrates due to the extensive migration of HSCs on flat substrates.

The same properties that allow microwells to extend the application of protein patterns to non-adherent cell types may also be used for testing the effect of protein combinations on stem cell differentiation. Combinatorial growth factor and ECM microarrays typically bear the risk of differential adhesion preferences of cells to different substrates, potentially affecting the outcome of an experiment (11). A micro-contact printing technology that combines microarrays and microwell fabrication with soft materials possessing tunable biophysical properties should enable the screening of different substrate properties and protein combinations with well-controlled cell numbers and thereby open up new possibilities to define functional components of stem cell niches.

Recent efforts to develop 3-D microarrays—where cells and test compounds are embedded into a hydrogel—indicate the need to expand screening possibilities to the third dimension (105). Apart from robotic spotting, other techniques might be well-suited for this purpose. Because the encapsulation of cells into a hydrogel eliminates the need to fix the hydrogel samples statically onto a substrate, encapsulated cells could also be cultured in suspension. One powerful approach could be the microfluidic fabrication of hydrogel microbeads containing cells and test compounds. Microfluidic-based droplet generation is based on the injection of an aqueous solution (the discontinuous phase) into an immiscible carrier fluid (the continuous phase), typically oil, inside a microfluidic chip. The immiscibility of the two phases leads to the well-controlled emulsification where the two phases meet and to the generation of up to 10,000 microdroplets per second with a very low size distribution (<2% variation) (106). Due to the very high throughput of microdroplet generation and the engineering of sophisticated on-chip droplet handling methods, microdroplets in combination with a bar-coding system represent a powerful alternative to conventional screening platforms. They were already successfully used in crystallization or cytotoxicity screenings (27,107) (Figure 6). However, microdroplet-based systems are currently limited to nonadherent cells because they lack a substrate for cells to adhere. The in-droplet gelation of a hydrogel would therefore not only extend the possibilities of microdroplets-based screenings to adherent cells, but also open the door for the high-throughput and consistent fabrication of microtissues (108).

In conclusion, the above stem cell culture systems, built at the interface of microfabrication and biomaterials technology, could greatly contribute in identifying the role of specific niche components and the niche architecture in regulating stem cell fate, including (symmetry of) cell division, self-renewal, and differentiation. These approaches currently represent highly simplified models of the in vivo niche, but they allow deconstructing the in vivo complexity and reconstructing it in a well-defined fashion (i.e., from the bottom up). By analyzing the dynamic responses of stem cells to these artificial niches, we should expect advances in the generation of adequate numbers of stem cells and the ability to control their directed differentiation in order to maximize their utility for cell-based therapeutics, as well as drug screening applications.

Acknowledgments

We are grateful to our collaborators in the Microsystems Institute of EPFL (namely Philippe Renaud, Ana Valero, Sebastian Maerkl, Luis-Miguel Fidalgo, Juergen Brugger and Kris Pataky) and in the Laboratory of Stem Cell Bioengineering (in particular, Samy Gobaa and Steffen Cosson). We apologize to all the scientists whose work we could not cite due to space restrictions.

Competing interests

The authors declare no competing interests.

