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Designing materials to direct stem-cell fate

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Abstract

Proper tissue function and regeneration rely on robust spatial and temporal control of biophysical and biochemical microenvironmental cues through mechanisms that remain poorly understood. Biomaterials are rapidly being developed to display and deliver stem-cell-regulatory signals in a precise and near-physiological fashion, and serve as powerful artificial microenvironments in which to study and instruct stem-cell fate both in culture and *in vivo*. Further synergism of cell biological and biomaterials technologies promises to have a profound impact on stem-cell biology and provide insights that will advance stem-cell-based clinical approaches to tissue regeneration.

Stem cells are defined by their ability to self-renew and produce specialized progeny[1,2]. Consequently, they are the most versatile and promising cell source for the regeneration of aged, injured and diseased tissues. Embryonic stem cells, induced pluripotent stem cells and adult stem cells are obtained from three different sources and have different advantages (Fig. 1). However, despite the remarkable potential clinical applications of each of these stem-cell populations, their use is currently hindered by hurdles that must be cleared[3] (Table 1). These obstacles may appear daunting, but nature has strategies to surmount them *in vivo*. Thus, a major goal is to develop new culture-based approaches, using advanced biomaterials, that more closely mimic what the body already does so well and promote differentiation of pluripotent cells or propagation of specialized adult stem cells without loss of 'stemness'.

An increasing emphasis on design principles drawn from basic mechanisms of cell–matrix interactions and cell signalling has now set the stage for the successful application of biomaterials to stem-cell biology. This application has the potential to revolutionize our understanding of extrinsic regulators of cell fate, as matrices can be made using technologies that are sufficiently versatile to allow recapitulation of features of stem-cell microenvironments, or niches, down almost to the molecular detail[4].

In the body, adult stem cells reside within instructive, tissue-specific niches that physically localize them and maintain their stem-cell fate[5,6] (Fig. 2). The key function of stem-cell niches is to maintain a constant number of slowly dividing stem cells during homeostasis by balancing the proportions of quiescent and activated cells. On insult (that is, injury, disease or damage), stem cells exit the niche and then proliferate extensively, self-renew and differentiate to regenerate the tissue. Within the niche, stem cells are thought to be exposed

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to complex, spatially and temporally controlled biochemical mixtures of soluble chemokines, cytokines and growth factors, as well as insoluble transmembrane receptor ligands and extracellular matrix (ECM) molecules (Fig. 2). In addition to understanding this biochemical signalling regulatory network, it is key to appreciate the biophysical properties of the niche, including matrix mechanical properties and architecture (topographical cues), to elucidate the role of niche elements[7,8].

To shed light on the mechanisms that regulate stem cells, approaches that allow the study of stem-cell function in response to isolated components of a complex system — that is, models that simplify it — are crucial. Biomaterials approaches, in combination with other technologies such as microfabrication and microfluidics, are well suited to assist studies of stem-cell biology through the creation of evolving systems that allow key variables to be systematically altered and their influence on stem-cell fate analysed. Thus, biomaterials technologies provide the exciting possibility of deconstructing and then reconstructing niches, allowing quantitative analysis of stem-cell behaviour in a manner not previously possible.

In this Review, we use specific examples to outline the various means by which biomaterials technologies have been, and could be, used to construct versatile model systems for stem-cell biology, as well as to develop carriers for stem cells and biomolecules, facilitating the *in vivo* use of stem cells in tissue engineering. We focus on hydrogels as one emerging and physiologically relevant class of biomaterial, although we acknowledge that other biomaterials have been, and will be, used in these applications. For a more comprehensive understanding of the diverse types and applications of biomaterials in stem-cell biology and bioengineering, we refer readers to several recent reviews[9–15]. We anticipate that insight will be gained from studies using biomaterials that allow the enhanced differentiation of embryonic stem cells and induced pluripotent stem cells into tissue-specific differentiated states and the propagation of adult stem cells without losing their stem-cell properties.

Designing 2D materials to control stem-cell fate *in vitro*

In vitro stem-cell research is carried out with cells cultured on flat substrates coated, for example, with collagen or laminin, on feeder-cell layers (that is, in co-culture experiments) or on or within hydrogels made from naturally derived ECM components (for example collagen or Matrigel). By far the most frequently used material for the culture of stem cells is rigid polystyrene tissue-culture plastic. Cells in plastic dishes are typically exposed to soluble factors in liquid media. These culture conditions are very different from the conditions experienced by cells in the body, where they are associated with anchored molecules presented in close proximity to surrounding cell surfaces and contained within an ECM that creates a relatively soft microenvironment. The constraints imposed on stem cells within the three-dimensional (3D) niche have effects that are still being explored and should not be ignored. With this goal in mind, two-dimensional (2D) biomaterial culture systems are highly advantageous as a simplified approach to deconstructing the niche and identifying and assessing the effects of individual niche components on stem-cell fate (Fig. 3).

Probing biochemical stem-cell–ECM interactions in two dimensions

The identification of ECM molecules with biological relevance to stem-cell regulation is a critical step towards defining the regulatory influences of the stem-cell niche. Biomaterials approaches have been explored to define the identity, concentration and patterns of soluble or tethered ECM molecules singly (Fig. 3a) and in combination (Fig. 3b). Several groups have made ECM arrays by means of robotic spotting to screen for a molecule or combinations of molecules that induce fate changes[16–19]. For example, arrays consisting of 192 unique combinations of ECM and signalling molecules have been printed onto slides

containing a thin coating of polydimethylsiloxane; and placental cadherin, epithelial cadherin, laminin and JAG1 (a ligand for the receptor NOTCH1) were each found to promote the conversion of mammary progenitor cells to myoepithelial or luminal epithelial fates[19].

Notably, not only is the rigidity of the tissue determined by the structure of the ECM (whether loosely or densely packed), but differences in density also result in different local concentrations of exposed ECM components, which in turn lead to differences in cell signalling and adhesion. In addition, the architecture of the ECM provides geometric cues to cells in the form of fibre diameter, length and crosslinking patterns, as well as surface irregularities (‘nanotopography’). Two-dimensional approaches should greatly improve our understanding of the relevance of these key ECM properties to stem-cell biology[8].

Probing cell–cell interactions in two dimensions

The effects of cell–cell interactions are usually studied by culturing two cell types together; however, using such co-culture strategies makes it difficult to discern the role of particular molecules, be they soluble or tethered. In tissues, secreted growth factors and cytokines are mostly tethered to ECM components such as proteoglycans, whereas receptor ligands are presented to stem cells at the surface of nearby support cells. In both cases, molecule immobilization is proposed to have the critical role of increasing protein stability, promoting persistent signalling and inducing receptor clustering[20]. For example, covalent attachment of fibroblast growth factor 2 (FGF2) to a synthetic polymer stabilized the growth factor and increased its potency 100-fold relative to FGF2 in solution. In response to the tethered FGF2, embryonic stem cells exhibited increased proliferation and activation of ERK1 (also known as MAPK3), ERK2 (MAPK1), JNK (MAPK8) and c-Fos signalling[21]. Similarly, when epidermal growth factor (EGF) was covalently tethered to a biomaterial scaffold, it was more effective than its soluble counterpart in inducing the spread of mesenchymal stem cells and preventing Fas-ligand-induced death[22]. Finally, immobilized leukaemia inhibitory factor (LIF), but not soluble LIF, led to prolonged activation of LIF targets and maintenance of embryonic stem cells in an undifferentiated state with the capacity to generate chimaeric mice even after culture for more than 2 weeks[23].

