4 Guiding Stem Cell Fate through Microfabricated Environments

Lisa R. Trump, Gregory Timp, and Lawrence B. Schook

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4.1 INTRODUCTION

The multipotent nature of stem cells provides enormous potential for clinical applications for treatment of disease, cancers, and for organ replacement. Despite decades of research, robust culture techniques that consistently permit isolation, expansion, and directed differentiation of stem and progenitor cells in adequate numbers remains a major hurdle to ensure full clinical usage of stem cell therapies. *In vivo*, stem cell

fate is governed by specialized microenvironments termed "niches." The stem cell niche consists of supporting cells, extracellular matrix (ECM), and extrinsic cues such as growth factors and cytokines that are spatially and temporally controlled to direct differentiation and maintain stem cell pools (Figure 4.1).[1,2] When removed from niches and cultured *in vitro*, stem cells rapidly lose self-renewal capabilities and undergo spontaneous differentiation due to the loss of intrinsic and extrinsic

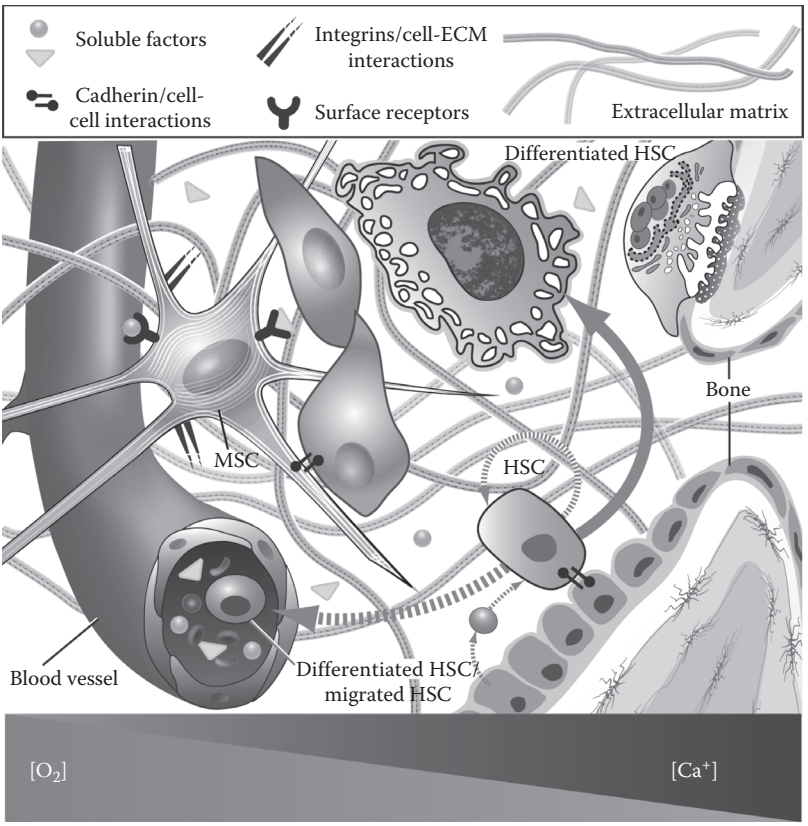

FIGURE 4.1 (See color insert.) The perivascular and endosteal bone marrow niche: example of signals and cues regulating stem cell function. The stem cell niche is a complex microenvironment that guides stem cell fate through a combination of extracellular matrix (ECM), cell–cell interactions, and extrinsic factors such as growth factors and cytokines. In the bone marrow, hematopoietic stem cells (HSCs) situated in the endosteal niche are physically anchored to the niche by cadherens junctions with osteoblasts (cell–cell interactions). In this location, cells are exposed to high Ca^{2+} concentrations, low oxygen tension, and a variety of autocrine, paracrine, and endocrine signals (extrinsic factors) and are attached to the ECM through integrin receptors. These signals and cues maintain the quiescence and self-renewal of HSCs. In the perivascular niche, however, HSCs are exposed to low Ca^{2+}, high oxygen tension, different cell–cell interactions, and ECM composition that promote migration and differentiation of stem cells. While a majority of these signals are found in a variety of stem cell niches, their utilization and the effects of niche components vary from niche to niche.

cues found in stem cell niches and physiological tissues. This loss of stem cell characteristics in culture *in vitro* severely limits the ability to expand and directly differentiate cells into sufficient numbers for clinical usage.

The ability to recapitulate aspects of physiological tissue environments is key to identifying and understanding the intrinsic and extrinsic cues directing stem cell self-renewal and differentiation. Currently, the understanding of spatial and temporal cues directing stem cell fate is generated from tissue culture systems where the cellular microenvironment is regulated in batch conditions. Typical *in vitro* cell culture techniques rely on the use of two-dimensional (2D), plastic surfaces such as petri dishes and tissue culture flasks to propagate, differentiate, and understand cell behavior in response to various small molecules and chemical stimuli. These conventional cell culture techniques are well established and inexpensive. However, traditional systems poorly recapitulate the complex physiochemical tissue environment and offer little control over cell seeding, cell–cell interactions, and biologically relevant presentation of soluble molecules. Removed from the niche and cultured in *in vitro*, stem cells display altered phenotypes and gene expression and have limited expansion and differentiation capabilities.[3–5] Furthermore, cell isolation techniques are unable to provide homogeneous populations of stem cells. Contaminating cells may secrete soluble molecules that can affect cellular function, select for a subpopulation of cells, or easily proliferate and overtake populations of stem cells. Since cellular responses are mostly measured on a population basis, responses of a small subset or limited population of cells may be masked.

Though recent efforts have increased our knowledge of stem cell biology, little is known about the combinatorial signals that guide stem cell fate. Thorough understanding of the combinatorial microenvironments that direct the behavior and differentiation properties of stem cells require robust culture systems that permit precise control over cell–cell interactions, ECM properties, and extrinsic factor delivery. To circumvent limitations of poorly controlled microenvironments found in traditional batch culture systems, cell biologists are looking toward tissue engineering and microfabrication technologies to design culture systems that more accurately recapitulate *in vivo* cellular microenvironments. These technologies combine biomaterial scaffolds with various engineering strategies that provide the ability to tailor cellular microenvironments and provide signals and cues spatially and temporally (Figure 4.2).[6] This chapter first briefly discusses how the various components of the stem cell niche guide cell behavior and then reviews the various microscale technologies currently used to recreate the stem cell niche *in vitro*.

4.2 THREE-DIMENSIONAL ENVIRONMENTS AND THE STEM CELL NICHE

Physiologic tissue environments are complex, three dimensional (3D) environments that direct cell function through ECM, cell–cell interactions, mechanical stimuli, and soluble factors. The concept of stem cell niches, first proposed by Schofield et al. in 1978, suggests that adult stem cells reside in defined compartments (i.e., "niches"), which balance stem cell self-renewal and differentiation to maintain tissue homeostasis and the stem cell pool.[2] To date, stem cell niches have been identified in a variety of

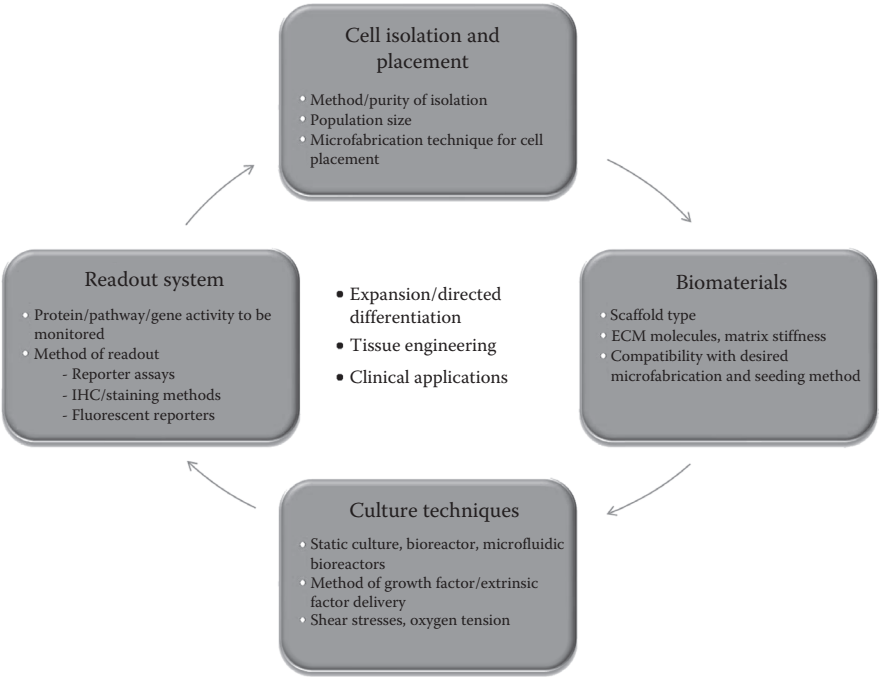

FIGURE 4.2 (See color insert.) Overview of processes to design environments that guide cell fate. First, the cell source or line, isolation, and optimal microfabrication technique for the study or application must be identified. Biomaterials selected for microfabrication techniques must be compatible with the cell and microfabrication technique of interest. Modifications of biomaterials such as the incorporation of ECM molecules or material stiffness can affect cellular processes. Culture techniques provide nutrient delivery and waste removal to microfabricated cells. Spatial delivery of growth factors, cytokines, and other extrinsic factors can be controlled by bioreactors and microfluidics systems. The culture technique or bioreactor type can affect nutrient delivery throughout scaffolds, provide shear stresses, affect cell seeding into scaffolds, or modulate ECM deposition by cells. Lastly, systems to monitor cellular activity in the microfabricated system must be identified. Assays can be end point, such as cellular staining, or measured in real time by reporter assays. Each of these factors must be taken into consideration when designing microfabrication experiments.

