Artificial Stem Cell Niches

By *Matthias P. Lutolf,** and *Helen M. Blau**

The authors dedicate this article to Bob Langer, a pioneer in bridging materials and biology.

Stem cells are characterized by their dual ability to reproduce themselves (self-renew) and specialize (differentiate), yielding a plethora of daughter cells that maintain and regenerate tissues. In contrast to their embryonic counterparts, adult stem cells retain their unique functions only if they are in intimate contact with an instructive microenvironment, termed stem cell niche. In these niches, stem cells integrate a complex array of molecular signals that, in concert with induced cell-intrinsic regulatory networks, control their function and balance their numbers in response to physiologic demands. This progress report provides a perspective on how advanced materials technologies could be used (i) to engineer and systematically analyze specific aspects of functional stem cells niches in a controlled fashion in vitro and (ii) to target stem cell niches in vivo. Such "artificial niches" constitute potent tools for elucidating stem cell regulatory mechanisms with the capacity to directly impact the development of novel therapeutic strategies for tissue regeneration.

1. Introduction

While embryonic stem cells generate diverse tissues, adult stem cells are specialized and essential for tissue maintenance and repair throughout life.[1] In adulthood, tissue homeostasis and regeneration are critically dependent on both the self-renewal and the differentiation capacity of stem cells. Due to these unique properties, the potential applications of adult stem cells are vast, in the treatment of both various genetic diseases and injuries due to trauma. However, to fully exploit this clinical potential, we must increase our knowledge of the regulatory mechanisms that govern stem cell behavior. To date only a few adult stem cell types are approved for clinical use. Bone marrow transplants that harbor hematopoietic stem cells (HSCs) have saved the lives of numerous leukemia and lymphoma patients and skin transplants have significantly alleviated disfigurement and increased the function of burn victims. Moreover, recent findings suggest that cells with stem cell-like properties may give rise to and maintain some cancers, including acute leukemia, brain, breast, and skin cancer. Thus, an increased understanding of stem cell regulatory mechanisms may not only augment treatment options in regenerative medicine but also spawn new strategies for the treatment of cancer.

To overcome the hurdles inherent in enlisting adult stem cells therapeutically or in targeting cancer stem cells for destruction, stem cell biologists are addressing fundamental questions regarding the precise cell-intrinsic and cell-extrinsic regulation of key stem cell functions. Which genes determine the unique properties of a stem cell? Do adult stem cells of diverse tissues share gene expression patterns? How can the regulation of these genes be manipulated to advantage? What genetic determinants distinguish stem cells from their more specialized progenitors and can this specialization be reversed? Stem cells are exposed to a multitude of diverse biochemical and biophysical cues present in their spatial vicinity. To what extent is stem cell function predetermined or subject to such extrinsic influences? How can extrinsic factors keep stem cells quiescent, activate them, or direct stem cell divisions to result in self-renewal or differentiation, leading to either maintenance, expansion, or depletion of the stem cell pool? Can these factors be exploited in vitro to control these stem cell functions? Can perturbation of certain soluble or immobilized factors typical of the stem cell microenvironment lead either to the promotion or to the arrest of cancer?

To address some of these questions, technologies for controlling adult stem cell behavior outside of tissues would constitute tremendous progress. Such methods would not only allow a reduction of costly animal experimentation, but would also simplify the complexity of existing experimental systems by reducing the numerous variables typical of a stem cell's native microenvironment, termed niche. Advanced biomaterials technologies, alone or in combination with other technologies, could greatly facilitate this type of "deconstruction" effort, enabling

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analyses of stem cell behavior in a manner previously not possible. Biomaterials could, for example, be exploited as modular toolboxes to construct simplified de novo niches with diverse biochemical and biophysical properties. An integration of biomaterials technologies with microfabrication platforms such as biomolecule patterning or microfluidics could open up new avenues for identifying stem cell regulators and investigating stem cell behavior at the single cell level and in high-throughput.

Here, we highlight recent progress and future opportunities in the design of engineered, completely artificial niches. We first summarize the fundamentals of adult stem cell function in niches, because it is necessary to establish a solid conceptual framework in order to consider potential opportunities for stem cell manipulation by engineering techniques. We then discuss emerging approaches of materials engineering in combination with stem cell biology that have already been realized. Finally, we provide a vision which describes prospects for the future generation of artificial niches. Although still in their infancy, niche model systems of the type described here will ultimately translate into novel strategies for tissue regeneration.

Note: Definitions of biological vocabulary can be found in the glossary at the end of this report.

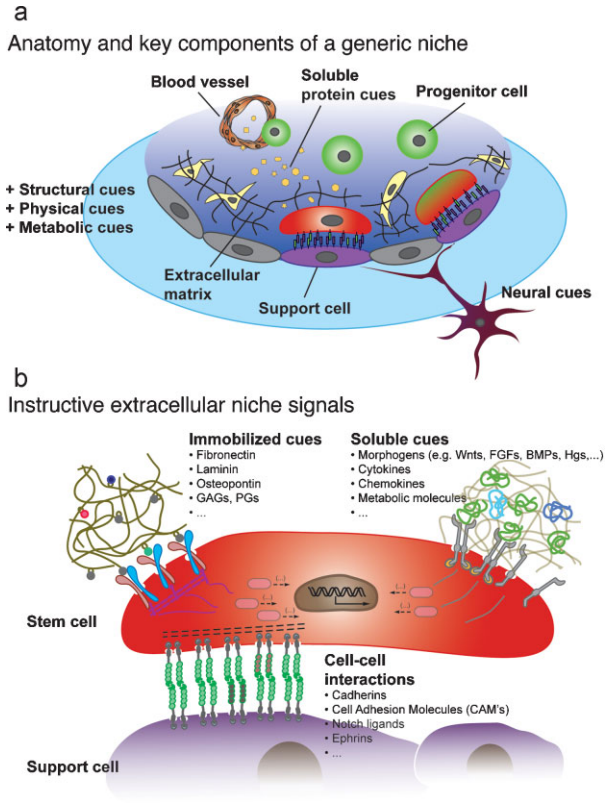

Figure 1. Architecture and composition of a stem cell niche. Adult stem cells are located within instructive microenvironments comprised of complex mixtures of extracellular cues delivered by support cells in close proximity (a). The main components of a niche are support cells and their secreted transmembrane cell–cell adhesion proteins, soluble factors, and the surrounding ECM (b).

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2. Biological Background

2.1. Adult Stem Cells Reside in Niches

Adult stem cell niches are anatomically defined locations comprised of complex mixtures of extracellular cues delivered by support cells in close physical proximity (Fig. 1).[2–7] Niches, in concert with cell-intrinsic regulatory networks, control multiple functions of adult stem cells, most importantly stem cell self-renewal, as discussed in some detail below. Indeed, the niche is so important that its absence leads to the loss of stem cell function, which in turn can have profound consequences, impacting tissue function and regeneration. Niches are not only common to many animal species, but are also found in plants.[8] Although the niche concept was postulated more than two decades ago for hematopoietic (blood) stem cells,[9] concrete proof of the existence and function of niches was only provided relatively recently in studies of fruit flies (e.g.,[10–13]). Stem cell niches are currently best understood in such model organisms, because they are relatively accessible, easily visualized by

microscopy, and readily manipulated using genetics. These niches have served as paradigms for more complex niches in mammals.

As described in several excellent reviews[4,6,7,14] mammalian niches have been identified and characterized in multiple tissues including the skin (in the bulge region of the hair follicle), intestine (in the epithelium), brain (in the hippocampus), bone marrow (on the endosteal surface and near blood vessels), and muscle (beneath the muscle fiber basal lamina). Although most mammalian niches remain somewhat ill defined because, in contrast to fruit flies, they are poorly accessible and therefore difficult to manipulate experimentally, niche components are being identified at a rapid pace. Evidence is accumulating that these factors play a critical role in regulating adult stem cell fate, as described below.

