Scaffolds for stem cells

Today, most people know at least something about stem cells. Embryonic stem cells enjoy regular mentions in news programs and magazines, probably because of the controversial way in which they are generated but also because of their huge potential in medicine. Here we distinguish between and define types of stem cells, discuss techniques used so far to create various cells and tissues from stem cells, and discuss how three-dimensional supports and stem cells have been and should be used to encourage the development of functional replacement tissue.

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Stem cells can be thought of as versatile, unspecialized cells that have the potential either to divide to make more stem cells or to differentiate into one or more cell type, usually in response to some kind of signal. Ultimately, these cells are used in the hope of addressing the shortfall in the quantity of tissue available for transplantation, either alone, as may be the case for replacing lost pancreatic beta cells in type 1 diabetes mellitus[1], or in combination with a scaffold, as may be necessary in the engineering of bone tissue[2]. The term 'stem cell' refers to a rather confusing assortment of different and distinct cell types all sharing this property, but for simplicity stem cells are usually divided into adult stem cells and embryonic stem cells.

Stem cells

Adult stem cells

Adult stem cells have been used for many years now, with particular success in the treatment of cancers of the blood system. Hematologists often point out that they pioneered stem cell biology long before the more recent explosion in scientific and public interest[3]. Throughout the 1950s and 1960s, these scientists demonstrated that transplantations of 'hematopoietic stem cells' (HSCs), isolated from the bone marrow, could generate a new immune system composed of many distinct specialist cell types in organisms in which the host immune system had been destroyed[4]. This culminated in 1963 when Mathé demonstrated the long term survival of a leukemia patient treated with HSCs[5]. Bone marrow transplantation is now a routine medical procedure.

Following these successes, Friedenstein *et al.*[6] noticed another cell type in bone marrow explants, initially called the fibroblast colony-forming cell because it stuck down on cell culture plastic, that was later shown to be a stem cell[7,8]. They are now referred to as marrow stromal cells or mesenchymal stem cells (MSCs) (Fig. 1). These cells resemble cells of the connective tissue (fibroblasts) and, in contrast to HSCs, can be grown easily in cell culture dishes. By changing the composition of the medium in which they are grown, MSCs can

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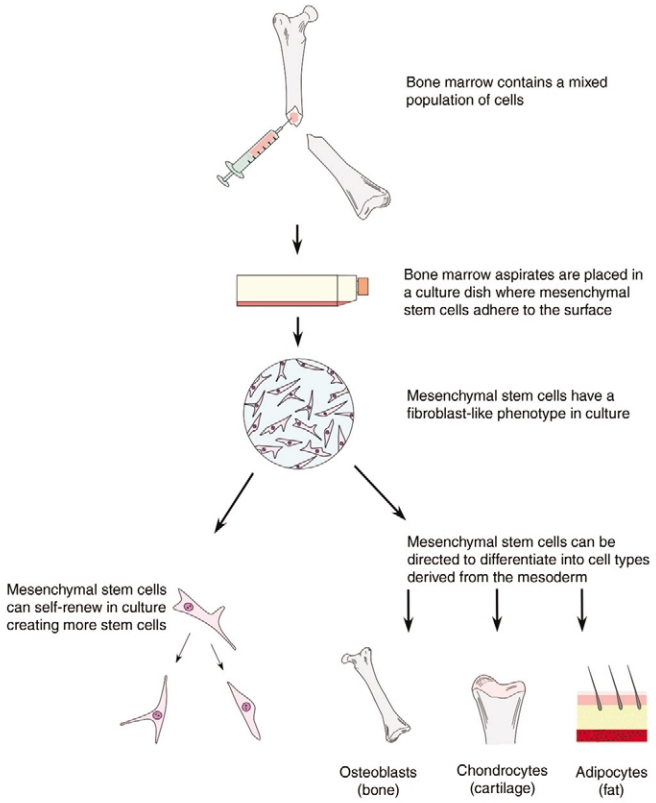