The function of receptor ligands associated with cell membranes also is contingent on the mode of presentation. When tethered, DLL1 (a ligand for the receptor NOTCH1) resulted in an increase, relative to soluble DLL1, in the number of human cord-blood $CD133^+$ ($PROM1^+$) cells capable of reconstituting the circulation in irradiated mice[24]. Similarly, when tethered, rat JAG1 enhanced NOTCH1 signalling and increased the differentiation of rat oesophageal stem cells[25].

These examples demonstrate the importance of ligand presentation in stem-cell fate and function. Testing single candidate molecules is instructive, but to discover novel ligands and cytokines that have effects on stem cells, an unbiased high-throughput biomaterials approach is desirable. Such an approach, using spots of 576 different combinations of 25 acrylate-based polymers in arrays on the nanolitre-scale, found combinations that influenced embryonic stem-cell attachment, proliferation and differentiation[26]. Ideally, high-throughput approaches could be devised to incorporate the many other biophysical and biochemical parameters described above.

Probing biophysical stem-cell–matrix interactions in two dimensions

Ageing, injury and disease are often associated with increased deposition and altered organization of ECM components such as collagen, resulting in significant changes to the stiffness of the matrix, which most likely potentiate pathogenesis, for example in the case of

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Duchenne muscular dystrophy[27–29]. Natural and synthetic matrices can be produced to create cell-culture substrates with known elastic modulus (or stiffness) (Fig. 3c) and, unlike plastic substrates, they also provide diffusion of soluble molecules to the basal surface, as well as the apical surface, and can be used to test the relevance of homeostatic and disease-related matrix stiffness to stem-cell behaviour. Notably, soluble factors in culture media always act in conjunction with the tissue-culture matrix, and together they affect cell fate.

In a landmark study, human mesenchymal stem cells assumed morphological patterns and gene expression patterns consistent with differentiation into distinct tissue-specific cell types when exposed to polyacrylamide gels with a range of stiffnesses typical of brain, muscle and bone[30]. This study highlighted the potent influence of matrix mechanical properties on stem-cell fate and led to the exploration of further links between stem-cell behaviour and matrix elasticity. Since then, substrate stiffness has been shown to modulate the proliferation and differentiation of embryonic stem cells and certain types of adult stem cell. Specifically, adult neural stem cells cultured on a relatively stiff synthetic matrix gave rise primarily to glial cells, whereas on a softer matrix that more closely resembled the compliancy of *in vivo* brain tissue, neurons were the predominant cell type[31]. Furthermore, the rate of adult skeletal-muscle stem-cell proliferation increased as substrate stiffness increased[32].

A major challenge in studies of this type is separating the effects of matrix stiffness from those of ligand density. To eliminate this variable, ‘tunable’ gel systems in which matrix stiffness and ligand density can be independently controlled are especially advantageous. Using one such tunable, synthetic cell-culture system, human embryonic stem cells have been propagated and maintained in an undifferentiated state in the absence of a feeder layer[33]. We predict that once the profound effects of the physical properties of culture substrate on stem-cell fate are fully appreciated, culture platforms based on soft biomaterials are likely to largely replace those made of the standard, rigid, tissue-culture plastic.

Within the niche, cell shape is defined, in part, by the constraints imposed by the surrounding ECM on cells during development and in adulthood[34,35]. Although some of these effects are probably due to alterations in the adhesive interactions and crosstalk between the ECM and the cell as they work to define each other, there is ample evidence suggesting that physical control of cell shape alone can act as a potent regulator of cell signalling and fate determination[36] (Fig. 3d). One remarkable demonstration of the influence of cell shape on cell function used micro patterned ECM islands allowing precise and reproducible control of the size of the cell attachment area[37]. Single mesenchymal stem cells cultured on small islands adhered poorly, had a rounded morphology and acquired an adipogenic fate, whereas on larger islands they were adherent, spread out, exhibited increased focal adhesions and cytoskeletal reorganization, and acquired an osteogenic fate[38]. Furthermore, human embryonic stem cells cultured on spatially restricted islands yielded dense $OCT4^{+}$ ($POU5F1^{+}$) pluripotent colonies, whereas on large islands embryonic stem cells differentiated[39]. Such studies are just beginning to shed light on the profound impact that matrix architecture, during development and pathogenesis, has on cell-shape-induced changes to cytoskeletal organization and signalling, and subsequent stem-cell specification and function.

High-throughput single-cell analyses in 2D microenvironments

Traditional *in vitro* experiments are conducted on cell ensembles. In these studies, measurements entail averaging responses across an entire population. Consequently, behaviours such as apoptosis, changes in cell-cycle kinetics, changes in self-renewal, and differentiation may be missed. For stem-cell analyses, this poses a significant problem, as many stem-cell populations are heterogeneous. As a result, rare stem cells in a heterogeneous mixture may be missed, or analyses may be skewed by the behaviour of

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rapidly growing progenitor cells, because in many cases stem cells are non-dividing or grow significantly slower than do progenitors. Conventional cell-culture platforms are not readily applicable to the investigation of stem cells at the single-cell level. For example, standard multiwell plates such as 96-well plates would require large amounts of expensive culture-media components and do not offer sufficient throughput. This problem has been solved by the introduction of microwell array cultures for cell biology (Fig. 4). These modular platforms permit the analysis of a large number of single, spatially confined cells. They have recently been successfully applied in stem-cell biology, using both embryonic stem cells and adult stem cells (see, for example, refs [40–48]).

Polymer-hydrogel networks such as those formed from polyethylene glycol (PEG) are useful in the production of microwell substrates, as they allow simultaneous and independent assessment of the effects of biophysical and biochemical properties on stem-cell fate at the clonal level. Currently available hydrogel-crosslinking chemistries and macromolecule architectures can generate a wide range of hydrogels with distinct and reproducible mechanical properties[49]. PEG is almost inert to protein adsorption, and proteins can be tethered to hydrogels by attaching a chemical moiety to proteins of interest and subsequently crosslinking it into the hydrogel network in a technique called microcontact printing[50] (Fig. 4a). By using a standard microfabrication technique with polydimethylsiloxane as a replica, it is possible to structure hydrogel arrays topographically to contain thousands of spatially segregated micropatterns, for example round microwells with proteins printed specifically at the bottom of each well[48].