tissues including the bone marrow,[7,8] skin,[9] hair follicles,[10] intestine,[11] brain,[12] and muscle.[13] The stem cell niche provides signals and cues that balance self-renewal, maintenance, and differentiation as well as protect cells from apoptosis and prevent depletion or overpopulation of stem cells. Cells are physically anchored within the niche by ECM proteins and supporting stromal cells, which in combination with soluble signals regulate the maintenance and self-renewal of stem cells. Figure 4.1 details the bone marrow niche and the various cues that maintain stem cell function. Within the niche, stem cells either undergo symmetric division to give rise to identical progeny (self-renewal), asymmetric diffusion to produce one stem cell and a differentiated progeny, division without differentiation, or remain quiescent.[1] Aberrations within the niche are thought to cause pathologies such as cancer, ageing, and degeneration of tissue function.[14,15]

4.2.1 Extracellular Matrix

The ECM is composed of a combination of proteoglycans, polysaccharides, and proteins that provide structural support to cells. The ECM varies in composition and stiffness from tissue to tissue and plays an integral role in maintaining cellular phenotypes and cell fate decisions. Cells attach to the matrix through integrin receptors on the cell surface that, when bound to their ECM ligands, activate cellular signaling cascades. Loss of cell–ECM interactions results in a specialized form of detachment-induced cell death termed "anoikis," which is derived from the Greek word for homelessness.[16] For example, when cultured in PEG matrices that do not permit cellular attachment, mesenchymal stem cells (MSCs) undergo anoikis. Restoration of cellular attachment by the cell-adhesive peptide Arg-Gly-Asp (RGD) increases viability of encapsulated cells by engaging cell integrin receptors.[17,18] Furthermore, several studies have reported that the stiffness and elasticity of the ECM affect stem cell processes. When cultured on stiff surfaces mimicking bone tissue, MSCs display hallmarks of osteoprogenitor differentiation, while culture on soft surfaces promotes adipose differentiation.[19] ECM interactions also govern cell shape and size, which affect cellular survival, proliferation,[20] and differentiation.[21] McBeath et al. patterned fibronectin ECM in various geometries onto tissue culture substrates and seeded human MSCs onto the ECM. Large islands of fibronectin moieties promoted cell spreading, whereas cells had a rounded phenotype on small ECM islands. Cells allowed to spread on large islands displayed osteoprogenitor commitment, while rounded cells differentiated into adipocytes. A recent study by Chowdhury et al. explored the effects of cyclic strain on embryonic stem cells and embryonic differentiated cells.[22] Cyclic stress induced cell spreading and down regulation of the stemness marker Oct 3/4, whereas embryonic differentiated cells demonstrated no genotypic or phenotypic changes from the cyclic stressors. It is hypothesized that the cell softness, defined as the ratio of strain to stress on the cells, affects a cells response to stress. As embryonic stem cells are significantly softer than embryonic differentiated cells, it is concluded that the ES cells showed responses to stressors due to greater cyclic strain.

4.2.2 Extrinsic Factors

Within the niche, stem cells are exposed to a mixture of extrinsic factors that influence cell fate decisions. Such factors include growth factors, cytokines, small proteins, and ions. The spatial and temporal presentation of extrinsic factors within the niche affects stem cell self-renewal and differentiation fates. Secreted factors arise from adjacent cells, from diffusion throughout the niche, or immobilized to ECM proteins. Soluble proteins that affect stem cells include Wnts, hedgehog proteins, fibroblast growth factors (FGFs), and the BMP/TGFβ superfamily. In the neural stem cell niche, for example, TGFβ secreted by nearby differentiated neurons suppress the division of neural stem cells (NSCs) within the niche. It is important to note, however, that the spatial and temporal presentation of soluble molecules can also affect stem cell activity. Immobilization of growth factors and small proteins by ECM proteins affect concentrations, stability, and bioavailability to niche cells. FGF-2 tethered to fibrinogen increases endothelial cell (EC) proliferation relative to

FGF-2 in solution.[23] Similarly, bone marrow MSCs exposed to biomaterial surfaces with tethered EGF promotes cell spreading and survival more strongly than soluble EGF.[24] Inorganic ion concentrations and gradients within the niche also affect stem cell behavior. Hematopoietic stem cells (HSCs) situated near the endosteal surface are exposed to high calcium levels from nearby osteoblasts and low oxygen tension. These conditions are thought to help maintain HSCs in the quiescent state. In contrast, HSCs situated closer to microvasculature are exposed to higher oxygen tensions and lower calcium ion levels, which promotes HSC division and differentiation.[25]

4.2.3 Cell–Cell Interactions

Stem cells represent a very small portion of adult tissues and exist as single cells or small clusters of cells and are in contact and respond to a variety of differentiated cell types within the niche. These interactions, mediated by adherens and gap junctions, influence stem cell fate. Chondrogenic differentiation of MSCs is facilitated *in vitro* by increasing cell–cell interactions via pellet culture.[26] Supporting cells such as stromal cells, vasculature, and basal lamina anchor stem cells within the niche and may direct cellular placement to soluble signals secreted by surrounding cells. Osteoblasts anchor HSCs to the perivascular niche through N-cadherins that are involved in maintaining the quiescent state. The proximity of HSCs to osteoblasts places them in high Ca^{2+} and low oxygen tension microenvironments as discussed above, as well as induces production of cytokines and growth factors. However, exposing HSCs to cocktails of these cytokines is not sufficient to maintain stemness, suggesting that direct HSC–osteoblast contact is required for maintenance of stem cell properties.[27] Changes in cell density or loss of adherens junctions initiate cell division or migration out of the niche. Loss of cadherin junctions between HSCs and osteoblasts induces loss of HSC and migration out of the niche.

4.3 ENGINEERING TECHNOLOGIES TO GUIDE STEM CELL FATE

To understand the requirements for cell microenvironments, cell biologists and tissue engineers developed microfabrication techniques that enable precise control over cell seeding onto substrates and biomaterials, as well as control spatial and temporal cues within the culture microenvironment. Borrowed from semiconductor and microelectronics industries, microfabrication technologies are able to pattern ECM proteins onto 2D substrates such as glass and 3D substrates and scaffolds to control cell adhesion and cell–cell interactions. Other microfabrication techniques offer the unique ability to mold 3D biomaterials into desired shapes and precisely place cells within biomaterial scaffolds. Either way, such techniques are reproducible and able to create objects from tens of microns to millimeters in size with high resolution. These microscale technologies promise advances in elucidating the *in vivo* function of stem cells and niche components, generation of tissue engineering constructs and for development of high throughput platforms for drug discovery and cell-based biosensors. The following sections will first briefly discuss properties of biomaterial scaffolds and then various 2D and 3D microfabrication technologies to recapitulate aspects of the stem cell niche microenvironments within cell culture surfaces or biomaterial scaffolds. Examples of various microfabrication technologies and their applications are further outlined in Table 4.1.

TABLE 4.1
Strategies to Engineer Various Components of the Stem Cell Niche

Niche Component	Engineering Strategies	Examples	References
Extracellular matrix			
Substrate stiffness	Scaffold/substrate type and design	Human ESCs cultured on PDMS surfaces of varying stiffness affected cellular spreading, growth rate, and osteogenic differentiation. Culture of cells on stiff surfaces increases the degree of cell spreading, attachment, and osteogenic differentiation as compared to softer substrates	Evans et al. (87, 2009, p. 1)
Ligand presentation and gradients	Inkjet printing	Patterns of collagen printed onto agarose films directed smooth muscle cell and primary neuron attachment in predefined patterns	Roth et al. [85, p. 3707]
	Microcontact printing	Microcontact printing techniques have been used to specifically place ECM ligands onto cell repellant surfaces to determine effects of ECM on cellular activity	McBeath et al. [21, p. 483], Offenhäusser et al. (88, 2007, p. 290)
	Microfluidic patterning	Microfluidic chips create gradients of Fc-tagged fusion proteins through laminar flow deposition	Cosson et al. (89, 2009, 3411)
	Photolithography	Two photon laser scanning photolithography micropatterned RGDs onto 3D hydrogel scaffolds to direct cell migration	Lee et al. (90, 2008, p. 2962)
Topography	Laser-guided direct writing	Direct writing techniques fabricate biomaterial scaffolds with precise 3D architecture and composition to guide cell patterning and behavior	Lewis et al. (91, 2004, p. 32)
	Photolithography	Photolithographic masks precisely pattern poly (ethylene glycol) diacrylate (PEGDA) scaffolds into desired architectures. Sequential patterning allows for development of 3D architectures	Hahn et al. (92, 2006, p. 2679)
Cell–cell interactions			
Direct cell placement in 3D	Optical tweezers	Time-shared optical tweezers used in conjunction with microfluidic devices allow precise 2D and 3D placement of *E. coli* bacterium within hydrogel scaffolds	Mirsaidov et al. [53, p. 2174]