2.2. Common Structural and Compositional Hallmarks of Stem Cell Niches

The structural and biochemical features of stem cell niches that are conserved from fruit flies to mammals are depicted in Figure 1. Stem cells are in intimate physical contact with support cells which provide short-range signals via soluble factors as well as via membrane-bound proteins. Stem cells are also surrounded by an extracellular matrix (ECM), a protein- and sugar-rich crosslinked gel network that provides structure and organization as well as biochemical and mechanical signals (Fig. 1a). Blood vessels are often found near niches (or are believed to constitute niches themselves, e.g., in the central nervous system[15] or bone marrow[16–18]), presumably serving to transport long-range signals and as a conduit for recruitment of circulating cells into the niche. In addition, stem cells in niches can respond to inputs from the nervous system, as demonstrated in the HSC niche.[19] Finally, metabolic signals such as calcium ions or reactive oxygen species in the niche can influence stem cell function. For example, HSCs that are located near the endosteal surface of the bone are exposed to high ionic calcium concentrations. Not surprisingly, they express high levels of receptors that can sense ionic calcium in the niche (calcium-sensing receptors). In the absence of these receptors, the stem cells lose their ability to find their way back into the niche.[20] Thus, the changing local metabolic conditions in the niche may reflect the specific state of a tissue to which stem cells adapt.

Niche components can be categorized into three main types of molecular signals that are presented to stem cells in a precise spatial organization. These signal types and their functions are briefly discussed in the following paragraphs including (i) integral membrane proteins, (ii) localized secreted ECM components, and (iii) soluble proteins such as growth factors and cytokines.

2.2.1. Niche Signals Provided by Membrane-Associated Proteins

Stem cells are in direct contact with the integral membrane proteins of support cells within the niche. Support cells may anchor the stem cells to their niches via adhesion proteins on the cell surface (Fig. 1a and b). These adhesive interactions retain the stem cells within the niche and help to position the stem cells in close proximity to self-renewal signals emanating from the support cells. Cell–cell interactions of this kind are governed, for example, by the family of cadherin proteins that form ''adherens junctions.'' The extracellular domain of these transmembrane receptors on support cells can interact either with stem cell-derived cadherins of the same kind (i.e., homophilic binding) or with different cadherins (i.e., heterophilic binding).[21] Loss of cadherin function in stem cell niches may lead to stem cell loss,[22] underscoring the importance of adhesive cell–cell interactions in the niche. It is believed that cell–cell adhesion interactions via transmembrane proteins such as cadherins are involved not only in adhesion, but also in directing stem cell self-renewal, although the underlying mechanisms remain poorly understood.

Support cells within the niche also provide transmembrane cues unrelated to adhesion. A case in point is Notch signaling via Notch receptors and their corresponding ligands Jagged or Delta. These transmembrane proteins are highly conserved and are expressed by stem cells and their support cells in various tissues. Indeed, Notch signaling is not only essential in controlling cell function throughout embryonic development,[23] but also in adult tissues for stem cell self-renewal and differentiation.[24–27] For example, in the central nervous system, the absence of Notch signaling has been implicated in a reduction of neural stem and progenitor cell numbers, suggesting that this pathway has an important function in maintaining stem cells.[24]

2.2.2. Niche Signals Provided by Localized Extracellular Matrix (ECM) Components

Like most cells in our tissues, stem cells in the niche are in contact with the ECM (Fig. 1a and b). The ECM can be either in the form of a two-dimensional sheet-like basal lamina, as is the case for epithelial stem cells[28] (with a lumen on the other side) or muscle satellite cells[29,30] (enveloped by a membrane), or a highly hydrated three-dimensional fibrillar polymer network that fully encompasses the cells, as in the case of HSCs.[31] Stem cells interact with these ECM components via adhesion protein receptors, such as integrins. These integral membrane proteins regulate many stem cell functions. They define cell adhesion, shape, and motility. Notably, upon transplantation into the peripheral blood, HSCs find their way back into the niche, a process that is termed homing, by migrating via mechanisms that involve integrin receptor–ligand interactions.[32–34] Indeed, the HSC niche is rich in ECM proteins such as osteopontin[35,36] and fibronectin[37] as well as heparan sulfate proteoglycans[38,39] such as the glycosaminoglycan hyaluronic acid.[40] In addition to adhesive functions, integrins also control the cell cycle, and are involved in signal transduction leading to stem cell maintenance or differentiation. For example, beta1-integrin signaling in the niche is involved in the maintenance of epidermal stem cells[41] or neural stem/progenitors[42] in a stem cell state.

2.2.3. ''Soluble'' Niche Effectors

Soluble molecules play an important role in directing stem cell fate and have been a predominant target for modulating stem cell behavior in vitro due to the ease with which they can be studied. Of a long list of soluble candidate molecules, developmental morphogen proteins that include Wnts, hedgehog proteins,

fibroblast growth factors (FGFs) or bone morphogenetic proteins (BMPs) are of particular relevance. These signals can be found in many niches across different species, from the fruit fly to mammals. For example, several proteins that belong to this family of molecules have emerged as candidates for regulating HSC self-renewal, including the classical stem cell factor or c-kit ligand,[43] Wnt-3a,[44,45] Sonic hedgehog (Shh),[46,47] or FGF-1.[48] BMP-signaling appears to regulate HSCs in an indirect fashion, by controlling the size of the osteoblast support cell pool.[49] Notably, many soluble proteins are bound to the ECM via electrostatic interactions involving heparan sulfate proteoglycans such as heparin, localizing their response to the niche and protecting them from rapid proteolytic inactivation.[50] As outlined above, in addition to secreted proteins, it should be noted that small metabolic molecules can provide important regulatory cues in stem cell niches.[20]

2.3. The Niche as Key Regulatory Entity of Adult Stem Cells

The complex ensemble of localized signals described above constitutes a stem cell niche. It acts on the stem cell to physically tether it, keep it in a relatively quiescent (non-dividing) state, protect it from rapid differentiation, and regulate its self-renewal.[51] The importance of the niche as a stem cell regulatory network is exemplified by the fact that loss of contact with the niche, loss of key biochemical signals in the niche, or a disruption of the physical structure of the niche can result in loss of stem cell function. This is most evident when adult stem cells are removed from their microenvironment and cultured in vitro where they tend to quickly differentiate. This behavior suggests that the "default fate" of many adult stem cells in the absence of a niche is specialization. Further evidence that molecular niche components are required to maintain stem cell function is provided by studies showing that in some tissues, empty niches are repopulated when stem cells home back to their protective milieu. This is best exemplified by the recruitment of either circulating HSCs back to their bone marrow niches or muscle stem cells (MuSCs) back to their basal lamina niches following transplantation into host tissues irradiated to deplete endogenous stem cells. Once in the niche, the HSCs and MuSCs proliferate to reestablish homeostatic conditions.[29,52] Notably, upon complete loss of stem cells, the niche has been shown in fruit flies[53] and mice[54] to instruct progenitor cells with a reduced self-renewal capacity to revert to a stem cell state. This observation also strongly implicates niches as key regulatory microenvironments that establish and maintain stem cell function. Moreover, these findings suggest that multipotency and long-term self-renewal are not functions that are cell-intrinsic and solely characteristic of stem cells, but can also be induced in other cells such as progenitors by extrinsic cues.