Using a hydrogel-culture approach in conjunction with time-lapse microscopy, the behaviour of haematopoietic stem cells in response to a panel of soluble and tethered molecules was assessed. Division patterns consistent with depletion (fast symmetrical division), asymmetrical self-renewal (asymmetrical cell division) and symmetrical self-renewal (symmetrical division) were observed and subsequently assayed *in vivo* for their ability to reconstitute the blood over a longer timescale. Remarkably, this study showed that exposure to single factors, such as WNT3A and neural cadherin, could induce self-renewal of haematopoietic stem cells *in vitro*[48]. Additionally, it provided strong support for the idea that *in vitro* stem-cell behaviour can be highly predictive of *in vivo* potential[46]. A similar approach can now be applied to any number of stem-cell types to identify previously unknown physical and chemical regulators and the relevant presentation of those molecules to elicit effects on stem-cell self-renewal and differentiation. The production of novel microwell arrays in which substrate stiffness, protein doses (such as in gradients) (Fig. 4b), and protein combinations (Fig. 4c) and their spatial arrangement (Fig. 4d) can all be controlled will be essential for the success of these studies.

In conclusion, deconstructing a complex 3D niche into 2D biomaterial model systems is a powerful and promising strategy for discovering new regulatory mechanisms governing stem-cell biology. The structural, biophysical and biochemical parameters of these model systems can be varied in myriad ways to identify and elucidate the effects of the components of putative stem-cell niches on stem-cell function. Given the precise control of nanometre-scale chemical and topographical features, as well as the possibility of computationally predicting fluid dynamics and transport conditions during cell culture, and the simplicity of collecting cells after culture, 2D platforms are poised to generate fresh insight into the biochemical and biophysical regulation of stem cells[51,52].

Designing 3D materials to control stem-cell fate *in vitro*

Whereas 2D approaches allow well-controlled analysis of the impact on stem cells of individual components of the niche, 3D approaches should allow reconstruction, and

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realization of the complexity, of the natural tissue (Fig. 5). In epithelial tissues (for example skin and gut), stem cells adhere to 2D, sheet-like basement membranes, but most stem-cell niches (for example those in bone marrow, brain and muscle) are 3D microenvironments composed of hydrated, crosslinked networks of ECM proteins and sugars. In three dimensions, stem cells can be exposed to a solid microenvironment that fully ensheathes them (Fig. 5a), in contrast to 2D platforms, in which cells are typically exposed to a solid, flat surface on the basal side and to liquid at the apical surface. However, although conceptually appealing, the construction of 3D artificial microenvironments is not simple[53]. There are chemical challenges in the production process, considerations of appropriate elasticity, and the need to overcome the physical constraints that impede migration or morphogenesis. First and foremost, in most cases, cell viability remains a problem; second, understanding the read-outs from such complex multicomponent systems is not straightforward. As a result, high-throughput analyses are currently not possible, and few of the many possible variables can be systematically explored. Nonetheless, progress is being made.

Several impediments to 3D culture must be overcome. First, to expose stem cells to an accurate 3D artificial environment, chemical approaches that allow the embedding of stem cells must be used. This is ideally performed *in situ* (that is, during the formation of the 3D material), which requires a mild and highly specific crosslinking chemistry so as not to compromise cell viability as a result of adverse side reactions. Several methods of forming synthetic or semi-synthetic hydrogel matrices under physiological conditions have been developed for this purpose and are reviewed in, for example, refs [54] and [55]. Some of these approaches explore not only highly specific chemical or enzymatic reactions but also physical mechanisms of crosslinking, such as the molecular self-assembly of small-molecule building blocks (including peptides, peptide amphiphiles and oligonucleotides). Each of these approaches has been demonstrated to yield viable encapsulated cells after crosslinking; the strategies differ primarily in the hydrogel-network structures that are produced and in how cells respond to these different network structures (of which some are porous and others are dense meshworks).

Second, the biophysical characteristics of the 3D environment are important. Cells embedded in a 3D environment can suffer from a lack of gases and nutrients. This problem is overcome by using scaffolds made of solids such as polymers with interconnected porosity and by using hydrogel networks with microscopic meshes, as such structures readily allow the diffusion of macromolecules. Third, substrate elasticity and materials with mechanical properties closely approximating those of natural stem-cell niches are desirable[28], as described above. Last, physical constraints that impede cell proliferation, migration and morphogenesis should be avoided. To avoid the potential problems of having physical barriers in three dimensions, materials that have matrix porosity on the scale of cellular processes can be designed. For example, nanofibrillar hydrogels that contain microscopic pores large enough to facilitate cell growth have been developed[56]. An attractive alternative approach uses polymer gels that can be synthesized to contain chemically crosslinked substrates for proteases naturally secreted by cells, for example during cell invasion. This feature allows a dynamic interplay between the cells and their microenvironment such that the cells locally degrade and then 'remodel' the matrix. For example, PEG-based hydrogels have been rendered chemically degradable through hydrolytic breakdown of ester bonds[57] and have been developed with cleavage sites for cell-secreted matrix metalloproteinases or plasmin[4]. This cell-regulatable breakdown of the matrix allows cell migration and proliferation in a manner determined by the cells.

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Probing stem-cell–matrix interactions in three dimensions

A long-standing question in stem-cell biology and tissue engineering is that of how the numerous components of the stem-cell niche govern stem-cell fate in three dimensions. This question is difficult to address *in vivo* or using any existing 2D *in vitro* approaches. A 3D stem-cell niche is extremely complex (Fig. 2), and the number of its physical, chemical and mechanical effectors is too great to define in practice. Even if the specific nature of its components were known, testing them systematically would not be possible. Thus, developing new approaches aimed at high-throughput screening of combinations of 3D microenvironmental variables, in a manner analogous to 2D ECM protein microarrays or other cellular arrays described above, is a major goal[58–60].

The production of high-throughput microarrays of 3D matrices could be possible using robotic liquid-dispensing and printing approaches in combination with biomaterial-crosslinking chemistries. Combinatorial mixtures of liquid precursors of hydrogel networks can be deposited in minute volumes and at high density onto solid substrates[61]. In one of the first examples of this emerging strategy[62], 3D PEG-hydrogel arrays were produced to screen for the individual and combinatorial effects of gel degradability, cell-adhesion-ligand type and cell-adhesion-ligand density on the viability of human mesenchymal stem cells. Increased PEG-network degradability and greater cell-adhesion-ligand density were both found to increase the viability of the stem cells in a dose-dependent manner.

Measures of cell viability constitute a minimal first step. It is necessary to design more-sophisticated ways of measuring stem-cell proliferation, asymmetrical and symmetrical division, self-renewal and differentiation into selected lineages that can be assessed in three dimensions. One challenge in this endeavour will be to analyse cellular responses in three dimensions, for which one focal plane for microscopic read-out is not sufficient. Ultimately, it would be desirable to investigate the role of the 3D microenvironment in controlling stem-cell fate on a more comprehensive (‘systems’) level, integrating the complete set of relevant variables. Importantly, when promising candidate microenvironments are identified through such studies, selected materials need to be further evaluated using *in vivo* approaches, for example by transplantation of cell–matrix constructs into mice.