Fig. 1 Diagram demonstrating how adult stem cells can be used in tissue engineering. Marrow is removed from the adult bone and placed in a culture dish. Adherent mesenchymal stem cells can then be expanded or directed to differentiate into bone, cartilage, or fat cells.

be selectively differentiated into bone cells (osteocytes), fat cells (adipocytes), and cartilage cells (chondrocytes)[8]. This property has made them an attractive choice for bone and cartilage tissue engineering, especially since they may be used to treat the person from whom they were isolated – as an 'autologous' transplant. There are also numerous examples of evidence in the literature that these cells can differentiate into other lineages, including heart cells[9] and neurons[10]. But they do have limitations – they can only divide a finite number of times (depending on the age of the donor)[11], which limits their supply, and they may accumulate genetic changes over time[12].

Stem cells are also known to be distributed around the body in various other 'niches'. For example, neural stem cells can be isolated from brain tissue, grown *in vitro*, and induced to differentiate into the three cell types of the brain – neurons, astrocytes, and oligodendrocytes[13,14]. They also appear to be capable of turning into other cell types – after injection into a developing mouse blastocyst, they can be found later in the adult organism in several tissues, including heart, kidney, and liver[15]. Similar stem cells are also thought to reside in other tissues as a repair mechanism against injury, for example in the skin[16]. Again, however, these stem cells cannot be grown easily *in vitro* and are thought to have a limited replicative capacity.

Embryonic stem cells

Embryonic stem cells (ESCs), on the other hand, are renowned for their ability to divide indefinitely and their capacity to differentiate into most, if not all, of the tissues of the body. This makes them a potentially far more versatile cell type than adult stem cells. In mammals, they were first isolated in 1981 from the blastocyst of the mouse, a ball of cells formed several days following fertilization. Because of technical and ethical hurdles, it was not until 1998 that Thomson and colleagues[17] were able to do the same in humans. Human ESCs can now be routinely cultured as preserved 'pluripotent' cells, which retain their ability to divide indefinitely in an undifferentiated state and, when stimulated with the right signals, to differentiate into all of the tissues of the adult (Fig. 2). This raises the exciting possibility that they may be able to provide an unlimited source of cells for tissue replacement.

Following this advance, many groups rushed to make clinically-important cell types using human ESCs as a starting point. It was known that human ESCs would differentiate spontaneously into many cell types, both *in vitro* in free-floating structures analagous to the early embryo called 'embryoid bodies' and following implantation into experimental animals[17], but methods were needed to turn them *selectively* into a cell type of interest. The simplest and most common strategies involved simply growing ESCs in a medium designed for the required cell type. For example, Bielby *et al.*[18] grew human ESCs in a medium containing β-glycerophosphate, vitamin C, and dexamethasone, which is used routinely for the growth of osteoblasts in cell culture experiments, and demonstrated the formation of bone nodules and cells that expressed bone-specific genes. Other researchers have used similar methods, usually by including a variety of growth factors in the medium, to make pancreatic β cells[19], neurons[20], cardiomyocytes[21], lung cells[22,23], and even eggs[24] and sperm[25]. Another simple strategy involves culturing human ESCs in the presence of the target cell or a cell type postulated to have a role in differentiation. In this way, Vats *et al.*[26], Mummery *et al.*[27] and Van Vranken *et al.*[28] have shown that human ESCs can be differentiated into chondrocytes, cardiomyocytes, and pneumocytes respectively. Genetic manipulation is also a useful technique for directing the differentiation of ES cells. Kim and colleagues[29] introduced the gene for *nurr1* into ESCs, and demonstrated differentiation into dopamine-producing neurons and an improvement in the condition of Parkinson's disease-afflicted rats following their implantation. Tai *et al.*[30] have shown that increased numbers of osteoblasts could be produced by ESCs over-expressing *osterix*. Extracellular matrix – the proteinaceous amalgam in which cells grow – is also thought to be an important factor in the differentiation of ESCs. For example, Coraux *et al.*[31] showed that the culture of ESCs on a matrix derived from skin fibroblasts could generate a tissue that looked remarkably like real skin.