References

1. **Daley, G.Q. and D.T. Scadden.** 2008. Prospects for stem cell-based therapy. Cell *132*:544-548.
2. **Morrison, S.J. and J. Kimble.** 2006. Asymmetric and symmetric stem-cell divisions in development and cancer. Nature *441*:1068-1074.
3. **Fuchs, E., T. Tumbar, and G. Guasch.** 2004. Socializing with the neighbors: stem cells and their niche. Cell *116*:769-778.
4. **Lutolf, M.P., P. Gilbert, and H.M. Blau.** 2009. Designing materials to direct stem-cell fate. Nature *462*:433-441.
5. **Beebe, D.J., G.A. Mensing, and G.M. Walker.** 2002. Physics and applications of microfluidics in biology. Annu. Rev. Biomed. Eng. *4*:261-286.
6. **Falconnet, D., G. Csucs, H.M. Grandin, and M. Textor.** 2006. Surface engineering approaches to micropattern surfaces for cell-based assays. Biomaterials *27*:3044-3063.
7. **Maerkl, S.J.** 2009. Integration column: Microfluidic high-throughput screening. Intgr. Biol. *1*:19-29.
8. **Lutolf, M.P. and J.A. Hubbell.** 2005. Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. Nat. Biotechnol. *23*:47-55.
9. **Flaim, C.J., S. Chien, and S.N. Bhatia.** 2005. An extracellular matrix microarray for probing cellular differentiation. Nat. Methods *2*:119-125.
10. **Soen, Y., A. Mori, T.D. Palmer, and P.O. Brown.** 2006. Exploring the regulation of human neural precursor cell differentiation using arrays of signaling microenvironments. Mol. Syst. Biol. *2*:37.
11. **LaBarge, M.A., C.M. Nelson, R. Villadsen, A. Fridriksdottir, J.R. Ruth, M.R. Stampfer, O.W. Petersen, and M.J. Bissell.** 2009. Human mammary progenitor cell fate decisions are products of interactions with combinatorial microenvironments. Intgr. Biol. *1*:70-79.
12. **Mei, Y., M. Goldberg, and D. Anderson.** 2007. The development of high-throughput screening approaches for stem cell engineering. Curr. Opin. Chem. Biol. *11*:388-393.
13. **Underhill, G.H. and S.N. Bhatia.** 2007. High-throughput analysis of signals regulating stem cell fate and function. Curr. Opin. Chem. Biol. *11*:357-366.
14. **Flaim, C.J., D. Teng, S. Chien, and S.N. Bhatia.** 2008. Combinatorial signaling microenvironments for studying stem cell fate. Stem Cells Dev. *17*:29-39.
15. **Ruiz, S.A. and C.S. Chen.** 2008. Emergence of patterned stem cell differentiation within multicellular structures. Stem Cells *26*:2921-2927.
16. **Bauwens, C.L., R. Peerani, S. Niebruegge, K.A. Woodhouse, E. Kumacheva, M. Husain, and P.W. Zandstra.** 2008. Control of human embryonic stem cell colony and aggregate size heterogeneity influences differentiation trajectories. Stem Cells *26*:2300-2310.
17. **Peerani, R., K. Onishi, A. Mahdavi, E. Kumacheva, and P.W. Zandstra.** 2009. Manipulation of signaling thresholds in "engineered stem cell niches" identifies design criteria for pluripotent stem cell screens. PLoS One *4*:e6438.
18. **Rosenthal, A., A. Macdonald, and J. Voldman.** 2007. Cell patterning chip for controlling the stem cell microenvironment. Biomaterials *28*:3208-3216.
19. **Figallo, E., C. Cannizzaro, S. Gerecht, J.A. Burdick, R. Langer, N. Elvassore, and G. Vunjak-Novakovic.** 2007. Micro-bioreactor array for controlling cellular microenvironments. Lab Chip *7*:710-719.
20. **Gómez-Sjöberg, R., A.A. Leyrat, D.M. Pirone, C.S. Chen, and S.R. Quake.** 2007. Versatile, fully automated, microfluidic cell culture system. Anal. Chem. *79*:8557-8563.
21. **Kamei, K., S. Guo, Z.T. Yu, H. Takahashi, E. Gschweng, C. Suh, X. Wang, J. Tang, et al.** 2009. An integrated microfluidic culture device for quantitative analysis of human embryonic stem cells. Lab Chip *9*:555-563.
22. **Méndez-Ferrer, S., D. Lucas, M. Battista, and P. Frenette.** 2008. Haematopoietic stem cell release is regulated by circadian oscillations. Nature *452*:442-447.
23. **King, K.R., S. Wang, A. Jayaraman, M.L. Yarmush, and M. Toner.** 2008. Microfluidic flow-encoded switching for parallel control of dynamic cellular microenvironments. Lab Chip *8*:107-116.
24. **Chau, L., M. Doran, and J. Cooper-White.** 2009. A novel multishear microdevice for studying cell mechanics. Lab Chip *9*:1897-1902.