Probing cell–cell interactions in three dimensions

Important components of stem-cell niches are the cells that abut stem cells, which are sometimes referred to as support cells or niche cells. These can include vascular cells, neural cells, and stromal cells such as fibroblasts. They not only provide instructive secreted signalling cues but also send signals through transmembrane proteins or bound matrix proteins. Although this type of cellular crosstalk is conceptually appreciated as being highly significant to stem-cell behaviour (to quiescence, activation and proliferation), the study in three dimensions of which factors have a critical role and how they act together is a nascent field.

Nonetheless, progress is being made in technologies that would allow the investigation of such cell–cell signalling interactions in near-physiological 3D microenvironments (Fig. 5b). One approach is based on the electropatterning of mammalian cells within hydrogels[50]. Electropatterning localizes live cells (possibly of any type) within hydrogels, such as photopolymerized PEG gels, by using dielectrophoretic forces. Large numbers of multicellular clusters of precise size and shape have been formed in three dimensions on one focal plane. By modulating cell–cell interactions in 3D clusters of various sizes, this microscale tissue organization was, for example, shown to influence the biosynthesis of bovine articular chondrocytes, with larger clusters producing smaller amounts of sulphated glycosaminoglycan per cell.

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Other work has combined gel patterning with microfluidic technology to analyse angiogenesis in 3D co-cultures[63]. Primary liver and vascular endothelial cells were cultured on each side wall of a collagen gel between two microfluidic channels. Morphogenesis of 3D hepatic cultures was found to depend on fluid flow across the nascent tissues. Vascular cells formed 3D capillary-like structures that extended across an intervening gel to the hepatocytes' tissue-like structures. This is a remarkable advance, as microvascular networks are considered to be important components of several stem-cell niches[6]. Thus, these approaches could prove useful in addressing fundamental questions in stem-cell biology.

3D biomolecule gradients in stem-cell biology

Morphogen gradients have long been known to regulate cell fate and tissue or organ development[64]. Biomolecule gradients are crucial regulatory components of dynamic tissue processes, not only during development but also during homeostasis and regeneration. Therefore, the creation of biomolecule gradients in 3D biomaterials systems has received increasing attention in stem-cell bioengineering (Fig. 5c). Such gradients could be shallow, such that a given cell experiences one concentration along its whole length, or steep, such that the cell experiences a different concentration at each end. Cells may migrate away from or towards a particular biomolecule concentration. Alternatively, when gradients are steep, cell polarity and asymmetry may be induced, just as in a stem-cell niche.

Arguably the most precise and robust way of generating a biomolecule gradient is through microfluidic technology[65], because microfluidics allows the well-controlled manipulation of very small amounts of fluid. Microfluidic gradient platforms have already been applied to stem-cell biology, albeit in two dimensions (see, for example, ref. [66]). However, 3D gradient systems are rapidly being developed[67,68]. One example is a microfluidics-based approach whereby cells within alginate gels could be exposed to desirable soluble gradients in 3D microenvironments[67]. Applied to adult stem-cell culture, such intricate control over the biochemical microenvironment in three dimensions is an important step towards the *in vitro* recapitulation of stem-cell microenvironments that are more complex. The advantages of combining biomaterials engineering with microfluidics for stem-cell applications are clear[69]: this combination offers the potential for arrays of individually addressable cell-culture chambers[70,71] in which artificial microenvironments are exposed to spatially and temporally controlled biomolecule gradients (temporal control allowing delivery at any time during an experiment). Because proteins can be tethered to gel networks, it should be possible to combine tethering and soluble gradients of protein morphogens to mimic the exposure of cells to both ECM-bound protein gradients and soluble gradients, to recreate a stem-cell niche in three dimensions more accurately.

Mimicking the spatial 3D niche heterogeneity

Stem cells sense and respond to the spatial heterogeneity of 3D microenvironments. Many *in vivo* stem-cell microenvironments are 'polarized' structures, in that they expose individual stem cells to differential niche components. An example is the niche of the satellite cell (the canonical muscle stem cell), which is located between the muscle-fibre membrane and the surrounding basement membrane (Fig. 2). An ideal 3D *in vitro* model of a stem-cell niche would allow recapitulation of this type of complex architecture and manipulation at a desired time during an experiment, for instance to address the question of whether microenvironmental polarity dictates when a cell is quiescent and when it is activated.

Application of hydrogel engineering using photochemistry suggests that the construction of such complex microenvironments in three dimensions will be possible and will allow impressive precision and control over the dynamics[72–74] (Fig. 5d). For example, in photopolymerized PEG hydrogels, photolabile building blocks have been synthesized[74]:

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these can be cleaved by a controlled light beam to modulate biophysical and biochemical gel properties locally at a given time. Mesenchymal stem cells were shown to respond to locally induced network changes in stiffness and cell-adhesion properties; in a densely crosslinked gel, the decrease in crosslinking density obtained through cleavage of the backbone of the photolabile chain induced a significant morphological change in the encapsulated stem cells (initially round in shape, they became more spread out). Moreover, the controlled manipulation of the concentration of cell-adhesive peptide ligands in the PEG gel led to inducible changes in chondrocyte differentiation. Differentiation into chondrocytes increased when an RGD peptide, which binds to integrins, was removed using light at a later time during 3D cell culture.

Microfluidic technology could also be used to mimic to some extent the spatial heterogeneity of stem-cell microenvironments[75]. Several 3D matrices (such as type I collagen, Matrigel or fibrin) containing cells were micropatterned within a single microfluidic channel, stably interfacing each other. Cell culture was performed over several weeks and led to spatially restricted development of multicellular structures within designed patterns. These new methods will be of use in studying a great number of questions in stem-cell biology.

From artificial niches to 3D *in vitro* ‘tissues’

The 3D approaches discussed above serve as powerful model systems to elucidate extrinsic stem-cell regulation, but they would not form an appropriate basis on which to reconstruct large-scale tissue models[76] using stem cells and biomaterials as building blocks, because they do not facilitate the modular and spatially well-controlled combination and positioning of these building blocks and they do not extend to scales of millimetres to centimetres. A technology known as bioprinting may be the method of choice in this endeavour, because theoretically it is feasible to combine layers of ECM and cell mixtures in modules of varying composition on a micrometre scale and in three dimensions. In bioprinting, custom-designed inkjet printers deposit, in a controlled layer-by-layer fashion, cells and biomaterials in almost picolitre-sized droplets at a rate of tens of thousands per second (see, for example, ref. [77]). On deposition on a substrate, these droplets can be polymerized to form a solid gel that could encapsulate stem cells or contain biomolecules with locally modular composition. Although the bioprinting field has arguably had little impact on stem-cell biology as yet, the results obtained so far with other cell types look promising. For example, viable 3D composites of embryonic neurons and astrocytes have been patterned in multilayered collagen[78]. Currently, bioprinting is cumbersome, mainly because a suitable ‘bio-ink’ (that is, a hydrogel system that can be rapidly crosslinked, with high spatial precision, and is simultaneously highly biologically active and permissive) is lacking. However, if this obstacle could be overcome, bioprinting could be a significant step towards achieving the long-standing goal of tissue engineers, namely the formation of functional tissues outside the human body.