The literature on the directed differentiation of ESCs into various cell types is now vast, with over 1600 papers published since 1998, most of which use variations or combinations of the methods given

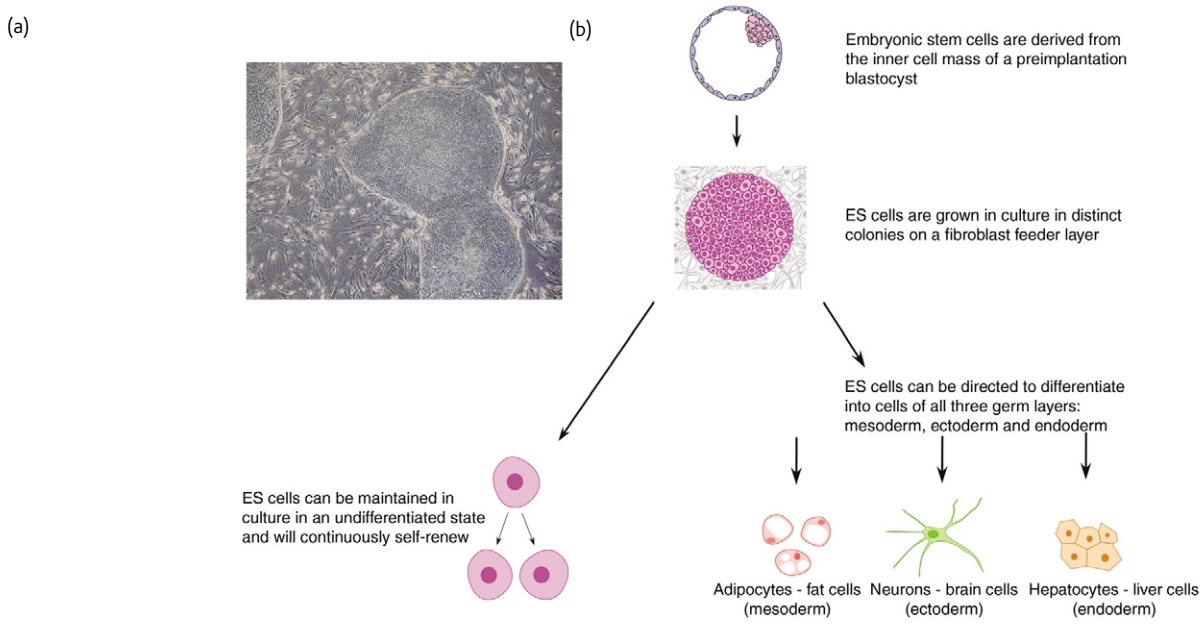

Fig. 2 (a) Photomicrograph of undifferentiated human ESCs (H1) grown on a mouse fibroblast feeder layer. (b) Diagram explaining the derivation and differentiation of ESCs. ESCs can be derived from the inner cell mass of a preimplantation blastocyst and expanded in culture on fibroblast feeder layers. Colonies of ESCs can be directed to differentiate into cell types from the three germ layers.

above. Despite this, however, the induction of differentiation to a specific cell type remains a largely hit-and-miss affair, and ESCs appear to have a malicious tendency to differentiate into a host of other cell types in addition to the cell type of interest. So most reviewers suggest that it is probably necessary to select the cell type of interest for most applications, either by sorting the cells using fluorescent antibodies or genetic markers, or by engineering a lethal marker into the cells that can be switched on should they not turn into the right kind of cell[32-34]. Perhaps for this reason, ESC research to date has lain firmly in the domain of cell biologists, who perform differentiation assays on cells grown on two-dimensional surfaces in cell culture dishes and flasks. Cellular differentiation and tissue development is, however, an inherently three-dimensional process and so to investigate differentiation and tissue formation *in vitro* fully, it may be necessary to turn to the field of tissue engineering, where three-dimensional cell culture systems have been used for many years.