25. **Kim, L., M.D. Vahey, H.Y. Lee, and J. Voldman.** 2006. Microfluidic arrays for logarithmically perfused embryonic stem cell culture. Lab Chip *6*:394-406.
26. **Maerkl, S.J. and S.R. Quake.** 2007. A systems approach to measuring the binding energy landscapes of transcription factors. Science *315*:233-237.
27. **Li, L., D. Mustafi, Q. Fu, V. Tereshko, D.L. Chen, J.D. Tice, and R.F. Ismagilov.** 2006. Nanoliter microfluidic hybrid method for simultaneous screening and optimization validated with crystallization of membrane proteins. Proc. Natl. Acad. Sci. USA *103*:19243-19248.
28. **Warren, L., D. Bryder, I.L. Weissman, and S.R. Quake.** 2006. Transcription factor profiling in individual hematopoietic progenitors by digital RT-PCR. Proc. Natl. Acad. Sci. USA *103*:17807-17812.
29. **Nagrath, S., L.V. Sequist, S. Maheswaran, D.W. Bell, D. Irimia, L. Ulkus, M.R. Smith, E.L. Kwak, et al.** 2007. Isolation of rare circulating tumour cells in cancer patients by microchip technology. Nature *450*:1235-1239.
30. **Gu, W., X. Zhu, N. Futai, B.S. Cho, and S. Takayama.** 2004. Computerized microfluidic cell culture using elastomeric channels and Braille displays. Proc. Natl. Acad. Sci. USA *101*:15861-15866.
31. **Thorsen, T., S.J. Maerkl, and S.R. Quake.** 2002. Microfluidic large-scale integration. Science *298*:580-584.
32. **Dittrich, P.S. and A. Manz.** 2006. Lab-on-a-chip: microfluidics in drug discovery. Nat. Rev. Drug Discov. *5*:210-218.
33. **McBeath, R., D.M. Pirone, C.M. Nelson, K. Bhadriraju, and C.S. Chen.** 2004. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. Dev. Cell *6*:483-495.
34. **Takayama, S., E. Ostuni, P. LeDuc, K. Naruse, D.E. Ingber, and G.M. Whitesides.** 2001. Subcellular positioning of small molecules. Nature *411*:1016.
35. **Fung, W.T., A. Beyzavi, P. Abgrall, N.T. Nguyen, and H.Y. Li.** 2009. Microfluidic platform for controlling the differentiation of embryoid bodies. Lab Chip *9*:2591-2595.
36. **Torisawa, Y.S., B. Mosadegh, G.D. Luker, M. Morell, K.S. O'Shea, and S. Takayama.** 2009. Microfluidic hydrodynamic cellular patterning for systematic formation of co-culture spheroids. Intgr. Biol. *1*:649-654.
37. **Lucchetta, E.M., J.H. Lee, L.A. Fu, N.H. Patel, and R.F. Ismagilov.** 2005. Dynamics of Drosophila embryonic patterning network perturbed in space and time using microfluidics. Nature *434*:1134-1138.
38. **Théry, M., A. Jiménez-Dalmaroni, V. Racine, M. Bornens, and F. Jülicher.** 2007. Experimental and theoretical study of mitotic spindle orientation. Nature *447*:493-496.
39. **Tien, J., C.M. Nelson, and C.S. Chen.** 2002. Fabrication of aligned microstructures with a single elastomeric stamp. Proc. Natl. Acad. Sci. USA *99*:1758-1762.
40. **Chiu, D.T., N.L. Jeon, S. Huang, R.S. Kane, C.J. Wargo, I.S. Choi, D.E. Ingber, and G.M. Whitesides.** 2000. Patterned deposition of cells and proteins onto surfaces by using three-dimensional microfluidic systems. Proc. Natl. Acad. Sci. USA *97*:2408-2413.
41. **Co, C.C., Y.C. Wang, and C.C. Ho.** 2005. Biocompatible micropatterning of two different cell types. J. Am. Chem. Soc. *127*:1598-1599.
42. **Derda, R., L. Li, B.P. Orner, R.L. Lewis, J.A. Thomson, and L.L. Kiessling.** 2007. Defined substrates for human embryonic stem cell growth identified from surface arrays. ACS Chem. Biol. *2*:347-355.
43. **Huang, Y.M., M. Uppalapati, W.O. Hancock, and T.N. Jackson.** 2008. Neutravidin micropatterning by deep UV irradiation. Lab Chip *8*:1745-1747.
44. **Tabata, T. and Y. Takei.** 2004. Morphogens, their identification and regulation. Development *131*:703-712.
45. **Ashe, H.L. and J. Briscoe.** 2006. The interpretation of morphogen gradients. Development *133*:385-394.
46. **Yan, D. and X. Lin.** 2009. Shaping morphogen gradients by proteoglycans. Cold Spring Harbor Perspectives in Biology *1*:a002493.
47. **Jeon, N.L., S.K.W. Dertinger, D.T. Chiu, I.S. Choi, A.D. Stroock, and G.M. Whitesides.** 2000. Generation of solution and surface gradients using microfluidic systems. Langmuir *16*:8311-8316.
48. **Keenan, T.M. and A. Folch.** 2008. Biomolecular gradients in cell culture systems. Lab Chip *8*:34-57.