(*continued*)

TABLE 4.1 (Continued)
Strategies to Engineer Various Components of the Stem Cell Niche

Niche Component	Engineering Strategies	Examples	References
	Dielectrophoresis	Dielectrophoresis techniques enable trapping of single cells and cell-laden hydrogels within 3D scaffolds	Albrecht et al. [66, 2007, p. 702]
	Plasmonic trapping	*S. cerevisiae* were arranged into arrays of defined architecture using plasmonic traps and microfluidic devices	Huang et al. [57, p. 6018]
	Bioreactors	Rotating wall vessel (RWV) bioreactors improve cell seeding density and uniformity within 3D scaffolds	Martin et al. [69, p. 80]
Direct cell placement in 2D	Microcontact printing	Microcontact printing of ECM modulates placement and cell–cell interactions by selective adhesion of cells to defined substrates	Ruiz et al. (93, 2008, p. 2921)
	Laser-guided direct writing	Optical forces directly placed chick neuronal cells onto glass surfaces in various 2D patterns with minimal loss in cellular viability	Odde et al. [58, p. 312]
	Inkjet printing	Chinese Hamster Ovary (CHO) Cells were specifically placed onto biomaterial substrates in predefined patterns via inkjet printing technologies	Xu et al. (94, 2005, p. 0210131)
Extrinsic factors			
Growth factor, culture medium, and inorganic ion delivery	Inkjet printing	Muscle-derived stem cells (MDSCs) cultured on patterns of BMP-2 printed onto fibrin substrates. Cells cultured on BMP-2-patterned substrates in myogenic conditions differentiate into osteoblasts, while unpatterned cells differentiate into myoblasts	Phillippi et al. [68, p. 127]
	Microfluidic bioreactors	Oxygen gradients of differing size and shape were created in specially designed microfluidics, where fluid flow was controlled by a computer-controlled gas mixer	Allen et al. (95, 2010) Adler et al. (96, 2010, p. 388)
		Pressure-driven laminar flow quickly switches solution streams presented to cell, enabling rapid microenvironmental changes and growth factor delivery	

TABLE 4.1 (Continued)
Strategies to Engineer Various Components of the Stem Cell Niche

Niche Component	Engineering Strategies	Examples	References
	Bioreactors	Mass transport of growth factors, ions, and oxygen is increased in several types of bioreactors, leading to increased cellular proliferation, matrix deposition, and differentiation	Martin et al. [69, p. 80]
Shear stresses	Bioreactors and microfluidic bioreactors	Shear stresses are modulated through changes in design, fluid flow, and fluid velocity throughout microfluidics and bioreactors	Martin et al. [69, p. 80]

4.3.1 Biomaterial Scaffolds

Biomaterial scaffolds serve as the foundation for many tissue engineering and microfabrication technologies. Made of natural or synthetic materials, such as alginate, collagen, poly(ethylene glycol) diacrylate (PEGDA), and polyesters, these materials form biocompatible networks that provide structural support to the cells, allow rapid diffusion of nutrients, metabolites, and small molecules to and away from encapsulated cells and are resistant to protein absorption. Many biomaterial scaffolds can be modified to include ECM molecules, vary mechanical stiffness, and tune degradation properties. Properties of biomaterial scaffolds vary based on application and have been shown to enhance osteogenic, neural, and adipose differentiation. When selecting biomaterial scaffolds for cell seeding or microfabrication technologies, cell type, fluid dynamics within the scaffold, material stiffness and surface chemistries, method of polymerization, delivery of bioactive molecules, and matrix degradation properties need to be taken into consideration. For extensive review of the properties and types of biomaterial scaffolds, see Chapter 10 of this book or a review by Dawson et al.[28] Several of the microfabrication technologies discussed below utilizes the tunable properties of biomaterial scaffolds to precisely engineer the cellular microenvironment.

4.3.2 Microfabrication Technologies

4.3.2.1 Photolithography

One of the first techniques used to pattern cells and substrates, photolithography utilizes materials that harden or soften in response to light irradiation. A schematic of the photolithography process is presented in Figure 4.3. In most cases, photolithographic micropatterns are created by spin coating glass, silicon, or quartz with a thin layer of liquid prepolymer solution termed photoresist. The spun photoresist is patterned by exposing and hardening the photoresist to light irradiation through

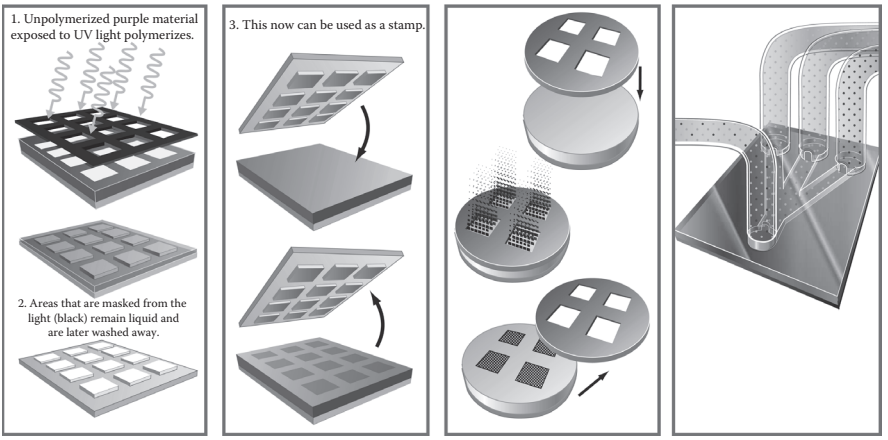

FIGURE 4.3 (See color insert.) Micropatterning techniques. (A) Photolithography. To create patterns using photolithography, a thin film of photopolymerizable material such as photoresist or poly (ethylene glycol) diacrylate hydrogel (PEGDA) is spun onto a glass substrate. The material is photopolymerized through a mask of the desired pattern. The material only hardens where exposed to light, and unexposed material is washed away. The resulting material can be used to directly culture cells, as a master to mold and shape 3D scaffolds, as a stamp for soft lithography (B) as a stencil (C), or as a microfluidic device (D). (B) Soft lithography. A master is formed in the desired pattern using photolithographic techniques and then filled with material such as PDMS, a soft elastomeric material that is commonly used for soft lithographic techniques. This stamp can be dipped directly into ECM molecules such as fibronectin, or functionalized with alkanethiols, and stamped onto substrates. (C) Stencil. The master created by photolithographic techniques can be used as a stencil to limit exposure of the substrate to molecules. (D) Microfluidic devices. Generally, microfluidics devices are formed in the same manner as stamps for soft lithography but have continuous channels to allow for fluid flow. These microfluidic devices can be used as guides to deposit ECM or cells, and then are peeled off the substrate. Additionally, they are used as bioreactors to control fluid flow, soluble molecule presentation, and cell deposition.

an opaque photomask of the desired pattern. Areas unexposed to light irradiation remain liquid and are removed. The resulting photoresist "master" is filled with the material of interest (e.g., ECM proteins), and the photoresist is removed from the substrate.[29] Cells are then patterned by selective adhesion to ECM proteins stamped on the culture substrate. Cell adhesion proteins utilized in photolithographic techniques include fibronectin, collagen, laminins, and Matrigel.[30]

Photolithography is able to accurately pattern substrates with a resolution from 2 to 500 μm.[30] Photomasks of the desired pattern are cheaply and easily fabricated with freely available computer software and printed onto transparencies, microfiche films, or quartz/chromium surfaces, depending on the resolution desired.[31] However, the photolithography process requires expensive clean room facilities, and many of the solvents used to process the photoresist can easily denature biological molecules and are toxic to living cells. Once created, the photoresist can be reused

for several experiments, but changing patterns or design requires fabrication of new photoresist masters that can be cost limiting.