Tissue engineering

Tissue engineers often focus their efforts on providing a three-dimensional environment, or scaffold, for cell attachment and growth, and hope that, by mimicking the *in vivo* environment, cells can be coaxed into creating a desired tissue type. The ultimate aim of tissue engineering is to make a three-dimensional cell-containing scaffold that can be implanted in the body to cure a disease or repair a defect (Fig. 3). The standard *in vivo* culture system – where cells are grown in a monolayer on a charged, flat, plastic surface – cannot replicate the complexity of the cells' natural environment and rarely supports the assembly of cells into a functioning tissue. Providing an appropriate scaffold that will lead to the development of a functional tissue is certainly not a simple matter, however, and tissue engineers have approached the problem in many ways, using a variety of materials.

Conventional scaffolds

Biomedical implants have been used since ancient times – for example, a Brazilian group recently reported that the ancient Incas successfully used Au plates to repair cranial defects[35]. Until the last few decades of the 20th century, the criteria used in choosing materials for implants has fundamentally changed very little and usually implant materials were chosen that were functional because of their inertness. Since the discovery in the 1960s that some glass ceramics actively bond to living bone, however, the focus has shifted away from inert materials and toward materials that are bioactive – those that deliberately elicit a specified response from the body. Currently, most scaffolds provide a three-dimensional environment in which tissue can grow and develop, so that it is able to reproduce the functions of the tissue it is intended to replace. Some scaffolds may be designed to be implanted without any cellular component[36] – instead they are designed to encourage tissue ingrowth and *de novo* tissue synthesis *in vivo* – while most are intended to have some kind of cellular component engineered *in vitro* before implantation (an example can be found elsewhere[37]). The latter strategies require that cells have access to nutrients and space to grow.