2.4. Niche-Controlled Fate Changes of Single Stem Cells

In principle, an individual adult stem cell in its niche can undergo four different fates (excluding death) (Fig. 2): a stem cell (i) remains quiescent (Fig. 2a), (ii) undergoes self-renewal divisions

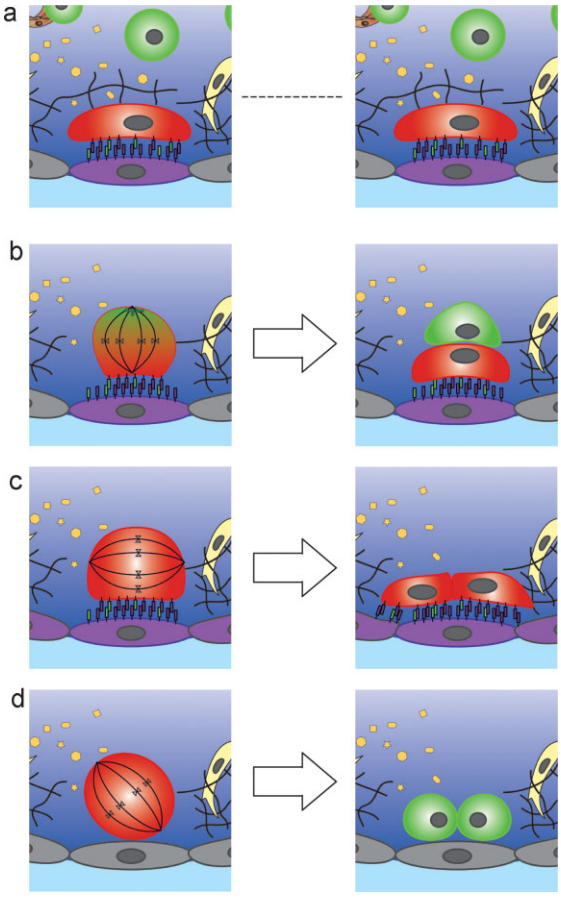

Figure 2. Stem cell fates in the niche. Stem cells are red, progenitor cells are green. Many types of adult stem cells are considered to be relatively quiescent in their niches (a) unless they are activated to proliferate in response to the demands of a tissue (e.g., stress or trauma). Quiescence is actively regulated by niche proteins such as cell–cell adhesion proteins. During homeostatic conditions, self-renewal divisions are asymmetric, generating one daughter cell retaining stem cell identity and another one being already partially differentiated (b). Asymmetric divisions can be controlled inside the cell via localization of cell fate determinants segregated to the cytoplasm of just one daughter cell. A hallmark of this type of division is the regulated orientation of the mitotic spindle that retains only one daughter in the niche. During development and/or stress the stem cell pool needs to expand via symmetric self-renewal divisions (c). Both daughter cells are exposed to the same niche environment and therefore keep their stem cell identity. A fourth possible fate is that of (symmetric) differentiation division, whereby both daughter cells lose stem cell function (d). This fate can be observed in many cases during in vitro culture of adult stem cells as well.

that result in one daughter stem cell and one differentiated cell (termed asymmetric divisions—asymmetric with regard to the identity of the two daughter cells; Fig. 2b), (iii) undergoes self-renewal divisions that result in two daughter stem cells (termed symmetric divisions; Fig. 2c), and (iv) undergoes divisions in the absence of self-renewal, resulting in two differentiated progeny, which could occur, for example, due to an absence of the niche or important niche cues (Fig. 2d). All of these fates must be actively regulated and coordinated by the niche in order to ensure an appropriate size of the stem cell pool

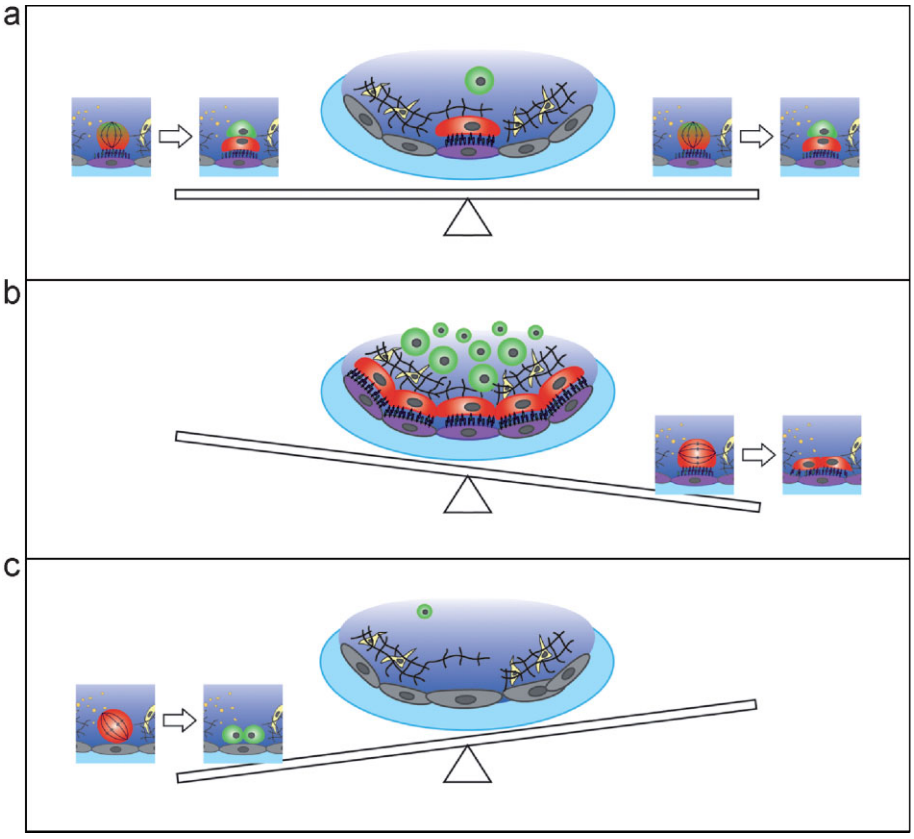

Figure 3. Control of the stem cell pool size. The ensemble of single cell fates (Fig. 2) determines the size of the stem cell pool in response to the demands of the tissue. Cell numbers in the niche need to be tightly controlled as an imbalance could have dramatic consequences for tissue function. Under homeostatic conditions, niche signals keep the number of stem cells in the niche constant (a). During stress or in pathological situations such as cancer, the demand is higher and symmetric self-renewal divisions dominate, allowing the stem cell pool to expand (b). Predominant symmetric differentiation divisions could lead to a depletion of the stem cell pool in the niche which would compromise regeneration (c).

during homeostasis and regeneration, and over the course of the lifetime of an organism (Fig. 3). An understanding of the regulatory mechanisms that direct these single-cell fate changes is not only of fundamental interest but also of paramount importance for therapeutic applications, as it could lead to the design of new methodologies (i) for culturing stem cells outside of their tissues or (ii) for stimulating and activating existing stem cells within tissues.

Studies of stem cell niches in fruit flies and worms have already helped to shed light on the mechanisms by which the delicate balance between self-renewal and differentiation is regulated, as described in detail elsewhere.[55–58] At the crux of these cell fate choices is the regulation of cell division. Under homeostatic conditions, stem cell numbers in the niche can be kept constant if the stem cells never divide (Fig. 2a)—notably, HSCs, for example, are considered to be relatively quiescent, and it has been estimated that they divide on average only ca. every 60 days[59]—or else via asymmetric divisions (Fig. 2b). Asymmetric stem cell divisions may be controlled by cell-intrinsic and/or cell-extrinsic mechanisms. In the first scenario, intracellular segregation of protein components such as the cell fate determinant protein Numb or cell polarity factors such as PAR proteins[56] may drive asymmetry. Conversely, the asymmetric positioning of daughter cells relative to external niche components may also induce asymmetric fates. In this case, both daughter cells may initially be equivalent, but their different microenvironments may impose two disparate identities. A general hallmark of asymmetric division is the orientation of the mitotic spindle (i.e., the part of the cytoskeleton that separates the chromosomes into the daughter cells during cell division). A perpendicular orientation with respect to the niche appears to determine whether one daughter cell remains in the niche and retains stem cell identity, while the other daughter cell moves away from the niche and starts to differentiate (Fig. 2b).[57] Adherens junctions at the interface between the niche and the stem cells have been implicated in controlling the orientation of the mitotic spindle.[60] Asymmetric segregation of cell fate-determinant proteins as well as perpendicular spindle orientation has been detected in mice, for example, in neural progenitors in the developing brain,[61] in satellite MuSCs[62,63] and in skin progenitors.[64] These findings suggest that asymmetric division may serve as an evolutionarily conserved mechanism to control, or limit, stem cell numbers.[58]