4.3.2.2 Soft Lithography

As a cheaper and more biocompatible alternative to photolithography, Whitesides and colleagues developed a patterning process termed soft lithography.[32] This method is termed soft lithography since it uses "soft" elastomeric materials such as poly (dimethylsiloxane) or PDMS. PDMS is a durable, biocompatible elastomer that is permeable to gases, optically transparent, and permissive for culturing cells. Once a PDMS master is formed, it can be used over an extended period of time with little degradation. In soft lithography techniques, a silicon master of the desired pattern is generated, usually by photolithographic techniques. PDMS is cast onto the silicon master and hardened. The resulting PDMS mold can then be used to directly culture cells or as a template to form microchannels filled with material or cells of interest.[33] Soft lithography techniques are generally able to pattern structures that are 500 nm or larger. Odom et al. improved the resolution of soft lithography patterns into the 50–100 nm range by using composite PDMS stamps.[34]

One of the most widely used soft lithography techniques, microcontact printing, utilizes PDMS molds created by soft lithography to stamp patterns onto tissue culture substrates. The simplest studies simply absorb ECM molecules onto the PDMS stamp and transfer them onto substrates. While this type of microcontact printing has been successful in patterning poly-L lysine,[35] laminin,[35] immunoglobulins,[36,37] and even lipid bilayers,[37] it may not be suitable for long-term biological studies due to the loose linkage of the stamped material and the substrate. To form stronger bonds between the protein and substrate, self-assembled monolayers (SAMs) of alkalinethiols are deposited onto gold surfaces. While non-patterned areas are rendered protein resistant, ECM that is added only absorbs to the SAMs. In most cases, the resolution of this technique is ~100 μm. However, using stamps made of polyolefin plastomers, Csucs et al. were able to stamp fibrinogen proteins using microcontact printing with nanometer-scale resolution.[38] Microcontact printing is cost effective and flexible, allowing various substrates and printing material. However, ligand density can vary from experiment to experiment since transfer efficiency of the stamp can vary. Furthermore, physioabsorbed ECM proteins can degrade from the substrate when in contact with the culture medium.

The elastomeric properties of PDMS stamps provide a unique ability to quickly and reversibly seal surfaces to form microfluidic devices. Microchannels formed by the bonding of PDMS to glass substrates can be used to selectively deliver ECM or cells onto the tissue culture substrate through capillary action. In addition, etching of culture surfaces can be achieved by flowing etching solutions through the microfluidic channels to form grooves that guide cell placement. In recent studies, biomaterial scaffolds have been patterned by flowing prepolymer solution into the microchannels, then polymerized to form 3D structures. Once patterned, the microfluidic device can be used as a culture vessel or is easily removed from the substrate. Drawbacks of microfluidic technology include limited spacing between microchannels—too little spacing between channels can compromise the structural integrity of the PDMS stamp. Due to the requirement of fluid flow, patterns

with continuous features can only be implemented. To overcome this problem, Khademhosseni et al. used microfluidics in combination with patterned substrates to trap cells in specific location within the microchannel. They successfully used this technique to pattern embryonic stem cells and MEF feeder cells.[39]

4.3.2.3 Optical Fabrication

Developed by Ashkin et al. in 1987, optical trapping techniques utilize tightly focused laser beams to manipulate dielectric particles.[40] Optical tweezers allow precise manipulation and placement of objects in both 2D and 3D. They have found many uses in biological applications, such as measuring molecular forces,[41] manipulating DNA,[42] cellular organelles,[43] viruses,[44] bacterium,[44] and more recently, mammalian cells with minimal damage to cell viability.[45]

A typical set up of optical tweezers consists of Nd:YAG or Ti:Sapphire lasers, beam steering optics, and an inverted microscope with high numerical aperture (NA) objectives. To form optical traps, a tightly focused laser beam is directed through a high NA lens onto a dielectric particle. Photon from the point of focus from the laser beam creates an electrical field that attracts dielectrical particles and traps the object near the focal point of the laser. Trapping forces depend on the size and shape of the particle in question as well as properties of the surrounding medium.[46–48] Average trapping forces are ~1 nN, which are sufficient for manipulating most bacterium and mammalian cells. Often, the manipulation of biological molecules requires the use of one or more optical traps. Acoustic optical deflectors (AODs) allows for time sharing of the laser beam between different positions in a planar field. The laser beam dwells at a position for a predetermined period of time before moving to the next position. As long as the "dark time" is faster than the Brownian motion and diffusion of the cell, the beam is able to fix the position of the object and is as effective as a continuous beam.[49]

In addition to directly manipulating cells, optical tweezers have been used to study the effects of mechanical forces on cells. Microbeads are attached to the cell surface through ligands and act as handles for the optical tweezers. Displacement of the microbeads on the cell membrane by optical tweezers generates stretching or bending forces. These techniques can recreate physiological forces from stretching, compression, and ECM stiffness. Wang et al. used this technique in combination with fluorescent resonance energy transfer (FRET) to study the mechanoactivation of Src, an important signal transduction molecule playing an important role in self-renewal and differentiation of stem cells, as well as in many cancers.[50]

Minimizing damage to cells during manipulation is a major factor in optical trapping design and optimization. Cellular viability is dependent on laser wavelength, laser power, and duration of exposure to the traps. Biological specimens typically absorb light in the near infrared range, and thus most lasers are often tuned in the 800–1000 nm range.[51] Vorobjev et al. reported faulty mitosis and abnormal chromosome bridges in PtK2 cells exposed to continuous wave optical traps in the 760–765 nm range and minimal damage to 700 and 800–820 nm light.[51] Similarly, Liang et al. demonstrated wavelength dependence on the growth of Chinese hamster ovary (CHO) cells. When exposed to 740–760 and 900 nm light, CHO cells had poor growth and cell division characteristics compared to nonirradiated controls. Light in

the 950–990 nm wavelengths resulted in the highest clonablilty of all wavelengths tested. In all cases, shorter exposure and lower power of traps result in increased cellular function.[52] Time-shared optical traps, as described above, reduce the exposure of biological samples to laser light as compared to continuous wave (CW) technology. Mirisaidov et al. discovered that, under the same wavelength and duration of trapping, *E. coli* bacterium displayed higher viability with time-shared optical traps than with CW traps.[53]

Optical trapping provides stringent control of the cell and its placement within an environment with a resolution of ~19 nm. To permanently fix cells in position for long-term studies, Jordan et al. and Akselrod et al. entrapped cells into 3D scaffolds with minimal effect on cellular viability.[54,55] Optical tweezers, however, require some knowledge of optical technologies for set up and use. As of yet, most cellular studies utilizing optical trapping are relatively low throughput and monitoring only 10–100 cells in a single experiment.

In recent years, a new field of optical trapping termed plasmonic trapping has emerged. When light is applied to metal nanoparticles, photons excite the electrons in the nanoparticles that form energy waves and strong electromagnetic fields. Plasmonic trapping was first coupled with optical tweezers for nanotechnology applications as a method to enhance optical gradient forces from optical tweezers, and therefore reducing the Brownian motion of nanoparticles in traps. Combining optical tweezers and plasmonic traps, the power required to manipulate and trap biological objects can be greatly reduced.[56] More recently, Huang et al. designed a plasmonic trapping device in a microfluidic system for lab-on-a-chip applications. Whereas cell viability was not explored, the team was able to successfully trap single nanoparticles and *S. cerevisiae* cells in plasmonic traps without the complex optical setup required for optical tweezers.[57] Still in its infancy, plasmonic trapping holds great potential as a new cell-patterning technique or to augment biological optical trapping setups.

Another form of optical trapping technologies to pattern cells is laser-guided direct writing (LGDW). Utilizing the same principles as optical trapping, a weakly focused laser beam is used to trap and direct cells down hollow fibers onto cell culture surfaces. This method provides single-cell manipulation with ~1 μm scale resolution.[58] Nahmias et al. have used LGDW to create vascular and sinusoid-like structures onto collagen scaffolds.[59] It is unknown what effects laser power has on cell viability. Unless the substrate is patterned with adhesion molecules, the cells will randomly spread on the substrate after patterning.

4.3.2.4 Dielectrophoresis

Dielectrophoresis (DEP) has emerged as a promising technique to identify and place cells and microparticles through their electrical properties, size, and shape of the entrapped specimen. When presented to a nonuniform electric field, all objects exert some dielectrical forces that can change the motion of the particle. The strength of the force and movement depends on the size, shape, and electrical properties of the object and the surrounding medium.[60,61] DEP technology has mainly been utilized in cell-sorting applications, as no modification or manipulation is required prior to sorting. Recently, DEP has undergone resurgence for micromanipulation and patterning of DNA, viruses, proteins, and cell applications.[62,63]

To pattern cells using DEP, electrodes are microfabricated into a microfluidic chip or other culture devices. Thousands of individual electrodes can be placed on a centimeter of surface area using common microfabrication techniques. Cells are introduced into the system and pulled toward the electrode surface through DEP forces. Fluid flow across the surface removes unpatterned cells. After trapping, cells can then either be encapsulated in 3D scaffolds or adherent cell lines can be cultured on the surface. As with optical trapping technology, the duration and intensity of electrical stimulation can affect biological activity of living cells. Grey et al. demonstrated DEP patterning of mammalian cells using bovine aortic endothelial cells.[64] The group patterned a $1 \times 1\,cm^2$ array within 5 min with minimal affects on cell viability. Suzuki et al. further modified the procedures by exposing C2C12 cells to DEP forces for 5 min to allow cell adherence, flushed the device, and electropatterned again with a second cell type.[65] Albrect et al. successfully patterned cell-laden alginate beads with DEP technologies.[66]

With DEP technology, the precise location of cells and microparticles can be patterned onto various substrates. The technology is rapid and easily scaled for larger experiments. However, there is little control over the exact cells that are patterned, and coculture experiments so far have only been established by engaging one set of electrodes, removing the cells, then flowing in the next cell type. Exposure to high power traps must be limited as they may result in cell death or local heating of the medium. In DEP applications, the cells must be suspended in low conductivity medium, as physiological medium has high electrical conductivity and will not allow DEP to occur. This medium may be toxic to living cells, so exposure to the medium must be limited. Alternatively, negative DEP occurs when the object is less polarizable than the surrounding medium, allowing patterning in physiological medium.[67]

4.3.2.5 Inkjet Printing

Another microfabrication technique that adapted technologies from an electronics industry is inkjet printing of biomaterials, scaffolds, and cells. Commercially available inkjet printers reproduce electronic images by depositing nanoliter-sized drops of ink onto the paper substrate. Inkjet patterning technologies utilize these same commercial inkjet printers and ink cartridges to deposit small drops of "ink" (i.e., proteins, alkanethiols, scaffold materials, and more recently, cells) onto "paper" (i.e., tissue culture substrate) into desired configurations. The resolution of inkjet printing is approximately 350 μm. While the resolution of inkjet printing is significantly lower than other microfabrication techniques, the configuration of deposited patterns is easily changed without the costs and time constraints of fabricating new masters[68] (Figure 4.4).