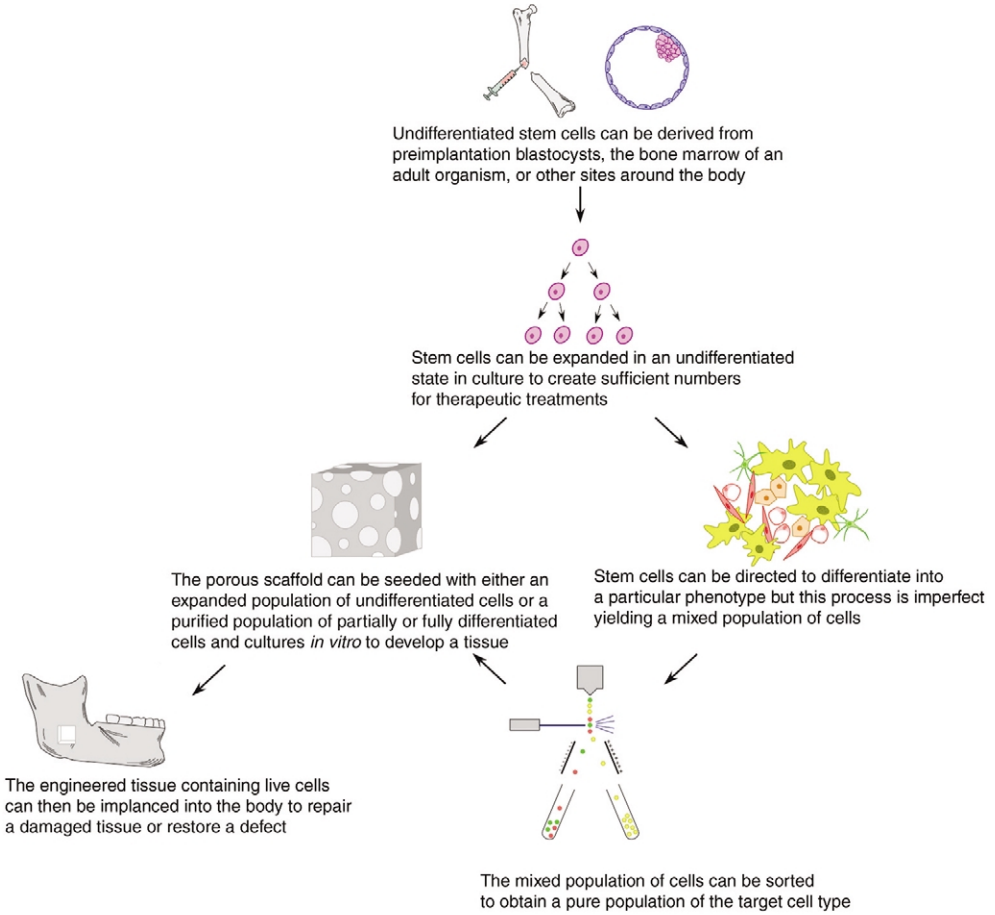

Fig. 3 Diagram outlining the potential for tissue engineering. Stem cells can be derived from embryos or adult tissues and expanded in culture. They can then be either seeded directly on a scaffold or differentiated in culture and sorted to obtain a purified population of a target cell type before seeding on the scaffold. Cell-seeded scaffolds can then be grown in culture to develop a desired tissue prior to implanting in the body.

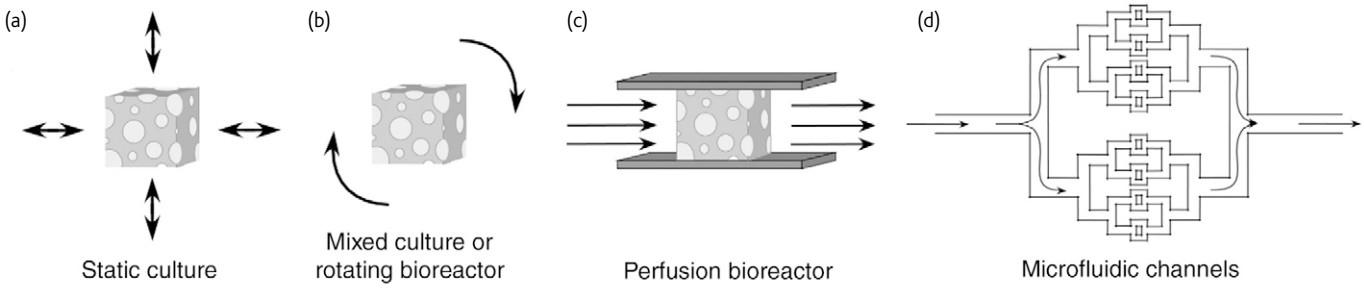

Fig. 4 Schematic illustration demonstrating four methods for achieving nutrient transport in an engineered tissue construct. (a) Construct is placed in static culture where it is reliant on simple diffusion for delivery of nutrients. (b) Construct is placed in either an environment where the media is mixed, such as a spinner flask, or in a rotating bioreactor where it achieves a zero-gravity state as fluid is moved around it. (c) Construct is placed in a system that forces fluid and nutrients to be continuously perfused directly through it. (d) Small channels are engineered into the construct allowing nutrients to be delivered in a similar manner to blood vessels in the body.

For this reason most scaffolds, regardless from which material they are made, are constructed with some kind of porous network and cultured with cells in a manner that encourages nutrient transport (Fig. 4). For instance, inorganic materials such as bioactive glasses and calcium phosphates have been used extensively for bone tissue engineering because of the similarities to and their ability to bond with bone's natural mineral backbone. Bioactive glasses can be sintered in powder form to create porous networks[38] or, when in solution, can simply be 'foamed' using soap and then gelled to make sol-gels[39]. Similarly, porosity can be engineered into polymers, such as polyesters (which