Designing materials systems to control stem-cell fate *in vivo*

Biomaterials technologies also offer exciting opportunities to control the fate of stem cells *in vivo*, that is, at a site of tissue damage. Two main modes of application have been proposed: one in which biomaterials are used as carriers for introducing stem cells into damaged, diseased or aged tissue, and one in which biomaterials are used to augment endogenous stem-cell function. Here we briefly discuss these two approaches, the challenges they entail, and the promise they hold for future applications. For a more comprehensive review of such strategies, we refer readers to recent reviews[7,79].

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Biomaterials-mediated *in vivo* delivery of stem cells and support cells

The transplantation of stem cells, or possibly any type of cell, for applications in regenerative medicine has serious limitations. First, survival and engraftment of transplanted stem cells is extremely poor (typically only a few per cent of all cells engraft); this is the main obstacle to the clinical translation of stem-cell biology. Second, in the absence of instructive cues in a disrupted biological environment characterized by abundant cell and tissue necrosis, such as in regenerating tissue, the fate of the engrafted cells may be poorly controlled. Biomaterials can be designed to act as carriers for the local delivery of stem cells, support cells or molecular niche cues. The biomaterials may markedly improve the impact of transplanted stem-cell populations. Many of the concepts described for *in vitro* use above could find useful application *in vivo*. For example, materials could be designed as multifunctional stem-cell microenvironments that affect tissue regeneration on multiple levels, including the following: delivering stem cells in a protective gel and enhancing viability; delivering support cells to increase the numbers and stimulate the function of endogenous stem cells; delivering diffusible cytokines to promote the mobilization of endogenous cells involved in repair, such as those that form blood vessels; displaying regulatory proteins to enhance survival and to stimulate self-renewal and expansion of the transplanted cells; and displaying regulatory proteins to stimulate tissue-specific differentiation for the purpose of large-scale tissue regeneration. We think that the spatial and temporal control of these features would enhance their utility in tissue regeneration, improving tissue function and overcoming the adverse effects of disease or ageing[80,81].

Biomaterials-controlled *in vivo* delivery of niche signals

Biomaterials concepts are also beneficial for the local and specific delivery of bioactive niche components. These components may be inhibitory or stimulatory molecules or drugs that might increase stem-cell numbers or function when delivered to the niche. This could be achieved by forming a scaffold that leads to timed drug (small chemical) or biomolecule delivery near a stem-cell niche or by targeted delivery of soluble microparticles or nanoparticles as carriers of such bioactive niche components[82]. Biofunctional polymer particles can now be engineered to be efficient in such applications. Specifically, they can be functionalized so that they bind to specific molecules on cells, are responsive to environmental signals such as proteases secreted by cells, or are delivered encapsulated in a manner that leads to temporally controlled release or cellular uptake[83,84].

The most challenging, but perhaps the ultimate, biomaterials goal is to create multicomponent, injectable materials designed to act as *de novo* niches *in vivo*. Heavily damaged, necrotic tissue may have lost microenvironments suitable for stem-cell occupancy, as is the case in aged or dystrophic muscles[80]. Artificial niches would need to incorporate appropriate ‘homing’ signals that could attract endogenous stem cells and localize them by means of known cell–cell or cell–matrix adhesive interactions. Then, once localized to these artificial niches, the cells would need to be exposed to tethered signals that control stem-cell function, in particular expansion by self-renewal division. Neighbouring vascular cells and neural cells would need access. Upon injury, the upregulation and release of proteases would enable the newly formed stem cells to escape the niche and contribute to differentiation and tissue regeneration. Cell transplantation has recently been used to show that the formation of a heterotopic haematopoietic microenvironment is possible[85]. Upon transplantation, MCAM (melanoma cell-adhesion molecule)-expressing subendothelial cells present in human bone-marrow stroma were shown to be capable of forming a miniature bone organ. In another example, macroporous polyester scaffolds pre-seeded with rat osteogenic cells were implanted into nude mice (which lack a thymus and therefore cannot mount an immune response to reject foreign, transplanted materials)[86]. This scaffold design led to the formation of an active haematopoietic marrow with stromal and haematopoietic

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compartments, of which the stromal compartment seemed to have attracted and retained endogenous haematopoietic precursor cells, thus acting as a functional artificial niche.

Future challenges

Both 2D and 3D biomaterials-based culture platforms have the potential to help researchers to identify novel biochemical and biophysical regulators of stem-cell fates (such as survival, quiescence, self-renewal and differentiation). Ultimately, these findings will translate into new biomolecule-based therapies to induce resident stem-cell function and promote the regeneration of aged, injured or diseased tissues *in vivo*[1].

A major hurdle for the advancement of most, if not all, of the described strategies lies not in the biomaterials field but rather in stem-cell biology. The identification of markers that specifically and robustly distinguish stem cells from their differentiated progeny (for example OCT4) has proved successful with embryonic stem cells but is particularly cumbersome with many adult stem-cell types such as haematopoietic stem cells, which currently require multiple positive and negative selective markers for robust identification. In addition to retrospective analyses by immuno histochemistry, prospective analyses would be a great advance here. In particular, there is a paucity of dynamic live-cell markers (for example stage-specific promoters driving the expression of fluorescent reporter genes with appropriate half-lives) that would allow gene expression changes to be monitored in real time in conjunction with morphological assessment.

Another current problem is the bottleneck in the analysis of the large data sets accumulated by exploring some of the biomaterials platforms described here. Although groups have presented computer-based algorithms to assay cell morphology and genealogical histories acquired by time-lapse microscopy[46,87,88], for the most part a large amount of manual correction is still required[48,89]. High-fidelity, fully automated analyses of cell behaviours (Fig. 6) (such as proliferation rate and division history, to generate genealogical histories; directed migration and velocity; and cell shape and size) could exponentially accelerate our understanding of stem-cell biology. However, although cells may express given markers and may have distinct proliferation behaviours, the only true test of *in vitro* data on stem-cell function is validation with an *in vivo* assay.

The rate at which biomaterials approaches are being applied to address questions in stem-cell biology ensures that new insight will be gained into the mechanistic regulation of stem-cell fate. However, although there is now a plethora of ingenious biomaterial platforms with which to analyse the influence of the biophysical and biochemical properties of stem-cell niches, these platforms have only just begun to be applied to directing stem-cell fate. Collaborative efforts between cell biologists and materials scientists are critical to answering the key biological questions and fostering interdisciplinary stem-cell research in directions of clinical relevance.