4.4 CULTURE HANDLING SYSTEMS

4.4.1 Bioreactors

Expansion of progenitor cells in traditional static cultures leads to a loss of proliferation and differentiation potential of stem and progenitor cells, therefore severely limiting the number of cells available for tissue engineering and stem cell therapies.

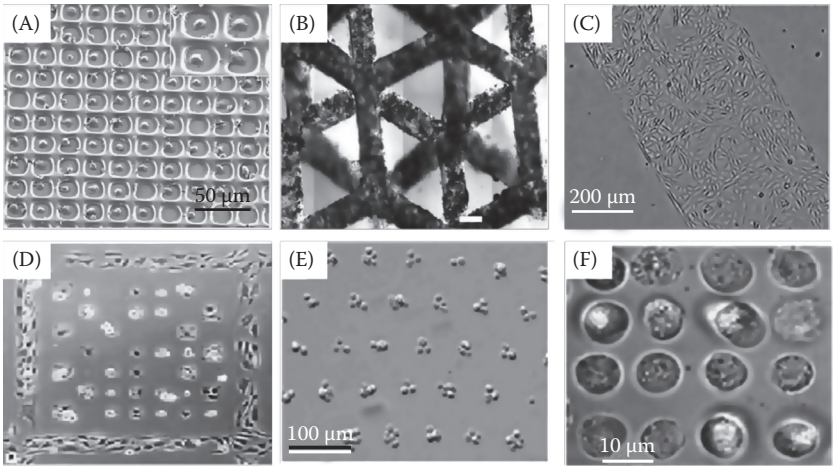

FIGURE 4.4 Microfabrication techniques to engineer the cellular microenvironment. (A) and (B) 2D and 3D photolithography. (A) Mouse 3T3 fibroblast cells seeded into poly (ethylene glycol) (PEG) wells created by photolithography. The PEG wells guided and patterned cellular adhesion to the glass surface. (Reprinted with permission from Revzin, A. et al., Surface engineering with poly (ethylene glycol) photolithography to create high-density cell arrays on glass, *Langmuir*, 19(23), 9855–9862. Copyright 2003 American Chemical Society.) (B) Hepatocytes were patterned into three dimensions by additive photolithography of photopolymerizable poly (ethylene glycol) diacrylate (PEGDA) hydrogels. (From Tsang, L. et al., *FASEB J.*, 21, 798, 2007. With permission.) (C) Inkjet Printing. Defined patterns of collagen were deposited onto cell-repellant agarose surfaces by inkjet printing. Smooth muscle cells (SMCs) were seeded onto the surface and adhered only to patterned collagen surfaces. (Reprinted from *Biomaterials*, 25(17), Roth, E.A. et al., Inkjet printing for high-throughput cell patterning, 3707–3715. Copyright 2004. With permission from Elsevier.) (D) Soft lithography/microcontact printing. Hexadecanethiolate and tri (ethylene glycol) were printed onto gold surfaces by microcontact printing. The ECM molecule fibronectin absorbs to the hexadecanethiolate but not onto tri (ethylene glycol). Bovine capillary endothelial cells (BCE) were patterned by selective adhesion to the fibronectin coated areas. (Reprinted from *Biomaterials*, 20(23–24), Kane, R.S., Takayama, S., Ostuni, E., Ingber, D.E., and Whitesides, G.M., Patterning proteins and cells using soft lithography, 2363–2376. Copyright 1999. With permission from Elsevier.) (E) Dielectrophoresis. Arrays of fibroblasts patterned through dielectrophoresis methodology and encapsulated in PEGDA hydrogels. (From Albrecht, D.R. et al., *Lab Chip— Roy. Soc. Chem.*, 5, 111–118, 2005. With permission.) (F) Optical Tweezers. Human monocytic U937 cells manipulated into a 4×4 array by optical tweezers and encapsulated in PEGDA hydrogels. (Trump, unpublished data.)

Once seeded into culture, cells require a proper balance of nutrients, oxygen, soluble molecules, and waste removal that is typically provided *in vivo* by the vasculature system. The static culture of cells in either 2D or 3D results in gradients of nutrients and small molecules. Further limiting the application of 3D scaffolds is that nutrients and oxygen can only penetrate the scaffold for a few hundred microns, leaving large constructs with a hypoxic and necrotic center surrounded by viable cells.[69] Static culture conditions so do not recapitulate the laminar flow and shear stress features

found in physiological tissue environments. These limitations severely inhibit the continued expansion of cells, the size of scaffolds available for use, and the differentiation capabilities of seeded stem cells. Another limitation to the clinical usage of 3D scaffolds and stem cells is the isolation, expansion, and differentiation of stem cells in sufficient quantities to seed into scaffolds or for cell based therapies. Bulk cultures of cells and scaffolds also result in local microenvironmental changes, which form concentration gradients that can affect cell behavior.

Bioreactors are cell culture vessels designed to provide strict control over culture conditions such as temperature, pH, oxygen levels, and for the perfusion of medium in large cultures of cells and 3D constructs. Many different types of bioreactors have been used for the culture and expansion of stem cells and 3D scaffolds, such as stirred flask bioreactors, rotating wall vessels, perfusion chambers, and microfluidic bioreactors. These bioreactors offer a distinct advantage over traditional cell culture as they provide automation, the ability to control and change culture parameters, and offer a more homogeneous environment for cell culture. Parameters can easily be changed from one experiment to another and are highly dependent on the objective of the experiment (i.e., expansion or differentiation). Continuous mixing of oxygen and nutrients in bioreactors reduces concentration gradients and increases nutrient diffusion throughout cellular colonies and constructs.[70]

Initial studies of stem cells in bioreactors reported the increased expansion and long-term maintenance of HSC over static culture systems. Murine HSCs cultured in stirred flask bioreactors showed a fivefold increase of the stemness marker Sca1+ and a fourfold increase in long-term culture initiating cells (LTC-IC) over 21 days of culture. Expansion of murine ESCs increased without the need for feeder layers or the loss of differentiation potential.[71] Bioreactors have also enabled the expansion of embryoid body culture to be scaled up. Cameron et al. reported increased expansion, more uniform morphology, and maintenance of differentiation potential of embryoid bodies cultured in stirred vessel bioreactors.[72]

Bioreactors have also been extensively used to seed progenitor cells onto 3D scaffolds. Important for the development of functional tissue engineering constructs, cell seeding of scaffolds remains a highly variable process. Bioreactor-based seeding methodologies have resulted in increased seeding densities and efficiencies and more uniform distributions of cells within the scaffold.[73] Seeding efficiency depends on cell type and density, scaffold type, and flow rates of culture medium. Both murine and human MSCs have been efficiently seeded onto a variety of scaffolds using stirred flask and perfusion bioreactors. In general, most studies have reported increased seeding efficiency, density, cell penetration, and overall more uniform distributions of cells throughout the scaffold using spinner and perfusion bioreactors. MSCs seeded and cultured onto 3D scaffolds increased expansion while maintaining differentiation capacity as compared to cells cultured in traditional culture vessels.[74]

High flow rates needed for efficient seeding and nutrient diffusion, however, can greatly affect cellular processes. Structural integrity of seeded scaffolds can be compromised by fluid channel formation at high flow rates. Even at low velocities, shear stresses imparted onto cells can be significant.[75] Often, the fluid flow required for efficient cell seeding and nutrient mixing is significantly higher than what is found in physiological tissues (100 and 0.1–10 μL/s, respectively).[70] Shear tolerance of cells and scaffolds

depends on the cell type, scaffold used, and experimental parameters. Mechanical loading imparted by shear can affect cellular differentiation. Higher fluid rates are conductive to osteogenic differentiation of MSCs, while lower rates facilitated expansion. Fluid rates also affect ECM distribution. Zhao et al. reported decreased collagen and laminin I deposition in cell-seeded scaffold.[70] Differentiation into osteoblasts was promoted at high flow rates. Similarly, bovine chondrocytes seeded on PGA scaffolds had increased GAG formation and synthesis, while net GAG accumulation throughout the scaffolds was reduced, most likely by release of GAG into culture medium in stirred flask bioreactors.[76] To protect cells from shear stressors in bulk culture systems, cells have been encapsulated in alginate microbeads and cultured in bioreactors.