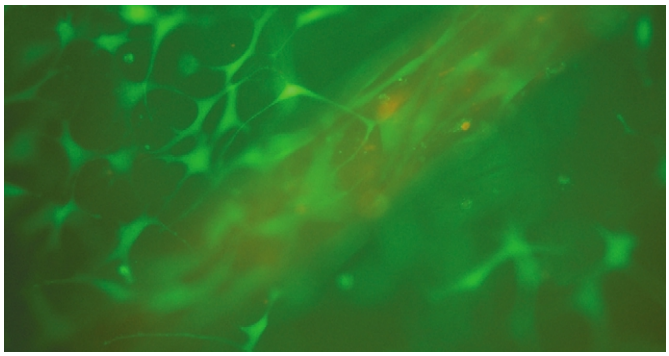

Fig. 5 Epifluorescence micrograph of a short collagen fiber/collagen hydrogel composite seeded with rat skin fibroblasts prior to hardening of the gel. Live cells appear green and have long projections into the gel matrix, while dead cells are small and round and stain red. The collagen fibers autofluoresce orange.

have the advantage of being biodegradable), either by foaming the polymer solution[40] or by molding the dissolved polymer around lumps of another material such as salt, allowing the polymer to harden and then leaching out the salt with water[41]. Porous networks can also be engineered into natural molecules – for example, collagen gels can be freeze-dried before cell seeding[42].

Alternatively, hydrogels can be used as scaffolds for cell growth and cell delivery. Since the gelling process is often nontoxic, cells can be introduced into the solution prior to gelation. In the case of alginate, a natural polymer made up of chains of guluronic and manuronic acid, calcium is usually added to cell/gel solutions, which crosslinks these chains and hardens the gel[43]. Likewise, collagen gels can be hardened by altering the pH of the solution[44,45] (Fig. 5) and poly(ethene) glycol can be solidified using light[46]. Hydrogels have different mechanical properties from other scaffolds, so the material must be selected on the basis of its properties, keeping in mind the intended application.

But all of these scaffolds have their disadvantages. Inorganic scaffolds such as ceramics and glasses tend to be too brittle and weak to be used in load-bearing applications, and even bioactive glasses, discovered more than 30 years ago, are limited to non-load-bearing applications such as the replacement of small bones in the middle ear[47]. Artificial polymers, on the other hand, may be viewed by the body as foreign material because they lack sticky surface molecules for cell adhesion. Their degradation products are, in the case of polyesters, acidic, and though not directly toxic, may create a possibly unphysiological acidic microenvironment. This is particularly important and often overlooked in bone tissue engineering – the natural mechanism by which bone is degraded *in vivo* by osteoclasts involves the formation of an acidic microenvironment! Collagen may be a better bet in this case, as the natural mechanism of bone formation involves the mineralization of a collagen scaffold created by osteoblasts – unsurprisingly collagen scaffolds are readily mineralized in tissue-engineering experiments[48]. Another problem with porous scaffolds is that because cells are seeded onto the internal porous matrix of the scaffold it becomes arguable whether the cells experience a truly three-dimensional environment – they merely 'see' a slightly curved two-dimensional surface. This can be solved to some extent by decreasing the pore size and adding surface texture, or embedding cells in a soft extracellular matrix, but then problems arise as to how to keep deeply embedded cells supplied with nutrients. Most importantly in the case of stem cells, there are challenges involved in providing the correct signals to encourage differentiation and to pattern cells as they differentiate into an organized tissue.

Micro- and nanopatterned scaffolds

To solve these problems, various groups have begun to investigate scaffold patterning – both at *microscale* resolution, possibly including channels so that cells can be supplied with nutrients and areas where different cell-types can be deposited, and at *nanoscale* resolution, where cells are supplied with the correct ligands to enhance and direct their function and to induce differentiation.