During development or when experiencing stress as in response to injury, adult stem cells in the niche must increase in numbers. This occurs via symmetric self-renewal divisions giving rise to two daughter stem cells (Fig. 2c). A symmetric division could, for example, be controlled by cell-extrinsic mechanisms, if both daughter cells encounter the same niche environment after division and remain in the niche as stem cells. In this case, divisions would be predicted to have a mitotic spindle orientation parallel to the niche, as seen in the epithelium of the skin.[64] Of note, the same stem cell populations appear to switch from symmetric divisions during embryonic development or regeneration in the adult to asymmetric cell divisions during late fetal development and homeostasis in later life, as observed, for example, in the developing brain.[65,66]

2.5. Balancing Cell Numbers in the Stem Cell Pool

The interplay between stem cells and their niches creates a dynamic and reciprocal system that leads to a balance in stem cell numbers in response to the physiological demands of the tissue (Fig. 3). Feedback loops of signaling interactions must play a key role. The size of the stem cell pool has been shown to correlate

with the size of the niche.[6] For example, when a change in numbers of support osteoblasts in the bone marrow niche was achieved,[49,67] accompanying changes in the HSC pool size were observed. Specific signals must keep the number of stem cells in the niche constant during homeostasis (Fig. 3a) and allow the stem cell pool to expand under physiological stress and in pathological situations such as cancer (Fig. 3b). Thus, one cause of cancer could be the loss of the tightly orchestrated regulation of cell numbers by overproduction of stem/progenitor cells via symmetric self-renewal divisions (Fig. 2c).

It is conceivable that multiple types of niches exist even for one particular stem cell type. For example, HSCs may transit from an endosteal niche (where they adhere to osteoblasts remaining quiescent) to a vascular niche (where they become activated due to interaction with endothelial cells on blood vessel walls). The nature of the two niches may serve to dictate the state of activity of the stem cell.[18,68–70] In addition, it is likely that niches are dynamic and can be formed or destroyed in response to physiological needs. In the absence of functional niches, symmetric differentiation divisions (Fig. 2d) could lead to a rapid depletion of the stem cell pool (Fig. 3c), therefore impairing further stem cell production and leading to loss of tissue function. This may explain why, to date, many adult stem cell populations cannot be grown in culture. Indeed, when plated on conventional cell culture substrates such as tissue culture plastic many types of adult stem cells undergo differentiation divisions due to the lack of instructive components from the niche essential to maintaining stem cell phenotype and function.

3. Engineering Concepts to Recapitulate or Manipulate Stem Cell Niches

3.1. Modeling the Niche In Vitro: Artificial Niches as Novel Platforms to Probe and Manipulate Stem Cell Fate

Progress in exploiting the unique self-renewal and differentiation potential of stem cells for applications in regenerative medicine has been impeded by our inability to control adult stem cell function in vitro. For example, a major limitation in the clinical use of HSCs is the shortage of donor cells available for transplantation to treat cancers of the blood. A promising route toward overcoming this hurdle involves the combination of stem cell biology with biomolecular materials engineering and microfabrication technologies in order to generate novel cell culture platforms that mimic crucial biochemical or structural aspects of the niche. These platforms can be particularly useful in the assessment of stem cell function at the single cell level and in high-throughput.[52,71] Several lines of research toward such artificial niches[72–75] that have emerged recently appear particularly promising and are briefly discussed in the following paragraphs (see also Table 1 for a summary).

3.1.1. Engineering Artificial Niches via Biomolecular Materials with Niche-Like Characteristics

Synthetic approaches that mimic specific physicochemical and biochemical characteristics of the niche using tunable biomolecular materials[76,77] have the potential to become a cornerstone in elucidating molecules and mechanisms that control adult stem cell fate outside the niche. Modular hydrogel networks[78] formed from synthetic building blocks are particularly well suited for this purpose. In contrast to the commonly used tissue culture plastic dish, hydrogel substrates mimic some of the physicochemical aspects of natural tissues, as they are soft and hydrated, typically comprising 95–99% of water. That the stiffness of the material on which stem cells are cultured alters their fate, or gene expression pattern, was clearly shown recently with mesenchymal stem,[79] lending credence to the notion that non-physiological rigid plastic surfaces likely favor the commitment of stem cells to undesired lineages. Furthermore, gels formed from hydrophilic polymer building blocks such as poly(ethylene glycol) (PEG) are resistant to protein adsorption and cell adhesion, but can be readily functionalized with desired bioactive signals.[80–82] Thus, biochemical complexity and functionality of hydrogel networks can be precisely controlled and constructed via a "bottom-up" approach starting from a relatively inert backbone material.[83,84]

Since many signaling cues in stem cell niches are presented in an immobilized fashion, approaches that allow the recapitulation of the natural presentation mode of niche proteins are receiving increasing attention. The well-controlled tethering of desired protein signals to a synthetic polymer is not an easy obstacle to overcome, as non-specific crosslinking may compromise the protein's bioactivity. Alberti et al.[85] have presented a solution to

Table 1. Summary of the key approaches used for recreating stem cell niches in vitro to manipulate stem cell fate.

Technology	Advantages or disadvantages						Selected examples [g]
	[a]	[b]	[c]	[d]	[e]	[f]	
Biomimetic/biomolecular stem cell substrates	√	—	—	—	√	—	[84,85]
Single stem cell microwell arrays	√	—	√	√	—	—	[52,94,95,97–99,101]
Niche protein microarrays	—	—	—	√	√	—	[103–107]
Microfluidic stem cell culture platforms	—	√	√	√	?	—	[116,117]
Micron-scale 2D niche protein patterning	√	—	√	√	—	—	[106,112]
Niche signal gradients (e.g., via μ-fluidics)	—	—	—	√	√	—	[115]
3D cell patterning (e.g., via inkjet printing)	—	√	?	√	?	?	[123]

[a] Low cost and robustness/simplicity. [b] Three-dimensionality. [c] Single cell resolution. [d] High-throughput. [e] Biochemical complexity, that is, exposure to more than one molecule. [f] Spatial control of niche signals at the scale of an individual stem cell or below. [g] The list is by no means exhaustive; we have picked representative examples for the different techniques.

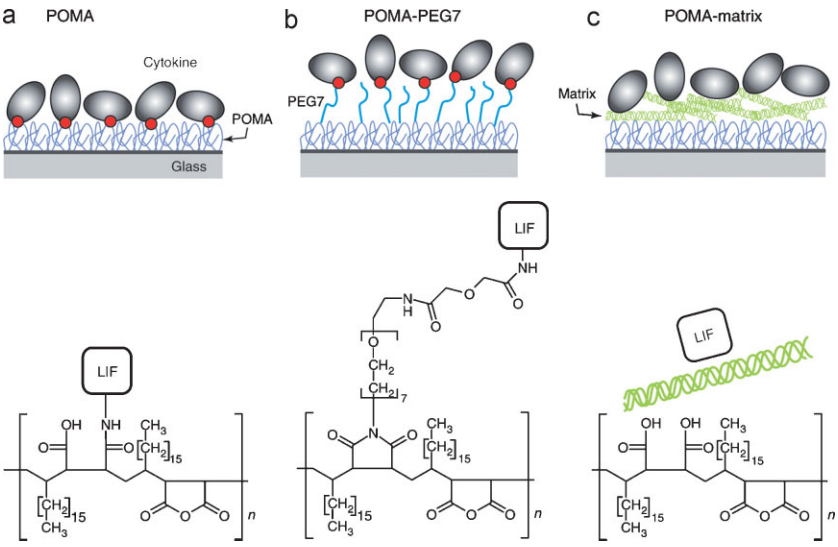

Figure 4. Tethering of LIF to maleic anhydride copolymer films as a means of recapitulating instructive microenvironments for embryonic stem cells. Different means of LIF immobilization can be realized via polymer layers (a–c), potentially affecting ligand accessibility and/or release for stem cells in physical contact. Red circles indicate covalent bonds. Chemical structures depict the polymer layer and the immobilization mode of LIF. Reproduced with permission from [85]. Copyright 2008 Nature Publishing Group.