49. **Chung, B.G., L.A. Flanagan, S.W. Rhee, P.H. Schwartz, A.P. Lee, E.S. Monuki, and N.L. Jeon.** 2005. Human neural stem cell growth and differentiation in a gradient-generating microfluidic device. Lab Chip *5*:401-406.
50. **Park, J.Y., S.K. Kim, D.H. Woo, E.J. Lee, J.H. Kim, and S.H. Lee.** 2009. Differentiation of neural progenitor cells in a microfluidic chip-generated cytokine gradient. Stem Cells *27*:2646-2654.
51. **von Philipsborn, A.C., S. Lang, Z. Jiang, F. Bonhoeffer, and M. Bastmeyer.** 2007. Substrate-bound protein gradients for cell culture fabricated by microfluidic networks and microcontact printing. Sci. STKE *2007*:pl6.
52. **Burdick, J.A., A. Khademhosseini, and R. Langer.** 2004. Fabrication of gradient hydrogels using a microfluidics/photopolymerization process. Langmuir *20*:5153-5156.
53. **Zaari, N., P. Rajagopalan, S.K. Kim, A.J. Engler, and J.Y. Wong.** 2004. Photopolymerization in microfluidic gradient generators: Microscale control of substrate compliance to manipulate cell response. Adv. Mater. *16*:2133-2137.
54. **Gunawan, R.C., J. Silvestre, H.R. Gaskins, P.J. Kenis, and D.E. Leckband.** 2006. Cell migration and polarity on microfabricated gradients of extracellular matrix proteins. Langmuir *22*:4250-4258.
55. **Cosson, S., S.A. Kobel, and M.L. Lutolf.** 2009. Capturing complex protein gradients on biomimetic hydrogels for cell-based assays. Adv. Funct. Mater. *19*:3411-3419.
56. **Yamada, K.M. and E. Cukierman.** 2007. Modeling tissue morphogenesis and cancer in 3-D. Cell *130*:601-610.
57. **Gerecht, S., J.A. Burdick, L.S. Ferreira, S.A. Townsend, R. Langer, and G. Vunjak-Novakovic.** 2007. Hyaluronic acid hydrogel for controlled self-renewal and differentiation of human embryonic stem cells. Proc. Natl. Acad. Sci. USA *104*:11298-11303.
58. **Lutolf, M.P.** 2009. Integration column: artificial ECM: expanding the cell biology toolbox in 3-D. Intgr. Biol. *1*:235-241.
59. **Jongpaiboonkit, L., W.J. King, G.E. Lyons, A.L. Paguirigan, J.W. Warrick, D.J. Beebe, and W.L. Murphy.** 2008. An adaptable hydrogel array format for 3-dimensional cell culture and analysis. Biomaterials *29*:3346-3356.
60. **Nelson, C.M., M.M. Vanduijn, J.L. Inman, D.A. Fletcher, and M.J. Bissell.** 2006. Tissue geometry determines sites of mammary branching morphogenesis in organotypic cultures. Science *314*:298-300.
61. **Gillette, B.M., J.A. Jensen, B. Tang, G.J. Yang, A. Bazargan-Lari, M. Zhong, and S.K. Sia.** 2008. In situ collagen assembly for integrating microfabricated three-dimensional cell-seeded matrices. Nat. Mater. *7*:636-640.
62. **Huang, C.P., J. Lu, H. Seon, A.P. Lee, L.A. Flanagan, H.Y. Kim, A.J. Putnam, and N.L. Jeon.** 2009. Engineering microscale cellular niches for three-dimensional multicellular co-cultures. Lab Chip *9*:1740-1748.
63. **Braschler, T., R. Johann, M. Heule, L. Metref, and P. Renaud.** 2005. Gentle cell trapping and release on a microfluidic chip by in situ alginate hydrogel formation. Lab Chip *5*:553-559.
64. **Mosadegh, B., C. Huang, J.W. Park, H.S. Shin, B.G. Chung, S.K. Hwang, K.H. Lee, H.J. Kim, et al.** 2007. Generation of stable complex gradients across two-dimensional surfaces and three-dimensional gels. Langmuir *23*:10910-10912.
65. **Choi, N.W., M. Cabodi, B. Held, J.P. Gleghorn, L.J. Bonassar, and A.D. Stroock.** 2007. Microfluidic scaffolds for tissue engineering. Nat. Mater. *6*:908-915.
66. **Kloxin, A.M., A.M. Kasko, C.N. Salinas, and K.S. Anseth.** 2009. Photodegradable hydrogels for dynamic tuning of physical and chemical properties. Science *324*:59-63.
67. **Albrecht, D.R., G.H. Underhill, T.B. Wassermann, R.L. Sah, and S.N. Bhatia.** 2006. Probing the role of multicellular