4.4.2 Microfluidics

In vivo cellular microenvironments are highly dynamic, and soluble factor concentrations can vary drastically on a scale of microns. In traditional cultures, soluble factors and medium exchange require the physical removal of culture medium and bolus delivery of soluble factors, resulting in a homogeneous mixture that is difficult to control in real time on a microscale level. Microfluidic bioreactors, on the other hand, allow soluble factor delivery and replacement in a matter of seconds, allowing real-time control over the cellular microenvironment. Fluid flow rates, pressures, and soluble factor concentration and delivery are easily manipulated using these devices.[77] Microfluidic bioreactors are formed by creating a master of desired pattern with soft lithography techniques and stamps are most commonly made of PDMS. The PDMS stamps contain one or more channel systems to direct nutrient, oxygen, and soluble factor flow on a microscale level. Fluid flow is typically controlled by syringe pumps that allow for rapid and pulsatile delivery of stimuli and can also maintain cultures for weeks at a time. Microfluidic devices designed with two or more channels permit the controlled mixing of soluble factors. Laminar flow in microchannels allows one or more streams of fluid to combine with limited mixing of streams. Thus, a single cell can be exposed to multiple microenvironments by placement at an intersection of streams carrying different soluble molecules.[78]

4.5 READOUT SYSTEMS

Once cells are exposed to various microenvironments, the activity of the cells in response to its stimuli can be assessed. There are several well-established techniques to monitor cellular activity. On a large scale, dynamic responses of the culture in whole can be measured by degree of expansion and apoptosis, morphological changes, migration, and differentiation. Stem cell differentiation has been readily identified through histochemistries such as alkaline phosphatase/von Kossa staining, oil red O, and safranin staining to elucidate bone, fat, and cartilage differentiation of MSCs.[26] Molecular characteristics can be measured by western blotting, RT-PCR, ELISA, and other well-established techniques. However, such measurements are end-point assays that require fixation or cell lysis. Since these methods measure the population as a whole, the signature of stem cells may be lost due to contaminating cell types. The discovery of green fluorescent protein (GFP) and its variants has enabled

single and live cell imaging to visualize cellular processes. GFP can be expressed as a fusion tag to proteins of interest to explore protein–protein interactions and gene activation and expression.[79,80] Specific organelles can be targeted, such as the staining of the actin cytoskeleton. Other assays have been developed using variants of GFP, such as FRET and fluorescent resonance after photobleaching (FRAP), which uses fluorescent protein pairs to monitor cellular interactions in real time.[81] Recently, to improve dynamic sensing of cellular activity, a mutated form of GFP has been developed. This GFP mutant shifts color spectrum as the protein matures. When the protein is first synthesized, the cell is green in color, but shifts to red fluorescence in a time dependent manner. Thus, readouts of gene expression dynamics and protein synthesis can be monitored in real time by the ratio of green to red fluorescence.[82]

4.6 CONCLUSIONS AND FUTURE DIRECTIONS

Tissue engineering and microfabrication technologies have arisen as invaluable tools to probe the spatial and temporal cues that govern stem cell fate. The ability to precisely pattern cells and external signals in 2D and 3D enables investigations into the roles of niche components on stem cell plasticity and differentiation. These approaches require (1) biocompatible scaffolds with defined mechanical properties, (2) microfabrication of scaffolds and signals into specific geometries, (3) controlled seeding and placement of cells and signals, (4) effective culture systems for nutrient delivery, and (5) readout systems to monitor cellular activity during culture. While extensive research has been conducted in establishing these technologies, the generation of functional stem cell niches and culture systems will require a multidisciplinary approach that combines and applies these technologies into functional stem cell environments. Combination of engineering approaches with traditional cell biology approaches will facilitate recapitulation of functional stem cell microenvironments and advance our knowledge of functional stem cell niches.

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REFERENCES

1. Watt, F. M.; Hogan, B. L. M., Out of eden: Stem cells and their niches. *Science* 2000, *287* (5457), 1427–1430.
2. Schofield, R., The relationship between the spleen colony-forming cell and the haemopoietic stem cell. A hypothesis. *Blood Cells* 1978, *4* (1–2), 7–25.
3. Birgersdotter, A.; Sandberg, R.; Ernberg, I., Gene expression perturbation in vitro—A growing case for three-dimensional (3D) culture systems. *Seminars in Cancer Biology* 2005, *15* (5 SPEC. ISS.), 405–412.

4. Doane, K. J.; Birk, D. E., Fibroblasts retain their tissue phenotype when grown in three-dimensional collagen gels. *Experimental Cell Research* 1991, *195* (2), 432–442.
5. Kale, S.; Biermann, S.; Edwards, C.; Tarnowski, C.; Morris, M.; Long, M. W., Three-dimensional cellular development is essential for ex vivo formation of human bone. *Nature Biotechnology* 2000, *18* (9), 954–958.
6. Khademhosseini, A.; Langer, R.; Borenstein, J.; Vacanti, J. P., Microscale technologies for tissue engineering and biology. *Proceedings of the National Academy of Sciences of the United States of America* 2006, *103* (8), 2480–2487.
7. Shi, S.; Gronthos, S., Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. *Journal of Bone and Mineral Research* 2003, *18* (4), 696–704.
8. Zhang, J.; Niu, C.; Ye, L.; Huang, H.; He, X.; Tong, W. G.; Ross, J.; Haug, J.; Johnson, T.; Feng, J. Q.; Harris, S.; Wiedemann, L. M.; Mishina, Y.; Li, L., Identification of the haematopoietic stem cell niche and control of the niche size. *Nature* 2003, *425* (6960), 836–841.
9. Tumbar, T.; Guasch, G.; Greco, V.; Blanpain, C.; Lowry, W. E.; Rendl, M.; Fuchs, R., Defining the epithelial stem cell niche in skin. *Science* 2004, *303* (5656), 359–363.
10. Ohyama, M.; Terunuma, A.; Tock, C. L.; Radonovich, M. F.; Pise-Masison, C. A.; Hopping, S. B.; Brady, J. N.; Udey, M. C.; Vogel, J. C., Characterization and isolation of stem cell-enriched human hair follicle bulge cells. *Journal of Clinical Investigation* 2006, *116* (1), 249–260.
11. Marshman, E.; Booth, C.; Potten, C. S., The intestinal epithelial stem cell. *BioEssays* 2002, *24* (1), 91–98.
12. Palmer, T. D.; Willhoite, A. R.; Gage, F. H., Vascular niche for adult hippocampal neurogenesis. *Journal of Comparative Neurology* 2000, *425* (4), 479–494.
13. Collins, C. A.; Olsen, I.; Zammit, P. S.; Heslop, L.; Petrie, A.; Partridge, T. A.; Morgan, J. E., Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. *Cell* 2005, *122* (2), 289–301.
14. Corre, J.; Mahtouk, K.; Attal, M.; Gadelorge, M.; Huynh, A.; Fleury-Cappellesso, S.; Danho, C.; Laharrague, P.; Klein, B.; Rème, T.; Bourin, P., Bone marrow mesenchymal stem cells are abnormal in multiple myeloma. *Leukemia* 2007, *21* (5), 1079–1088.
15. Conboy, I. M.; Conboy, M. J.; Wagers, A. J.; Girma, E. R.; Weismann, I. L.; Rando, T. A., Rejuvenation of aged progenitor cells by exposure to a young systemic environment. *Nature* 2005, *433* (7027), 760–764.
16. Frisch, S. M.; Francis, H., Disruption of epithelial cell-matrix interactions induces apoptosis. *Journal of Cell Biology* 1994, *124* (4), 619–626.
17. Nuttelman, C. R.; Tripodi, M. C.; Anseth, K. S., Synthetic hydrogel niches that promote hMSC viability. *Matrix Biology* 2005, *24* (3), 208–218.
18. Benoit, D. S. W.; Tripodi, M. C.; Blanchette, J. O.; Langer, S. J.; Leinwand, L. A.; Anseth, K. S., Integrin-linked kinase production prevents anoikis in human mesenchymal stem cells. *Journal of Biomedical Materials Research Part A* 2007, *81* (2), 259–268.
19. Winer, J. P.; Janmey, P. A.; McCormick, M. E.; Funaki, M., Bone marrow-derived human mesenchymal stem cells become quiescent on soft substrates but remain responsive to chemical or mechanical stimuli. *Tissue Engineering Part A* 2009, *15* (1), 147–154.
20. Chen, C. S.; Mrksich, M.; Huang, S.; Whitesides, G. M.; Ingber, D. E., Geometric control of cell life and death. *Science* 1997, *276* (5317), 1425–1428.
21. McBeath, R.; Pirone, D. M.; Nelson, C. M.; Bhadriraju, K.; Chen, C. S., Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. *Developmental Cell* 2004, *6* (4), 483–495.
22. Chowdhury, F.; Na, S.; Li, D.; Poh, Y. C.; Tanaka, T. S.; Wang, F.; Wang, N., Material properties of the cell dictate stress-induced spreading and differentiation in embryonic stem cells. *Nature Materials* 2010, *9* (1), 82–88.