this problem that entails tethering protein cues by immobilizing them to maleic anhydride copolymer thin-film coatings (Fig. 4). In one striking example, tethered leukemia inhibitory factor (LIF), an essential self-renewal protein signal for embryonic stem cells in feeder-free cultures, led to retention of pluripotency for at least 2 wk in the absence of soluble LIF in the culture medium (i.e., the standard culture condition). Biomaterial surfaces have also been engineered to mimic HSC niches, with the goal of inducing stem cell expansion.[86–89] For example, Suzuki and coworkers showed that the immobilization of a Fc-chimeric Delta1 fusion protein, in synergy with adsorbed fibronectin and soluble cytokines, led to a fivefold expansion of CD133+ cord blood cells, as scored by blood reconstitution in mice. Thus, the choice of a substrate that can be molecularly engineered and selectively functionalized with regulatory cues, and that recapitulates the physiochemical characteristics of niches may be important in controlling stem cell behavior outside of their natural microenvironment.

3.1.2. Technologies to Explore Stem Cell Biology at the Single Cell Level and in High-Throughput

Responses of cells to specific signals in culture are traditionally assessed using *cell ensembles*. As a result, the average behavior of the entire mixed population is monitored, potentially masking a key response by an individual cell, such as apoptosis, a change in cell cycle kinetics, or a division leading to self-renewal or differentiation. For adult stem cell cultures, this poses a significant problem, as these cells can only be isolated with limited purity, even when the most advanced phenotypic marker combinations and flow cytometry tools are utilized. As a result, analyses of the behavior of rare stem cells may be skewed by the behavior of more prevalent progenitor cells, since stem cells are often non-dividing or proliferate significantly slowly than progenitors.[52,90]

Unicellular systems employing standard multiwell plates (e.g., 96-well plate) are advantageous for studying stem cells, as they allow the cells to be analyzed and followed over time at the single cell level as clones. On the other hand, such large well formats are inefficient as they are low-throughput (only limited cell numbers can be analyzed) and require relatively large amounts of expensive culture medium components. Micron-scale technologies have been successfully combined with biomaterials science to generate arrays of microwells[91–98] that permit the analysis of a large number of individual, spatially confined cells (Fig. 5). These platforms have recently been utilized to investigate the differentiation of embryonic stem cells,[95–99] and to explore the fate of individual adult stem cells in high-throughput. For example, Dykstra et al.[100] tracked the dynamic behavior of single mouse HSCs on PDMS microwell

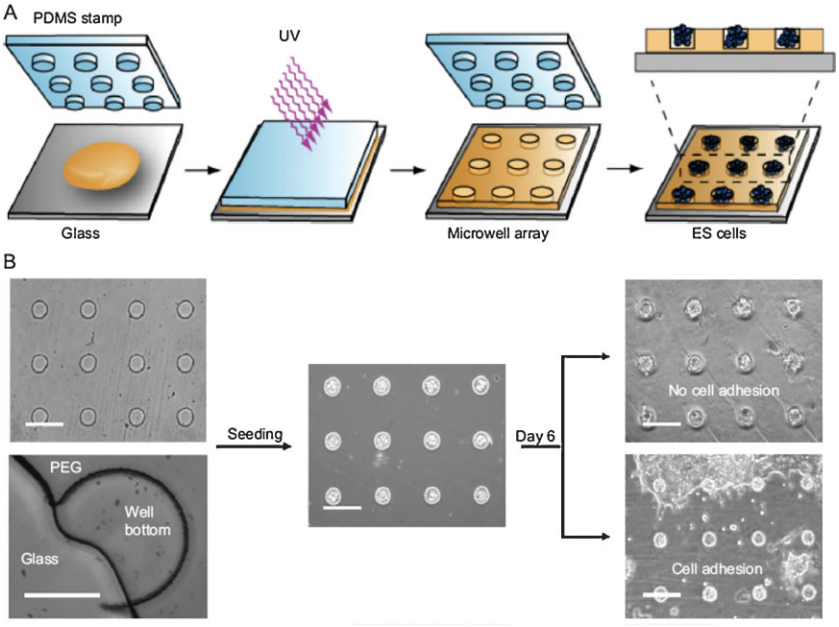

Figure 5. Fabrication of a microwell array platform for stem cell culture. Micromolding of photocrosslinkable PEG-diacrylate precursors to form microwell arrays on which stem cell (here: ES cells) can be cultured (a). A PDMS stamp with protruding features is used. Phase contrast images show a 50 μm microwell before and after seeding with ES cells (b). Cell constraining by the microwell results in homogeneous size distribution of cell spheres. A non-adhesive substrate is critical to restrict cell growth to the individual microwells. Reproduced with permission from [98]. Copyright 2008 Elsevier.

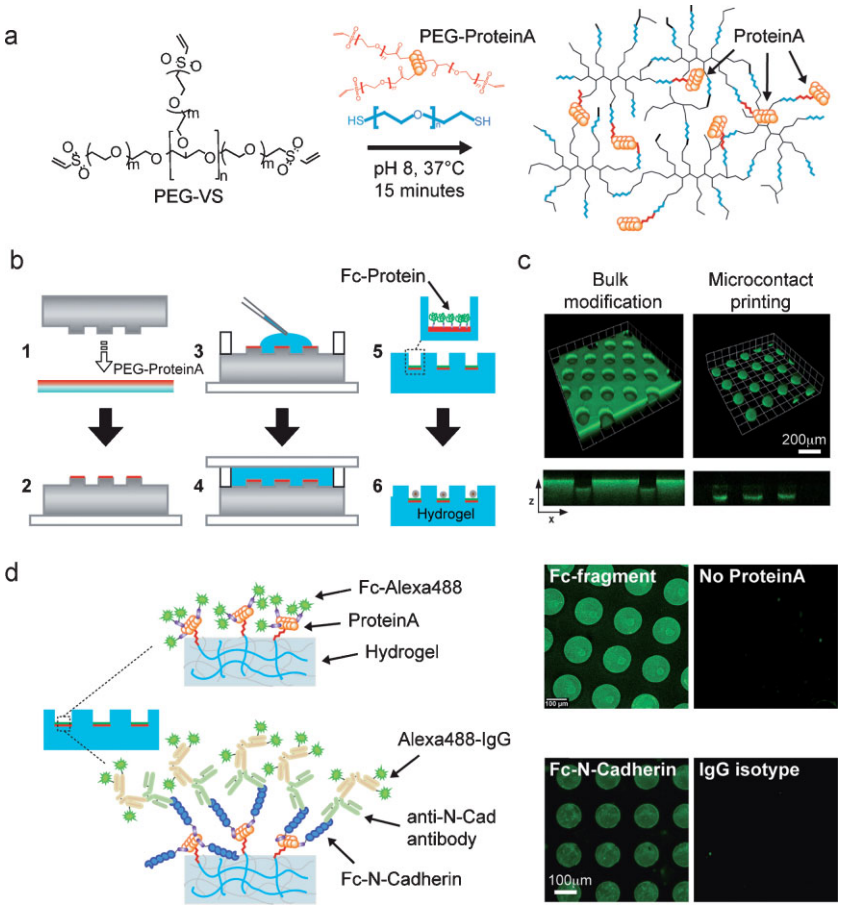

Figure 6. Microcontact printing to generate artificial niches via site-selective attachment of niche proteins. A heterofunctional PEG linker is used to covalently attach ProteinA to PEG hydrogel networks (a). Subsequently, ProteinA site-selectively anchors Fc-chimeric proteins on hydrogel microwell arrays. Overview of multistep process to locally immobilize Fc-chimeric proteins to the bottom of hydrogel microwells (b). Proof-of-principle experiments demonstrating the spatial control of protein (here: FITC-labeled BSA) immobilization afforded by the hydrogel microcontact printing process (c). Scale bar = 200 μm. Anchoring on the bottom of individual microwells (right panel) rather than on the entire surface of the microwell array (left panel) can be achieved. 3D confocal micrographs of projection of 84 stacks acquired at a constant slice thickness of 1.8 m. Immobilization of Fc-chimeric proteins via selective binding to ProteinA. Alexa-conjugated Fc-fragment and Fc–N-cadherin were tethered and detected via fluorescence microscopy according to the schemes (d, left and middle panels). Scale bar = 100 μm. As negative controls (right panels), microwell arrays are shown that are not tethered with ProteinA or treated with isotype control primary antibody. Reproduced with permission from [52]. Copyright 2008 Royal Society of Chemistry.