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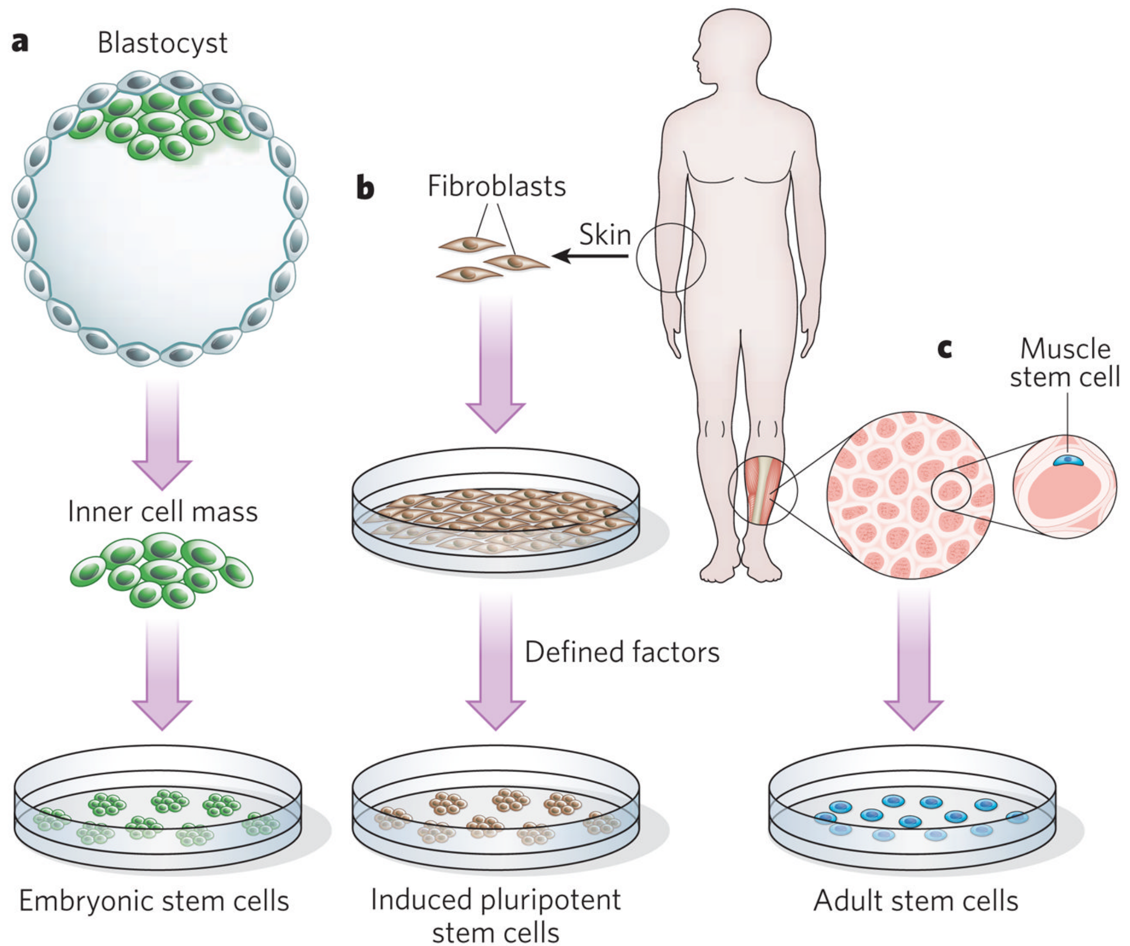

Figure 1. Origins, promises and challenges of stem cells

a, Embryonic stem cells, which are derived from blastocysts (formed at an early stage of embryogenesis), provided the first human source of pluripotent cells that could be differentiated to generate any cell type. **b**, Induced pluripotent stem cells, which have all of the properties of embryonic stem cells, were first generated by introducing genes encoding four proteins into somatic cells, such as skin fibroblasts[90]. Embryonic stem cells and induced pluripotent stem cells have a seemingly unlimited self-renewal potential in culture, but the absence of methods to direct these cells into a single tissue-specific lineage robustly and reproducibly and to avoid the risk of tumour formation reliably have restricted their use in humans. Induced pluripotent stem cells overcome the problem of immune tolerance and the ethical issues faced by the use of embryonic stem cells and adult stem cells in patients, but current methods to reprogram somatic cells and to generate induced pluripotent stem cells are extremely slow and inefficient. **c**, Resident tissue-specific adult stem cells (for example muscle stem cells) lack the plasticity of embryonic stem cells and induced pluripotent stem cells but are not tumorigenic. They are primed for, and extremely efficient at, generating progeny that differentiate into specialized cell types. It is difficult to induce the self-renewal of adult stem cells in culture and to propagate the cells to yield clinically useful numbers *in vitro*, underscoring the importance of elucidating the role of the endogenous microenvironment in the regulation of stem-cell fate. A cross-sectional view of muscle fibres (red) surrounded by basement membrane (white) is shown, together with a muscle stem cell (blue); these stem cells reside on top of muscle fibres, beneath the basement membrane.

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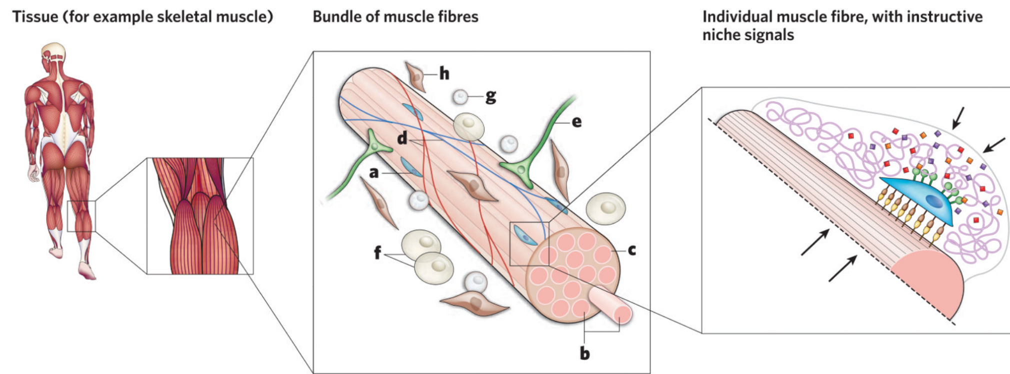

Figure 2. Biochemical and biophysical properties of stem-cell niches

Adult stem cells reside in tissue-specific microenvironments, called niches. Niches protect stem cells and regulate their functions. First described in *Drosophila melanogaster* and *Caenorhabditis elegans* ovary and testis, niches have now been characterized in many other tissues, including skeletal muscle (left panel). Muscle stem cells (**a**) reside on post-mitotic, multinucleated muscle fibres (**b**) and are ensheathed by a basement membrane (**c**) (central panel). The complexity of this stem-cell niche is increased by the presence of many other, non-muscle, cell types, including endothelial and blood cells in the vasculature (**d**), motor neurons (**e**), adipocytes (**f**), and circulating immune cells (**g**) and fibroblasts (**h**). Within the niche (right panel), spatially and temporally controlled biochemical mixtures of soluble and tethered chemokines, cytokines and growth factors (diamonds), as well as ECM molecules (purple) and ligands presented by muscle fibres (yellow), interact with transmembrane receptors displayed by muscle stem cells (brown and green) to regulate stem-cell fate. It is also becoming clear that the biophysical properties of the stem-cell microenvironment are crucial components of the niche; arrows indicate forces imposed on stem cells by the resistance of the ECM and surrounding tissue.