23. Sahni, A.; Sporn, L. A.; Francis, C. W., Potentiation of endothelial cell proliferation by fibrin(ogen)-bound fibroblast growth factor-2. *Journal of Biological Chemistry* 1999, *274* (21), 14936–14941.
24. Fan, V. H.; Tamama, K.; Au, A.; Littrell, R.; Richardson, L. B.; Wright, J. W.; Wells, A.; Griffith, L. G., Tethered epidermal growth factor provides a survival advantage to mesenchymal stem cells. *Stem Cells* 2007, *25* (5), 1241–1251.
25. Adams, G. B.; Chabner, K. T.; Alley, I. R.; Olson, D. P.; Szczepiorkowski, Z. M.; Poznansky, M. C.; Kos, C. H.; Pollak, M. R.; Brown, E. M.; Scadden, D. T., Stem cell engraftment at the endosteal niche is specified by the calcium-sensing receptor. *Nature* 2006, *439* (7076), 599–603.
26. Pittenger, M. F.; Mackay, A. M.; Beck, S. C.; Jaiswal, R. K.; Douglas, R.; Mosca, J. D.; Moorman, M. A.; Simonetti, D. W.; Craig, S.; Marshak, D. R., Multilineage potential of adult human mesenchymal stem cells. *Science* 1999, *284* (5411), 143–147.
27. Shiozawa, Y.; Takenouchi, H.; Taguchi, T.; Saito, M.; Katagiri, Y. U.; Okita, H.; Shimizu, T.; Yamashiro, Y.; Fujimoto, J.; Kiyokawa, N., Human osteoblasts support hematopoietic cell development in vitro. *Acta Haematologica* 2009, *120* (3), 134–145.
28. Dawson, E.; Mapili, G.; Erickson, K.; Taqvi, S.; Roy, K., Biomaterials for stem cell differentiation. *Advanced Drug Delivery Reviews* 2008, *60* (2), 215–228.
29. Park, T. H.; Shuler, M. L., Integration of cell culture and microfabrication technology. *Biotechnology Progress* 2003, *19* (2), 243–253.
30. Lee, J. Y.; Shah, S. S.; Zimmer, C. C.; Liu, G. Y.; Revzin, A., Use of photolithography to encode cell adhesive domains into protein microarrays. *Langmuir* 2008, *24* (5), 2232–2239.
31. Whitesides, G. M.; Ostuni, E.; Takayama, S.; Jiang, X.; Ingber, D. E., Soft lithography in biology and biochemistry. *Annual Review of Biological Engineering* 2001, *3*, 335–373.
32. Xia, Y.; Whitesides, G. M., Soft lithography. *Annual Review of Materials Science* 1998, *28* (1), 153–184.
33. Kane, R. S.; Takayama, S.; Ostuni, E.; Ingber, D. E.; Whitesides, G. M., Patterning proteins and cells using soft lithography. *Biomaterials* 1999, *20* (23–24), 2363–2376.
34. Odom, T. W.; Love, J. C.; Wolfe, D. B.; Paul, K. E.; Whitesides, G. M., Improved pattern transfer in soft lithography using composite stamps. *Langmuir* 2002, *18* (13), 5314–5320.
35. James, C. D.; Davis, R. C.; Kam, L.; Craighead, H. G.; Isaacson, M.; Turner, J. N.; Shain, W., Patterned protein layers on solid substrates by thin stamp microcontact printing. *Langmuir* 1998, *14* (4), 741–744.
36. Bernard, A.; Delamarche, E.; Schmid, H.; Michel, B.; Bosshard, H. R.; Biebuyck, H., Printing patterns of proteins. *Langmuir* 1998, *14* (9), 2225–2229.
37. Groves, J. T.; Boxer, S. G., Micropattern formation in supported lipid membranes. *Accounts of Chemical Research* 2002, *35* (3), 149–157.
38. Csucs, G.; Künzler, T.; Feldman, K.; Robin, F.; Spencer, N. D., Microcontact printing of macromolecules with submicrometer resolution by means of polyolefin stamps. *Langmuir* 2003, *19* (15), 6104–6109.
39. Khademhosseini, A.; Ferreira, L.; Blumling III, J.; Yeh, J.; Karp, J. M.; Fukuda, J.; Langer, R., Co-culture of human embryonic stem cells with murine embryonic fibroblasts on microwell-patterned substrates. *Biomaterials* 2006, *27* (36), 5968–5977.
40. Ashkin, A.; Dziedzic, J. M.; Yamane, T., Optical trapping and manipulation of single cells using infrared laser beams. *Nature* 1987, *330* (6150), 769–771.
41. Visscher, K.; Schnltzer, M. J.; Block, S. M., Single kinesin molecules studied with a molecular force clamp. *Nature* 1999, *400* (6740), 184–189.
42. Wang, M. D.; Yin, H.; Landick, R.; Gelles, J.; Block, S. M., Stretching DNA with optical tweezers. *Biophysical Journal* 1997, *72* (3), 1335–1346.

43. Shelby, J. P.; Edgar, J. S.; Chiu, D. T., Monitoring cell survival after extraction of a single subcellular organelle using optical trapping and pulsed-nitrogen laser ablation. *Photochemistry and Photobiology* 2005, *81* (4), 994–1001.
44. Ashkin, A.; Dziedzic, J. M., Optical trapping and manipulation of viruses and bacteria. *Science* 1987, *235* (4795), 1517–1520.
45. Uchida, M.; Sato-Maeda, M.; Tashiro, H., Micromanipulation: Whole-cell manipulation by optical trapping. *Current Biology* 1995, *5* (4), 380–382.
46. Mazolli, A.; Maia Neto, P. A.; Nussenzveig, H. M., Theory of trapping forces in optical tweezers. *Proceedings of the Royal Society A Mathematical, Physical and Engineering Sciences* 2003, *459* (2040), 3021–3041.
47. Berns, M. W., Optical tweezers: Tethers, wavelengths, and heat. *Methods in Cell Biology* 2007, 82, 457–466.
48. Sun, B.; Roichman, Y.; Grier, D. G., Theory of holographic optical trapping. *Optics Express* 2008, *16* (20), 15765–15776.
49. Brouhard, G. J.; Schek III, H. T.; Hunt, A. J., Advanced optical tweezers for the study of cellular and molecular biomechanics. *IEEE Transactions on Biomedical Engineering* 2003, *50* (1), 121–125.
50. Wang, Y.; Lu, S., The application of FRET biosensors to visualize Src activation. In *Small Animal Whole-Body Optical Imaging Based on Genetically Engineered Probes*, The International Society for Optical Engineering: San Jose, CA, 2008.
51. Vorobjev, I. A.; Liang, H.; Wright, W. H.; Berns, M. W., Optical trapping for chromosome manipulation: A wavelength dependence of induced chromosome bridges. *Biophysical Journal* 1993, *64* (2), 533–538.
52. Liang, H.; Vu, K. T.; Krishnan, P.; Trang, T. C.; Shin, D.; Kimel, S.; Berns, M. W., Wavelength dependence of cell cloning efficiency after optical trapping. *Biophysical Journal* 1996, *70* (3), 1529–1533.
53. Mirsaidov, U.; Scrimgeour, J.; Timp, W.; Beck, K.; Mir, M.; Matsudaira, P.; Timp, G., Live cell lithography: Using optical tweezers to create synthetic tissue. *Lab on a Chip—Miniaturisation for Chemistry and Biology* 2008, *8* (12), 2174–2181.
54. Akselrod, G. M.; Timp, W.; Mirsaidov, U.; Zhao, Q.; Li, C.; Timp, R.; Timp, K.; Matsudaira, P.; Timp, G. L., Laser-guided assembly of heterotypic three-dimensional living cell microarrays. *Biophysical Journal* 2006, *91* (9), 3465–3473.
55. Jordan, P.; Leach, J.; Padgett, M.; Blackburn, P.; Isaacs, N.; Goksör, M.; Hanstorp, D.; Wright, A.; Girkin, J.; Cooper, J., Creating permanent 3D arrangements of isolated cells using holographic optical tweezers. *Lab on a Chip—Miniaturisation for Chemistry and Biology* 2005, *5* (11), 1224–1228.
56. Righini, M.; Zelenina, A. S.; Girard, C.; Quidant, R., Parallel and selective trapping in a patterned plasmonic landscape. *Nature Physics* 2007, *3* (7), 477–480.
57. Huang, L.; Maerkl, S. J.; Martin, O. J. F., Integration of plasmonic trapping in a microfluidic environment. *Optics Express* 2009, *17* (8), 6018–6024.
58. Odde, D. J.; Renn, M. J., Laser-guided direct writing of living cells. *Biotechnology and Bioengineering* 2000, *67* (3), 312–318.
59. Nahmias, Y.; Schwartz, R. E.; Verfaillie, C. M.; Odde, D. J., Laser-guided direct writing for three-dimensional tissue engineering. *Biotechnology and Bioengineering* 2005, *92* (2), 129–136.
60. Chiou, P. Y.; Ohta, A. T.; Wu, M. C., Massively parallel manipulation of single cells and microparticles using optical images. *Nature* 2005, *436* (7049), 370–372.
61. Morgan, H.; Hughes, M. P.; Green, N. G., Separation of submicron bioparticles by dielectrophoresis. *Biophysical Journal* 1999, *77* (1), 516–525.
62. Chou, C.-F.; Tegenfeldt, J. O.; Bakajin, O.; Chan, S. S.; Cox, E. C.; Darnton, N.; Duke, T.; Austin, R. H., Electrodeless dielectrophoresis of single- and double-stranded DNA. *Biophysical Journal* 2002, *83* (4), 2170–2179.