arrays and were able to correlate proliferative behavior in vitro with in vivo function, that is, the multilineage reconstitution of the blood. As a result of the single cell resolution achieved in this study, the investigators detected previously unrecognized characteristics of proliferating HSCs that correlated with self-renewal divisions. Cordey et al.[101] recently described a novel hydrogel microwell array platform which they used to study the derivation of multicellular spheres (''neurospheres'') from single neural stem cells (NSC). Compared to conventional neurosphere culture methods on plastic dishes, the viability of single NSC on soft hydrogels more than doubled. Effective confinement of single proliferating NSC to microwells led to neurosphere formation of vastly different sizes, a high percentage of which maintained stem cell phenotypes after 1 week in culture. These data suggest that analyses of single cells in arrays of microwells will have broad utility in the study of many rare adult stem cell or cancer stem cell populations.

To more closely mimic the biochemical and physicochemical complexity of natural stem cell niches, a further integration of materials technologies and protein patterning into such microwell platforms will be crucial. In an effort to recapitulate cell–cell interactions typical of HSC niches without the complexity of coculture, we have recently combined hydrogel microwell array fabrication with protein microcontact printing (Fig. 6).[52] Protein tethering was achieved by attaching a heterofunctional PEG linker to a protein of interest and then crosslinking this conjugate into the gel network as it is formed. To ensure site-selectivity in protein immobilization, we focused on genetically engineered Fc-chimeric proteins that could be linked via binding to an intermediate auxiliary protein, ProteinA, that contains four high-affinity binding sites ($K_a = 10^8$ per mole) for the Fc-region of human, mouse, and rabbit immunoglobulins (Fig. 6a). To specifically functionalize gels and immobilize proteins only at the bottom of microwells, rather than homogeneously distributing proteins across the entire array (bulk modification), the commonly used micromolding process (Fig. 6a) was augmented by adding a protein microcontact printing step (Fig. 6b): PEG-functionalized ProteinA was adsorbed onto the posts of the PDMS stamp (steps 1 and 2) and the hydrogel polymerized onto the ProteinA/PDMS (steps 3 and 4), transferring both the topographic pattern and protein pattern onto the gel surface (steps 5 and 6). Immunofluorescence microscopy revealed that microcontact printed proteins, such as a BSA-FITC model protein were localized at the bottom of the microwells (Fig. 6c). When ProteinA was used, Fc-chimeric proteins such as N-Cadherin (N-Cad) were also shown via immunostaining to be effectively tethered (Fig. 6d). We found that selective modification of microwells with adhesion proteins such as fibronectin or Fc-chimeric vascular cell adhesion molecule-1 (VCAM-1) ensured efficient confinement and tracking of stem cells over long culture periods, whereas the cells escaped from the microwells within a few hours when the bulk of the surface was modified.

The incorporation of microcontact printing in the hydrogel microwell array platform ensures versatility, as ProteinA can be incubated with *any* Fc-chimeric protein or protein mixture of interest, yielding microwells with the properly oriented, immobilized protein localized to the bottom of each well. Using the Fc-chimera-ProteinA platform, individual HSCs were exposed to selected proteins previously implicated by others to be components of the HSC niche that were displayed on locally

functionalized gel surfaces at the bottoms of microwells. By monitoring the proliferation of single HSCs by time-lapse microscopy, we demonstrated that single HSCs can undergo self-renewal divisions in vitro in response to selected proteins in these artificial niches. A reduction in proliferation kinetics or an increase in asynchronous division of single HSCs in microwells in response to the soluble Wnt-3a protein or tethered N-Cad protein correlated well with subsequent serial long-term blood reconstitution in mice. These results validate the hydrogel microwell platform as a broadly applicable paradigm for dissecting the regulatory role of specific signals within a complex stem cell niche.

Ochsner et al.[102] recently demonstrated that it is possible to form arrays of microwells with smaller dimensions, comparable to those of single cells, so that each cell is closely circumscribed by its own protein-functionalized microwell. If it were possible to pattern the microwell surfaces with different types of proteins (or protein mixtures) on these arrays, a better simulation of a stem cell niche with spatially well-defined polarization of protein cues may become possible.

3.1.3. Protein Microarrays to Dissect Niche Complexity

A major challenge in stem cell research is to unravel the complexity of molecular signaling that governs adult stem cell behavior. Conventional experimental paradigms in which one signal at a time is probed, enable a distinction of the roles of specific molecules. However, as an adjunct to this approach, a means of recapitulating the complexity of the niche and examining the effects of protein–protein interactions is also desirable. Several groups have begun to address this challenge by screening the effects of unique combinations of multiple putative microenvironmental signals on stem cell fate.[89,103–106] Mixtures of protein signaling cues such as ECM components, morphogens, and other signaling proteins were microarrayed on flat substrates using robotic spotting technology. Stem cells can be exposed to such multicomponent artificial niches, and their response quantified at the single cell level via multiparameter analysis involving, for example, immunocytochemistry of cell surface markers. These high-throughput analyses of signaling networks have begun to characterize the effects of combinations of stem cell regulatory proteins on self-renewal and differentiation. In one key study by Soen et al.,[104] primary human neural precursor cells were cultured on printed protein arrays to explore the extent and direction of differentiation into neurons and glia. Costimulation with two specific developmental morphogens (Wnt and Notch ligands) maintained stem cells in an undifferentiated-like state, suggesting that this combination of signals might occupy the native niche, in contrast to BMP-4 which led to the expression of differentiation markers. In another seminal study by LaBarge et al.,[106] protein microarrays were utilized to dissect the instructive function of the microenvironment on human mammary progenitor cell regulation. In combination with organotypic 3D culture models and micropatterned substrates, the investigators functionally identified previously unrecognized combinatorial microenvironments of cell-extrinsic mammary gland proteins (including the Notch ligand Jagged-1, P- and E-Cadherin) as well as ECM molecules (including Laminin-1) that led the cells to convert into different breast cell types. Bhatia and coworkers, who pioneered this approach, recently expanded the breadth of these platforms by compartmentalizing protein arrays using gaskets to produce a multiwell plate (Fig. 7). This advance allows both the interactions of ECM components and soluble growth factors on stem cell fate to be probed simultaneously.[107] A potential disadvantage of these approaches is the nature of the glass substrate used, which is rigid and not hydrated. The application of robotic spotting technologies to hydrogels could allow the effects of combinatorial signaling to be studied with stem cells grown under physicochemical conditions more closely resembling niches.