organization in three-dimensional microenvironments. Nat. Methods *3*:369-375.
68. **Mittal, N., A. Rosenthal, and J. Voldman.** 2007. nDEP microwells for single-cell patterning in physiological media. Lab Chip *7*:1146-1153.
69. **Valero, A., J.N. Post, J.W. van Nieuwkasteele, P.M. Ter Braak, W. Kruijer, and A. van den Berg.** 2008. Gene transfer and protein dynamics in stem cells using single cell electroporation in a microfluidic device. Lab Chip *8*:62-67.
70. **Seger-Sauli, U., M. Panayiotou, S. Schnydrig, M. Jordan, and P. Renaud.** 2005. Temperature measurements in microfluidic systems: heat dissipation of negative dielectrophoresis barriers. Electrophoresis *26*:2239-2246.
71. **Nilsson, J., M. Evander, B. Hammarström, and T. Laurell.** 2009. Review of cell and particle trapping in microfluidic systems. Anal. Chim. Acta *649*:141-157.
72. **Charnley, M., M. Textor, A. Khademhosseini, and M.P. Lutolf.** 2009. Integration column: microwell arrays for mammalian cell culture. Intgr. Biol. *1*:625-634.
73. **Ochsner, M., M.R. Dusseiller, H.M. Grandin, S. Luna-Morris, M. Textor, V. Vogel, and M.L. Smith.** 2007. Microwell arrays for 3-D shape control and high resolution analysis of single cells. Lab Chip *7*:1074-1077.
74. **Kurth, I., K. Franke, T. Pompe, M. Bornhäuser, and C. Werner.** 2009. Hematopoietic stem and progenitor cells in adhesive microcavities. Intgr. Biol. *1*:427-434.
75. **Ungrin, M.D., C. Joshi, A. Nica, C. Bauwens, and P.W. Zandstra.** 2008. Reproducible, ultra high-throughput formation of multicellular organization from single cell suspension-derived human embryonic stem cell aggregates. PLoS One *3*:e1565.
76. **Karp, J.M., J. Yeh, G. Eng, J. Fukuda, J. Blumling, K.Y. Suh, J. Cheng, A. Mahdavi, et al.** 2007. Controlling size, shape and homogeneity of embryoid bodies using poly(ethylene glycol) microwells. Lab Chip *7*:786-794.
77. **Mohr, J.C., J.J. de Pablo, and S.P. Palecek.** 2006. 3-D microwell culture of human embryonic stem cells. Biomaterials *27*:6032-6042.
78. **Napolitano, A.P., D.M. Dean, A.J. Man, J. Youssef, D.N. Ho, A.P. Rago, M.P. Lech, and J.R. Morgan.** 2007. Scaffold-free three-dimensional cell culture utilizing micromolded nonadhesive hydrogels. BioTechniques *43*:494-500.
79. **Hwang, Y.S., B.G. Chung, D. Ortmann, N. Hattori, H.C. Moeller, and A. Khademhosseini.** 2009. Microwell-mediated control of embryoid body size regulates embryonic stem cell fate via differential expression of WNT5a and WNT11. Proc. Natl. Acad. Sci. USA *106*:16978-16983.
80. **Wang, W., K. Itaka, S. Ohba, N. Nishiyama, U.I. Chung, Y. Yamasaki, and K. Kataoka.** 2009. 3-D spheroid culture system on micropatterned substrates for improved differentiation efficiency of multipotent mesenchymal stem cells. Biomaterials *30*:2705-2715.