63. Pethig, R., Dielectrophoresis: Using inhomogeneous AC electrical fields to separate and manipulate cells. *Critical Reviews in Biotechnology* 1996, *16* (4), 331–348.
64. Gray, D. S.; Tan, J. L.; Voldman, J.; Chen, C. S., Dielectrophoretic registration of living cells to a microelectrode array. *Biosensors and Bioelectronics* 2004, *19* (7), 771–780.
65. Suzuki, M.; Yasukawa, T.; Shiku, H.; Matsue, T., Negative dielectrophoretic patterning with different cell types. *Biosensors and Bioelectronics* 2008, *24* (4), 1043–1047.
66. Albrecht, D. R.; Underhill, G. H.; Mendelson, A.; Bhatia, S. N., Multiphase electropatterning of cells and biomaterials. *Lab chip* 2007, *7*, 702–709.
67. Thomas, R. S.; Morgan, H.; Green, N. G., Negative DEP traps for single cell immobilisation. *Lab on a Chip — Miniaturisation for Chemistry and Biology* 2009, *9* (11), 1534–1540.
68. Phillippi, J. A.; Miller, E.; Weiss, L.; Huard, J.; Waggoner, A.; Campbell, P., Microenvironments engineered by inkjet bioprinting spatially direct adult stem cells toward muscle- and bone-like subpopulations. *Stem Cells* 2008, *26* (1), 127–134.
69. Martin, I.; Wendt, D.; Heberer, M., The role of bioreactors in tissue engineering. *Trends in Biotechnology* 2004, *22* (2), 80–86.
70. Zhao, F.; Grayson, W. L.; Ma, T.; Irsigler, A., Perfusion affects the tissue developmental patterns of human mesenchymal stem cells in 3D scaffolds. *Journal of Cellular Physiology* 2009, *219* (2), 421–429.
71. Zandstra, P. W.; Eaves, C. J.; Piret, J. M., Expansion of hematopoietic progenitor cell populations in stirred suspension bioreactors of normal human bone marrow cells. *Biotechnology* 1994, *12* (9), 909–914.
72. Cameron, C. M.; Hu, W. S.; Kaufman, D. S., Improved development of human embryonic stem cell-derived embryoid bodies by stirred vessel cultivation. *Biotechnology and Bioengineering* 2006, *94* (5), 938–948.
73. Wendt, D.; Marsano, A.; Jakob, M.; Heberer, M.; Martin, I., Oscillating perfusion of cell suspensions through three-dimensional scaffolds enhances cell seeding efficiency and uniformity. *Biotechnology and Bioengineering* 2003, *84* (2), 205–214.
74. Braccini, A.; Wendt, D.; Jaquiery, C.; Jakob, M.; Heberer, M.; Kenins, L.; Wodnar-Filipowicz, A.; Quarto, R.; Martin, I., Three-dimensional perfusion culture of human bone marrow cells and generation of osteoinductive grafts. *Stem Cells* 2005, *23* (8), 1066–1072.
75. Tada, S.; Tarbell, J. M., Interstitial flow through the internal elastic lamina affects shear stress on arterial smooth muscle cells. *American Journal of Physiology—Heart and Circulatory Physiology* 2000, *278* (5), H1589–H1597.
76. Martin, I.; Obradovic, B.; Freed, L. E.; Vunjak-Novakovic, G., Method for quantitative analysis of glycosaminoglycan distribution in cultured natural and engineered cartilage. *Annals of Biomedical Engineering* 1999, *27* (5), 656–662.
77. Yarmush, M. L.; King, K. R., Living-cell microarrays. *Annual Review of Biological Engineering* 2009, 11, 235–257.
78. Eriksson, E.; Sott, K.; Lundqvist, F.; Sveningsson, M.; Scrimgeour, J.; Hanstorp, D.; Goksör, M.; Granéli, A., A microfluidic device for reversible environmental changes around single cells using optical tweezers for cell selection and positioning. *Lab on a Chip—Miniaturisation for Chemistry and Biology* 2010, *10* (5), 617–625.
79. Chalfie, M.; Tu, Y.; Euskirchen, G.; Ward, W. W.; Prasher, D. C., Green fluorescent protein as a marker for gene expression. *Science* 1994, *263* (5148), 802–805.
80. van Roessel, P.; Brand, A. H., Imaging into the future: Visualizing gene expression and protein interactions with fluorescent proteins. *Nature Cell Biology* 2002, *4* (1), E15–E20.
81. Ha, T.; Enderle, T.; Ogletree, D. F.; Chemla, D. S.; Selvin, P. R.; Weiss, S., Probing the interaction between two single molecules: Fluorescence resonance energy transfer between a single donor and a single acceptor. *Proceedings of the National Academy of Sciences of the United States of America* 1996, *93* (13), 6264–6268.

82. Terskikh, A.; Fradkov, A.; Ermakova, G.; Zaraisky, A.; Tan, P.; Kajava, A. V.; Zhao, X.; Lukyanov, S.; Matz, M.; Kim, S.; Weissman, I.; Siebert, P., "Fluorescent timer": Protein that changes color with time. *Science* 2000, *290* (5496), 1585–1588.
83. Revzin, A. et al., Surface engineering with poly (ethylene glycol) photolithography to create high-density cell arrays on glass. *Langmuir* 2003, *19* (23), 9855–9862.
84. Tsang, L. et al., Fabrication of 3D hepatic tissues by additive photopatterning of cellular hydrogels. *FASEB Journal* 2007, *21* (3), 798.
85. Roth, E. A. et al., Inkjet printing for high-throughput cell patterning. *Biomaterials* 2004, *25* (17), 3707–3715.
86. Albrecht, D. R. et al., Photo- and electropatterning of hydrogel-encapsulated living cell arrays. *Lab on a Chip—The Royal Society of Chemistry* 2005, *5*, 111–118. http://dx.doi.org/10.1039/b406953f
87. Evans, N. D.; Minelli, C.; Gentleman, E.; LaPointe, V.; Patankar, S. N.; Kallivretaki, M.; Chen, X.; Roberts, C. J.; Stevens, M. M., Substrate stiffness affects early differentation events in embryonic stem cells. *European Cells and Materials.* 2009, *18*, 1–14.
88. Offenhausser, A.; Bocker-Meffert, S.; Decker, T.; Helpenstein, R.; Gasteier, P.; Groll, J.; Moller, M.; Reska, A.; Schafer, S.; Schulte, P.; Vogt-Eisele, A., Microcontact printing of proteins for neuronal cell guidance. *Soft Matter* 2007, *3*, 290–298.
89. Cosson, S.; Kobel, S. A.; Lutolf, M. P., Capturing complex protein gradients on biomimetic hydrogels for cell-based assays *Advanced Functional Materials*. 2009, *19*, 3411–3419.
90. Lee, S.; Moon, J. J.; West, J. L., Three-dimensional micropatterning of bioactive hydrogels via two-photon laser scanning photolithography for guided 3D cell migration. *Biomaterials* 2008, *20*, 2962–2968.
91. Lewis, J. A.; Gratson, G. M., Direct writing in three dimensions. *Langumir* 2004, *7*, 32–39.
92. Hahn, M. S.; Taite, L. J.; Moon, J. J.; Rowland, M. C.; Ruffino, K. A.; West, J. L., Photolithographic patterning of polyethylene glycol hydrogels. *Biomaterials* 2006, *27*, 2519–2524.
93. Ruiz, S. A.; Chen, C. S., Emergence of patterned stem cell differentiation within multicellular structures. *Stem Cells* 2008, *26*, 2921–2927.
94. Xu, Tao, Joyce Jin, Cassie Gregory, JJ Hickman, Thomas Boland. Inkjet printing of viable mammalian cells. *Biomaterials* 2005, *26*, 93–99.
95. Allen, P. B.; Milne, G.; Doepker, B. R.; Chiu, D. T., Pressure-driven laminar flow switching for rapid exchange of solution environment around surface adhered biological particles. *Lab chip* 2010, *10*, 727–733.
96. Adler, Micha, Mark Polinkovsky, Edgar Gutierrez, Alex Groisman. Generation of oxygen gradients with arbitrary shapes in a microfluidic device. *Lab chip* 2010, *10*, 388–391.