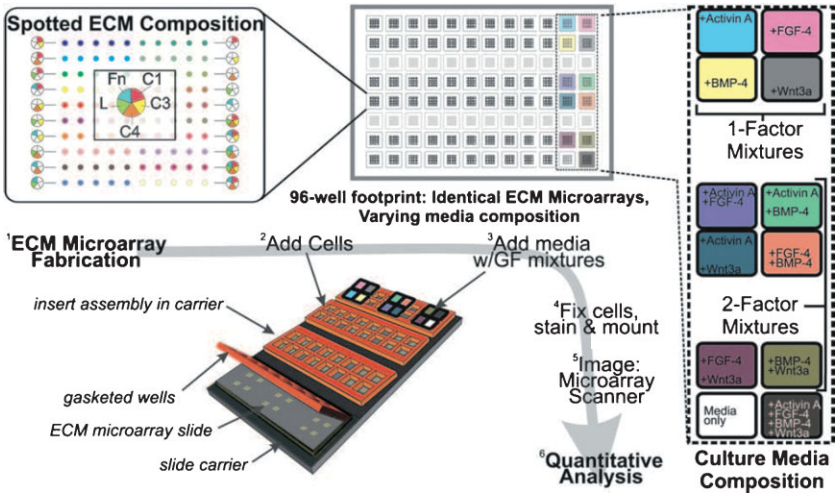

Figure 7. Multiwell ECM microarray with control over tethered and soluble signaling cues. Multiwell plate (top middle) in which each well accommodates a unique medium composition over an array of 100 ECM spots. Twenty spotted mixtures of ECM proteins including collagen I, human collagen III, mouse collagen IV, mouse laminin, and human fibronectin were spotted in five replicates. A multiwell format corresponding to a 96-well footprint was created by assembling gasketed well structures on the printed arrays (bottom middle). Each well can thus be exposed to medium containing a combination of soluble growth factors as well (right). Embryonic stem cells were seeded on the arrays, attached to the ECM protein domains and their fate was assessed after culture. Reproduced with permission from [107]. Copyright 2008 Mary Ann Liebert, Inc. Publishers.

3.1.4. Mimicking the Spatial Complexity of Niche Signals

In the organism, niche signals are presented in a complex but spatially discrete "polar" manner, such that each side of a stem cell may be exposed to a different microenvironment (Fig. 2). This signal polarization has been shown in fruit flies to lead to intracellular protein segregation with important consequences for the outcome of cell divisions, as we have discussed earlier. Novel protein spotting approaches can be envisioned that allow control of protein deposition at the subcellular scale, more closely mimicking the intricate native spatial organization of niche cues in two dimensions. Dip-pen lithography could, for example, be used to deposit desired protein cues in a very well-

controlled manner with the help of an atomic force microscope tip.[108] Stencil lithography, a versatile shadow-mask patterning technique, can be used to produce complex, (sub)micron scale cell adhesion patterns on a variety of backgrounds—including elastomeric substrates such as PDMS.[109] Alternatively, multiple cantilever-based patterning tools[110] or inkjet-printing approaches[111] could be used to transfer desired biomolecules from a reservoir to a desired substrate through micron-scale channels. Using microcontact printing, Bornens and coworkers[112] recently demonstrated the utility of such precisely controlled protein patterns for cell biology. Indeed, they were able to control the cell division axis by the geometry of the pattern. This approach could be particularly useful in studying the signals that induce asymmetric divisions of stem cells, a process which is characterized by a regulated orientation of the cell division axis (see Section 1, Fig. 2).

Signal gradients play an important role in regulating stem cell function, for example, triggering stem cell homing back to the niche via certain protein cytokine gradients. It is likely that the niche comprises gradients of soluble and ECM-tethered biomolecules, whereby fate decisions, such as the choice between self-renewal or differentiation, is controlled by the distance of the cell from the niche, just as in morphogenesis during development. Microfluidic technologies offer an elegant means of recapitulating spatial biological complexity, such as biomolecule gradients. Numerous examples of soluble and substrate-adsorbed protein gradients formed using microfluidic systems have been described in the last few years,[113] most of which have employed non-physiological glass or plastic surfaces. However, Burdick et al.[114] have demonstrated that adhesion ligand gradients can also be formed on soft hydrogels. Biomolecule gradients formed via microfluidics have already been applied to the study of adult stem cell regulation. For example, human NSC were cultured in a gradient-generating microfluidic device, and were exposed to concentration gradients of growth factor mixtures containing epidermal growth factor (EGF), fibroblast growth factor 2(FGF2) and platelet-derived growth factor-AB (PDGF-AB).[115] These experiments revealed that the extent of proliferation and differentiation was directly correlated with growth factor concentrations. Recently, Choi et al.[116] described an elegant microfluidics-based platform that exposed cells to gradients of soluble factors within 3D alginate gels. The application to adult stem cells of this technology, which allows control over the biochemical microenvironment in 3D, would constitute an important step toward the in vitro recapitulation of the stem cell niche. In this manner, it should be possible to elucidate the variables that either prevent stem cell differentiation and retain ''stemness'', or direct stem cell differentiation and the acquisition of specific specialized cell fates. The possibility of microarraying and constructing individually addressable cell culture chambers makes microfluidics systems very attractive for stem cell culture, as demonstrated by Lii et al. (Fig. 8).[117] We have no doubt that these systems will have a significant impact on stem cell biology in the coming years.

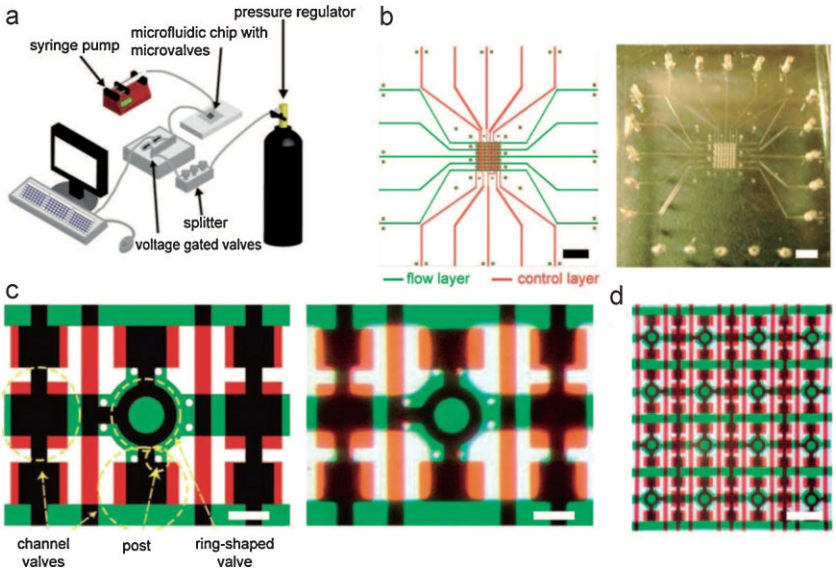

Figure 8. Design of a microfluidic setup for stem cell culture within controlled 3D microenvironments. Schematic diagram of the experimental setup (a). A two-layered PDMS chip is connected to a regulated gas tank via voltage-gated valves. For media perfusion through all microchambers, a syringe pump is connected to the chip. For injection of reagents or cell–matrix mixtures into specific microchambers, the path of the fluid flow is controlled through the switching of the voltage-gated valves. Design of the microfluidic chip as shown by an overlay of both the control and flow layers (left), and picture of the corresponding chip after assembly (right) (b). Scale bars = 2 mm. Design of a single chamber (left), and brightfield image of the device loaded with two different food coloring dyes (green in fluidic flow layer and red in control layer) (right) (c). The microvalves are located at enlarged crossings of a control channel with a flow channel when viewed from the top. Scale bar = 100 μm. Brightfield image of the 16 microchambers loaded with green dye in the flow layer and red dye in the control layer (d). Scale bar = 300 μm. Reproduced with permission from [117]. Copyright 2008 American Chemical Society.