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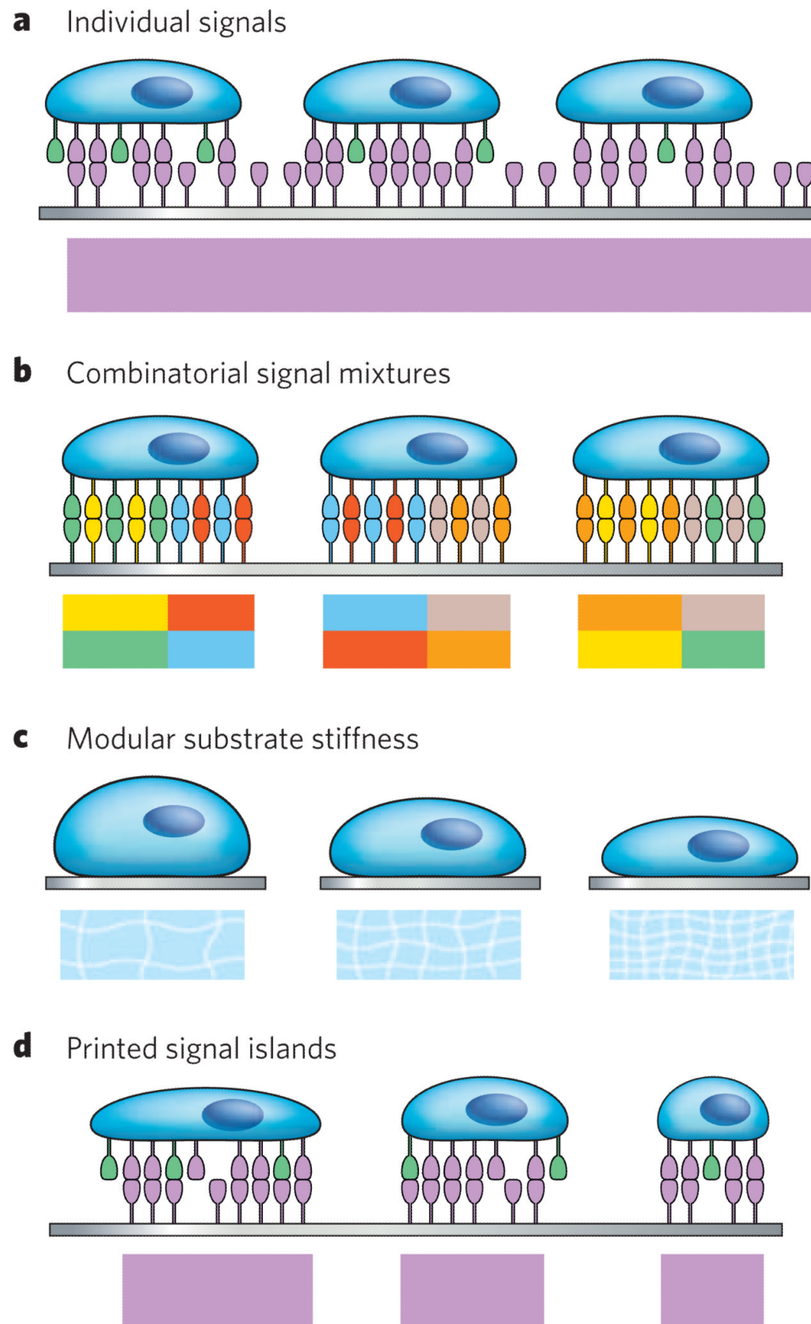

Figure 3. Engineering 2D artificial stem-cell niches

The top part of each panel shows stems cells exposed to a specific, engineered 2D microenvironment (viewed from the side), and the bottom part shows a schematic of the microenvironmental features (viewed from above), represented as blocks of colour matching the signals that are present. The substrates (grey) encompass various materials, such as plastics, glass or hydrogels, except for in panel **c** (in which soft materials such as hydrogels are depicted). **a**, Individual signal molecules are displayed on the substrate. **b**, Combinatorial mixtures of signals that are generated, for example, by robotic protein spotting can be presented to stem cells. **c**, The desired substrate stiffness can be controlled by, for example, differential crosslinking of hydrogel networks. **d**, Microcontact printing of cell-adhesion or

cell-regulatory proteins on inert surfaces allows control of protein spot size and, therefore, cell shape.

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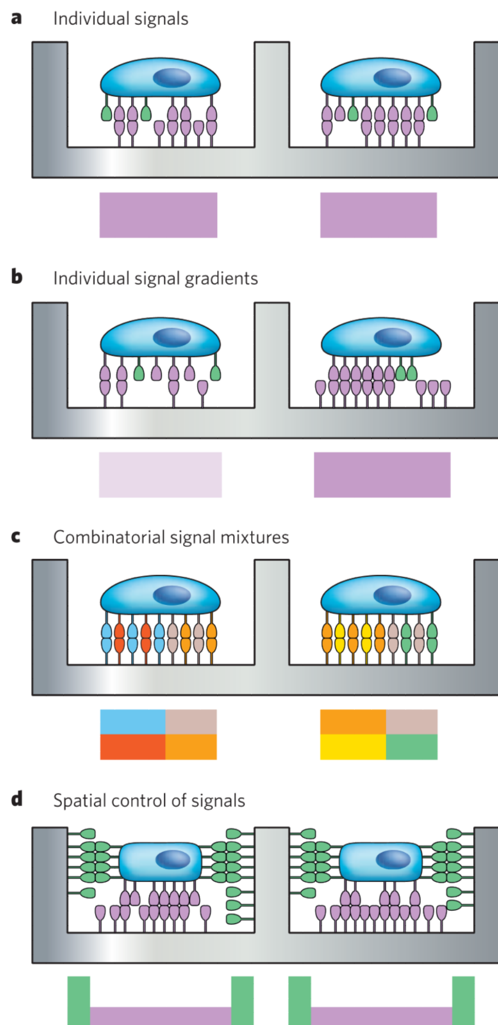

Figure 4. Engineering 'pseudo-3D' models of stem-cell niches

Microwell arrays allow the confinement of single stem cells and analysis of entire stem-cell populations at the individual cell level, overcoming the problem of heterogeneity of stem-cell populations. **a**, Microwell arrays can be readily engineered so that individual niche signals are presented at a certain concentration on the bottom of the well, by using manual microcontact printing. **b, c**, Robotic protein spotting on the microwell bottom should allow control of protein doses in each microwell, including the generation of protein gradients (**b**) or the production of combinatorial protein mixtures (**c**). **d**, Patterning approaches can be designed to allow the spatial arrangement of niche cues at the level of an individual, encapsulated stem cell. The top part of each panel shows stem cells exposed to a specific, engineered pseudo-3D microenvironment (viewed from the side), and the bottom part shows a schematic of the particular microenvironmental features (viewed from above (**a–c**) or from the side (**d**)).