81. **Cordey, M., M. Limacher, S. Kobel, V. Taylor, and M.P. Lutolf.** 2008. Enhancing the reliability and throughput of neurosphere culture on hydrogel microwell arrays. Stem Cells *26*:2586-2594.
82. **Friedrich, J., C. Seidel, R. Ebner, and L.A. Kunz-Schughart.** 2009. Spheroid-based drug screen: considerations and practical approach. Nat. Protocols *4*:309-324.
83. **Lutolf, M., R. Doyonnas, K. Havenstrite, K. Koleckar, and H.M. Blau.** 2009. Perturbation of single hematopoietic stem cell fates in artificial niches. Intgr. Biol. *1*:59-69.
84. **Kobel, S., M. Limacher, S. Gobaa, T. Laroche, and M.P. Lutolf.** 2009. Micropatterning of hydrogels by soft embossing. Langmuir *25*:8774-8779.
85. **Nguyen, D., S. Sa, J.D. Pegan, B. Rich, G. Xiang, K.E. McCloskey, J.O. Manilay, and M. Khine.** 2009. Tunable shrink-induced honeycomb microwell arrays for uniform embryoid bodies. Lab Chip *9*:3338-3344.
86. **Dykstra, B., D. Kent, M. Bowie, L. McCaffrey, M. Hamilton, K. Lyons, S.J. Lee, R. Brinkman, and C. Eaves.** 2007. Long-term propagation of distinct hematopoietic differentiation programs in vivo. Cell Stem Cell *1*:218-229.
87. **Graf, T. and M. Stadtfeld.** 2008. Heterogeneity of embryonic and adult stem cells. Cell Stem Cell *3*:480-483.
88. **Chang, H.H., M. Hemberg, M. Barahona, D.E. Ingber, and S. Huang.** 2008. Transcriptome-wide noise controls lineage choice in mammalian progenitor cells. Nature *453*:544-547.
89. **Kalmar, T., C. Lim, P. Hayward, S. Muñoz-Descalzo, J. Nichols, J. Garcia-Ojalvo, and A. Martinez Arias.** 2009. Regulated fluctuations in nanog expression mediate cell fate decisions in embryonic stem cells. PLoS Biol. *7*:e1000149.
90. **Ema, H., H. Takano, K. Sudo, and H. Nakauchi.** 2000. In vitro self-renewal division of hematopoietic stem cells. J. Exp. Med. *192*:1281-1288.
91. **Chin, V.I., P. Taupin, S. Sanga, J. Scheel, F.H. Gage, and S.N. Bhatia.** 2004. Microfabricated platform for studying stem cell fates. Biotechnol. Bioeng. *88*:399-415.
92. **Dykstra, B., J. Ramunas, D. Kent, L. McCaffrey, E. Szumsky, L. Kelly, K. Farn, A. Blaylock, et al.** 2006. High-resolution video monitoring of hematopoietic stem cells cultured in single-cell arrays identifies new features of self-renewal. Proc. Natl. Acad. Sci. USA *103*:8185-8190.
93. **Khademhosseini, A., J. Yeh, G. Eng, J. Karp, H. Kaji, J. Borenstein, O.C. Farokhzad, and R. Langer.** 2005. Cell docking inside microwells within reversibly sealed microfluidic channels for fabricating multiphenotype cell arrays. Lab Chip *5*:1380-1386.
94. **Wang, Y., G. Salazar, J. Pai, H. Shadpour, C. Sims, and N. Allbritton.** 2008. Micropallet arrays with poly(ethylene glycol) walls. Lab Chip *8*:734-740.
95. **Lechler, T. and E. Fuchs.** 2005. Asymmetric cell divisions promote stratification and differentiation of mammalian skin. Nature *437*:275-280.