3.1.5. Toward Rebuilding Complex Hierarchical, ''Tissue-Like'' Structures

The approaches described above to recreate stem cell niches have invariably focused either on two-dimensional or on pseudo-three-dimensional (such as in topographically structured surfaces) microenvironmental configurations. Conventional 3D approaches, such as stem cell encapsulation within porous scaffolds or hydrogel matrices, lack the necessary hierarchical spatial organization of complex stem cell microenvironments. As a consequence, we are still limited with regards to the possibilities of controlling stem cell behavior outside of the body to form larger, tissue-like morphogenetic ''structures.'' Pioneered by Mironov, Boland, Forgacs and coworkers, emerging ''bioprinting'' technologies are currently being scrutinized to recreate the 3D spatial organization of tissues or organs (e.g.,[118–120]). Although there is still a long way to go until microenvironments for stem cells that are conducive for tissue development or stem cell maintenance in 3D can be produced, inkjet printing of cells, biomaterials, or bioactive

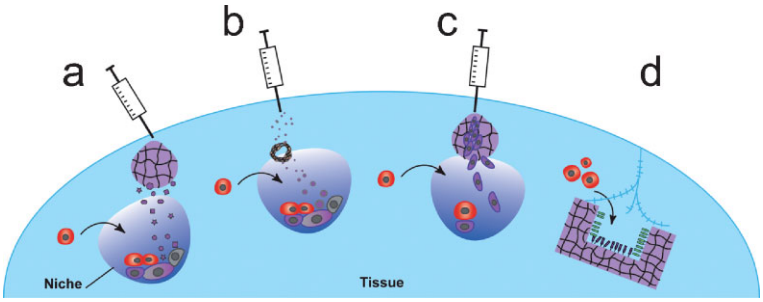

Figure 9. Therapeutic manipulation of the natural niche using biomaterials-based approaches. Local delivery of bioactive niche components or inhibitory/stimulatory molecules from a solid (injectable) biomaterial scaffold (a). Targeting the niche via micro- or nanoparticles that carry and delivery bioactive molecules to manipulate the niche (b). Local delivery of support cells to augment or manipulate stem cell fates in vivo (c). Cell delivery could be facilitated using (injectable) biomaterials carriers that likely improve the survival and engraftment of the transplanted cells. Implanted, multi-component artificial niche that could possibly attract stem cells to populate it (d).

molecules has already been successfully demonstrated. Campbell and coworkers[121] have presented an interesting 2D approach for creating artificial stem cell niches using inkjet printing technology. By creating spatially defined patterns of immobilized BMP2, they succeeded in instructing mesenchymal progenitor cells to differentiate into subpopulations of osteogenic (on BMP patterns) or myogenic (off pattern) cells simultaneously on the same fibrin hydrogel. If this approach could be expanded to the third dimension, it would represent a truly remarkable step forward in realizing the long-standing dream of tissue-engineers, that is, the formation of functional tissues outside the human body. Apart from inkjet printing, electropatterning of cells[122] within hydrogels, as demonstrated by Albrecht et al.,[123,124] could constitute an interesting alternative to reconstruct multicellular tissue organization. These and other 3D bioprinting approaches can be expected to produce not only valuable tools for fundamental biological research and high-throughput platforms for drug discovery, but could also translate into effective tissue engineering therapies in the not so distant future.

3.2. Outlook: In Vivo Biomaterials Strategies for Therapeutic Targeting of the Niche

The characteristic dynamic responsiveness of stem cells to extrinsic signals makes the niche a prime target for regenerative medicine and tissue engineering.[125] It has been suggested that instead of targeting the stem cells directly, the support cells in the niche could be manipulated or transplanted to indirectly alter endogenous stem cell numbers, in order to improve tissue function and overcome the adverse effects of disease or aging.[26,126] Moreover, if tumor-propagating cancer stem cells are dependent on signals from a niche,[127,128] it is conceivable that therapeutic ablation of components of a cancer stem cell niche could provide a promising path toward fighting cancer.

We propose that biomaterials-related approaches could play a key role in advancing our efforts to target the niche in order to augment endogenous stem cell function as a therapeutic strategy (Fig. 9). Several approaches are conceivable. First, biomaterials concepts could be devised to locally and specifically deliver bioactive niche components or stimulatory molecules (drugs) to a particular stem cell niche to increase stem cell numbers for example. This could be realized either from the "bulk" (solid) phase (Fig. 9a), by forming a drug/biomolecule-releasing scaffold in close proximity to a stem cell niche, or via targeted delivery of soluble micro- or nanoparticles (Fig. 9b).[129] Biofunctional polymer particles can now be engineered to render them efficient for such applications, as they can be functionalized to confer molecular targeting, environmental responsiveness, controlled drug release, or designed cell uptake as demonstrated by Hubbell and coworkers and others.[130–132] Secondly, biomaterials concepts could be harnessed to locally deliver cellular components of stem cell niches (Fig. 9c). "Smart" cell delivery systems which have been shown to improve the survival, engraftment, and fate of transplanted cells are now available, as has been reviewed elsewhere.[133] Finally and maybe most excitingly, it is conceivable that multicomponent, potentially injectable materials could be designed to act as de novo niches in vivo (Fig. 9d). If appropriate "homing" signals were present to attract endogenous stem cells to these artificial niches and localize the stem cells via known cell–cell or cell–matrix adhesive interactions, and signals were present which could control stem cell function, such efforts could have an important impact in the near future. An example of such an attempt was conducted by Gomi et al.,[134] who subcutaneously implanted macroporous polyester scaffolds pre-seeded with rat osteogenic cells into nude mice. This scaffold design led to the formation of an active hematopoietic marrow with stromal and hematopoietic compartments, the former of which appeared to have attracted and retained hematopoietic precursor cells, thus acting as a functional artificial niche.

4. Conclusions

Two decades ago it was widely believed that stem cells were regulated intrinsically, and were impervious to extrinsic signals. Potent approaches employing genetic engineering in developmental organisms and transgenic mouse models have shed light on the importance of the microenvironment, or niche, and on the extrinsic factors involved in stem cell regulation. In the foreseeable future, the synthesis of biomaterials science, microfabrication technology and stem cell biology will provide tools that will have the potential to revolutionize our understanding of how stem cell fate is controlled. By simplifying the niche and analyzing the dynamic responses of stem cells to well-defined artificial microenvironments, the role of specific niche components and niche architecture in regulating fundamental behaviors such as mechanisms of cell division, self-renewal, and differentiation can be elucidated. This may well lead to the generation of adequate numbers of stem cells and the ability to precisely control their differentiation in order to maximize their utility, not only as cell-based therapeutics for tissue regeneration and replacement, but also in the treatment of some cancers that arise due to defects in stem cell regulatory mechanisms.

5. Glossary

Adult stem cell: A stem cell that is derived from an adult tissue. This stem cell is restricted to form the specialized cells of the tissue that it is derived from. These cells are responsible for tissue regeneration throughout life.
Asymmetric division: Generation of two daughter cells with disparate function from a single stem cell division.
Cancer stem cell: Cell within a cancer with stem cell properties, i.e., self-renewing and able to differentiate. These cells are believe to have the potential to maintain a cancer.
Clonal analysis: Investigation of properties of single cells, often necessary to prove stem cell function (i.e., self-renewal and multipotency).
Coculture: The mixing of different (normally two) cell types in culture. Can be regarded as a simple, but effective means to recapitulate stem cell niche interactions in vivo.
Commitment: Engaging in differentiation, goes along with loss of self-renewal.
Embryonic stem cell: Pluripotent stem cell lines derived from early embryos before formation of the tissue germ layers.
Homing of stem cells: The inherent ability of stem cells to find their way back to their niche.
Long-term reconstitution: Renewal of a tissue by transplanted cells over the entire lifetime.
Pluripotent: Ability to form all types of differentiated cells of an organism, including germ cells. Embryonic stem cells are, for example, pluripotent.
Multipotent: Ability to form the types of differentiated cells of an entire tissue. Adult stem cells are considered multipotent.
Progenitor cell: Generic term for any dividing cell with the capacity to differentiate. In contrast to stem cells, progenitor cell are already restricted in their self-renewal ability.
Regenerative medicine: Reconstruction of diseased or injured tissue by activation of endogenous cells or by cell transplantation.
Self-renewal: Cell division leading to at least one daughter cell equivalent to the mother stem cell.
Homeostasis: Property of a tissue to maintain within a stable, constant state.

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