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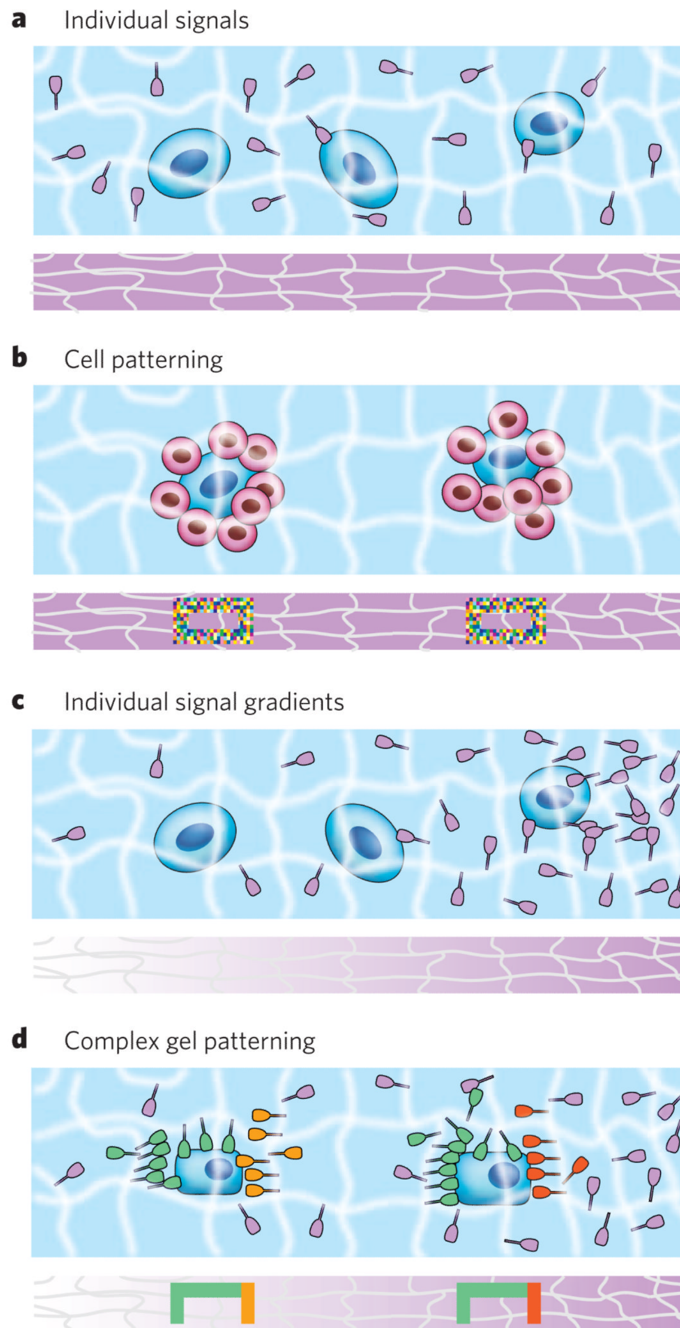

Figure 5. Engineering 3D *in vitro* models of stem-cell niches

Mild and selective hydrogel-crosslinking chemistries are necessary for a true 3D embedding of stem cells in an artificial microenvironment that more closely mimics natural stem-cell niches. Polymer-hydrogel networks can be engineered with tailor-made biochemical and biophysical characteristics. **a**, Individual niche signals can be tethered to gel networks to probe their function in stem-cell behaviour. **b**, Three-dimensional micropatterning technologies such as electropatterning allow the arrangement of cells in 3D hydrogels in a spatially well-controlled manner. Using this technique, single stem cells could be patterned in three dimensions in contact with support cells (pink) that provide many regulatory niche cues. **c**, Niche cues could be displayed as large-scale gradients (which is currently only

possible with non-tethered signals). **d**, Hydrogel networks can now be precisely micropatterned in three dimensions; for example, by light-controlled modification of biochemical gel characteristics (such as niche-signal availability) or biophysical gel characteristics (such as gel-crosslink density). The laser from a confocal microscope allows high spatial resolution, as well as dynamic control of 3D gel patterning. The top part of each panel shows cells exposed to a specific, engineered 3D microenvironment (viewed from the side), and the bottom part shows a schematic of the particular microenvironmental features (viewed from above (**a–c**) or from the side (**d**)).

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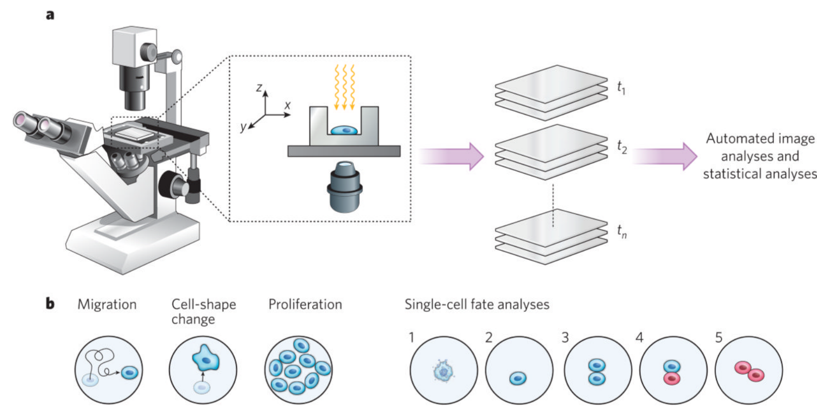

Figure 6. Quantitative investigations of *in vitro* stem-cell fates using live-cell microscopy

a, Time-lapse microscopy is a powerful way of probing the behaviour of live stem cells in artificial niches. Stem cells are imaged at various time points (t_1 to t_n) and locations to generate time-lapse movies, and automated image analysis and statistical analyses are used to quantify the dynamic cells' behaviour. **b**, A number of different read-outs, corresponding to different stem-cell functions, are available. Together with cell migration, changes in cell shape and changes in proliferation kinetics, the recording and automated analyses of changes in the fate of individual stem cells are crucial. Illustrated are cell death (1); quiescence (that is, non-cycling; 2); symmetrical self-renewal divisions (proliferation behaviour imposed in response to stress or trauma; 3); asymmetrical self-renewal divisions generating one daughter cell that retains stem-cell identity and one already partly differentiated (a behaviour thought to be dominant during homeostatic conditions; 4); and symmetrical depletion divisions, in which both daughter cells lose stem-cell function (the default behaviour of adult stem cells grown *in vitro*; 5).

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Table 1

Current promises and limitations of stem-cell populations

Feature	Embryonic stem cells	Adult stem cells	Induced pluripotent stem cells
Artificial system	Yes	No	Yes
Pluripotent	Yes	No	Yes
Efficient differentiation	No	Yes	No
Expansion in culture	Yes	No	Yes
Rare cell type	No	Yes	Yes
Immune compatible	No	No	Yes
Teratoma risk	Yes	No	Yes