96. **Rieger, M.A., P.S. Hoppe, B.M.. Smejkal, A.C. Eitelhuber, and T. Schroeder.** 2009. Hematopoietic cytokines can instruct lineage choice. Science *325*:217-218.
97. **Eilken, H.M., S. Nishikawa, and T. Schroeder.** 2009. Continuous single-cell imaging of blood generation from haemogenic endothelium. Nature *457*:896-900.
98. **Kachouie, N., L. Kang, and A. Khademhosseini.** 2009. Arraycount, an algorithm for automatic cell counting in microwell arrays. BioTechniques *47*:x-xvi.
99. **Roach, K.L., K.R. King, B.E. Uygun, I.S. Kohane, M.L. Yarmush, and M. Toner.** 2009. High throughput single cell bioinformatics. Biotechnol. Prog. *25*:1772-1779.
100. **Rowat, A.C., J.C. Bird, J.J. Agresti, O.J. Rando, and D.A. Weitz.** 2009. Tracking lineages of single cells in lines using a microfluidic device. Proc. Natl. Acad. Sci. USA *106*:18149-18154.
101. **Faley, S.L., M. Copland, D. Wlodkowic, W. Kolch, K.T. Seale, J.P. Wikswo, and J.M. Cooper.** 2009. Microfluidic single cell arrays to interrogate signalling dynamics of individual, patient-derived hematopoietic stem cells. Lab Chip *9*:2659-2664.
102. **Kobel, S.A., A. Valero, J. Latt, P. Renaud, and M.P. Lutolf.** 2010. Optimization of microfluidic single cell trapping for long-term on-chip culture. Lab on a Chip. doi 10.1039/b918055a.
103. **Tan, W.H. and S. Takeuchi.** 2007. A trap-and-release integrated microfluidic system for dynamic microarray applications. Proc. Natl. Acad. Sci. USA *104*:1146-1151.
104. **Engler, A.J., S. Sen, H.L. Sweeney, and D.E. Discher.** 2006. Matrix elasticity directs stem cell lineage specification. Cell *126*:677-689.
105. **Lee, M.Y., R.A. Kumar, S.M. Sukumaran, M.G. Hogg, D.S. Clark, and J.S. Dordick.** 2008. Three-dimensional cellular microarray for high-throughput toxicology assays. Proc. Natl. Acad. Sci. USA *105*:59-63.
106. **Clausell-Tormos, J., D. Lieber, J.C. Baret, A. El-Harrak, O.J. Miller, L. Frenz, J. Blouwolff, K.J. Humphry, et al.** 2008. Droplet-based microfluidic platforms for the encapsulation and screening of Mammalian cells and multicellular organisms. Chem. Biol. *15*:427-437.
107. **Brouzes, E., M. Medkova, N. Savenelli, D. Marran, M. Twardowski, J.B. Hutchison, J.M. Rothberg, D.R. Link, et al.** 2009. Droplet microfluidic technology for single-cell high-throughput screening. PNAS 106:14195-14299.
108. **Magyar, J.P., M. Nemir, E. Ehler, N. Suter, J.C. Perriard, and H.M. Eppenberger.** 2001. Mass production of embryoid bodies in microbeads. Ann. N.Y. Acad. Sci. *944*:135-143.

Received 2 March 2010; accepted 4 March 2010.

Address correspondence to Matthias P. Lutolf, Laboratory of Stem Cell Bioengineering (LSCB) and Institute of Bioengineering (IBI), Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland. e-mail: matthias.lutolf@epfl.ch