To address the first challenge, a number of new technologies are being investigated. Photopolymerizable poly(ethene) glycol hydrogels have been used as a material for encapsulating cells in three dimensions – these gels can be mixed with cells as a liquid and light can be used to solidify them[46]. Recently, several groups have used this property to add an element of patterning into hydrogels[49-51]. In these cases, a layer of cell-containing gel is selectively exposed to light in a pattern dictated by a light-blocking mask. Following gelation of this layer, another layer of cells, possibly containing another cell type, can be poured over this solid mold and gelled using another pattern. To illustrate this technique, Liu and Bhatia[50] encapsulated cells in recognizable shapes and layered different cell types in geometric patterns (Fig. 6). Another interesting technique involves the use of photolithography, a technique used extensively to make electronic components. This simply involves exposing the

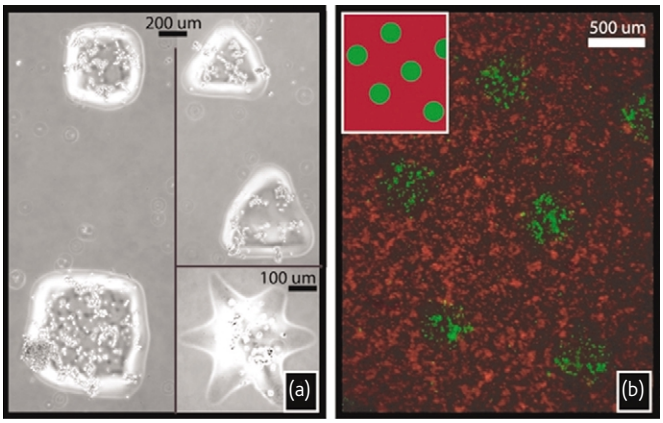

Fig. 6 Illustration of patterned cell-containing hydrogels. (a) HepG2 cells, a liver cancer cell line, encapsulated in various three-dimensional shapes in photopolymerized PEG hydrogels. (b) Cells labeled with red or green dyes, partitioned in distinct areas within a three-dimensional hydrogel layer. (Reprinted with permission from[50]. © 2002 Springer.)

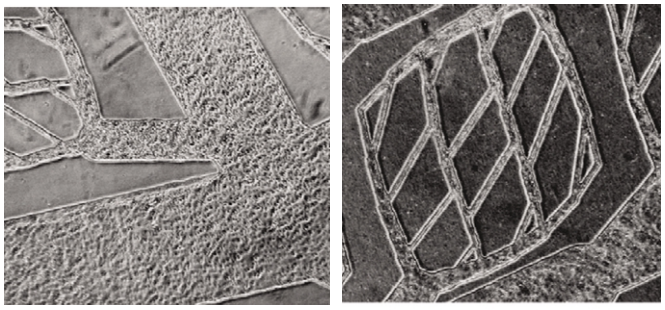

Fig. 7 Cells growing in microfabricated networks in PDMS. Channels are constructed by selectively exposing a photoresist to light to create an intricate, raised relief pattern. This relief pattern is then used to indent PDMS, which is then adhered to a glass slide. Cells and medium can be perfused through the channels and cells adhere to the walls. The cells here are HMEC-1, an immortalized endothelial cell line. (Reprinted with permission from[53]. © 2004 Springer.)

surface of a 'photoresist' mounted on a Si wafer to ultraviolet light. The exposed areas can be hardened – again in a pattern determined by a mask – and the patterned surface can be used to mold a soft substrate. This technique has been used extensively for microfluidic applications, where poly(dimethyl siloxane) (PDMS) is indented and adhered to a glass slide to make a series of channels (the technique has been reviewed elsewhere[52]). But more recently, tissue engineers have started to realize that this could be an excellent way of introducing something akin to a vascular system in a biomaterial. For example, Shin *et al*.[53] simulated a vascular network in a PDMS microfluidic device and showed the growth of endothelial cells – blood vessel cells – on the walls of the 'vascular tree' (Fig. 7), while Tan and Desai[54] made a fibroblast-containing scaffold based on collagen. The ability to develop three-dimensional, perfusable scaffolds has great potential in both tissue engineering and bioreactor technology as it could provide a way to keep deeply-embedded cells supplied with nutrients within a scaffold.

Another possible way of patterning scaffolds more intricately might involve rapid prototyping (RP). Put simply, RP encompasses a range of different techniques, all of which have the property of producing a physical object based on a computer design[55]. Most of these devices are analogous to printers and can print scaffolds using a variety of materials. Arguably, the most interesting, however, are devices that appear to be able to print cells and matrices in combination[56,57], which could allow precise control over tissue microstructure in the future.

Developing technologies such as these allow us to control the immediate surroundings of cells in a three-dimensional environment more precisely, and may provide a more authentic environment in which to direct the differentiation of cells to form a coherent tissue. But these approaches do not directly consider the chemical interactions that go on between a cell and its substrate (Fig. 8). To address this 'nanoscale' problem, tissue engineers are beginning to fabricate bioactive scaffolds, where the surface of the scaffold is engineered to stimulate cell function.

One such method involves using self-assembling amphiphilic peptides. These molecules are engineered with hydrophilic heads and hydrophobic tails that, under the correct conditions, can self-assemble into a network of 'nanofibers' with the heads sticking out into solution and the tails hidden in the core of the thread (Fig. 9)[58]. Networks

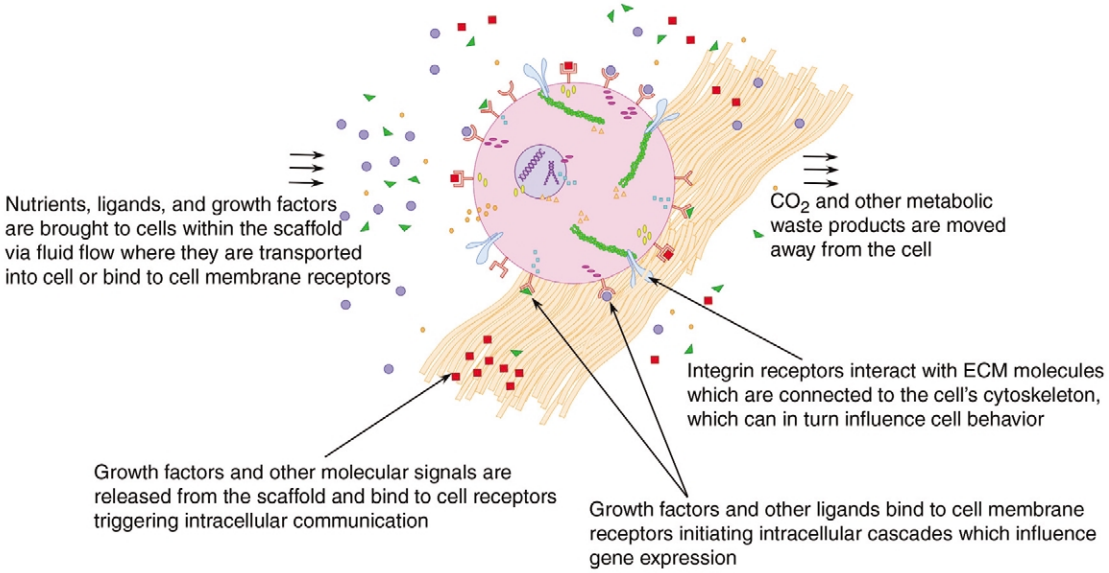

Fig. 8 Diagram demonstrating the relationship between cells and scaffolds. The microenvironment created around a cell adherent to a tissue-engineering scaffold is complicated. Nutrient transport brings growth factors, ligands, and other signals that can bind to cell receptors. The degrading scaffold can likewise release chemical messengers that bind to membrane receptors and influence intracellular communication and cellular processes such as gene transcription. Cells also attach to the scaffold via integrin receptors. Integrin receptors are closely connected to the cell's cytoskeleton and relay further information to the cell thereby affecting cell function.

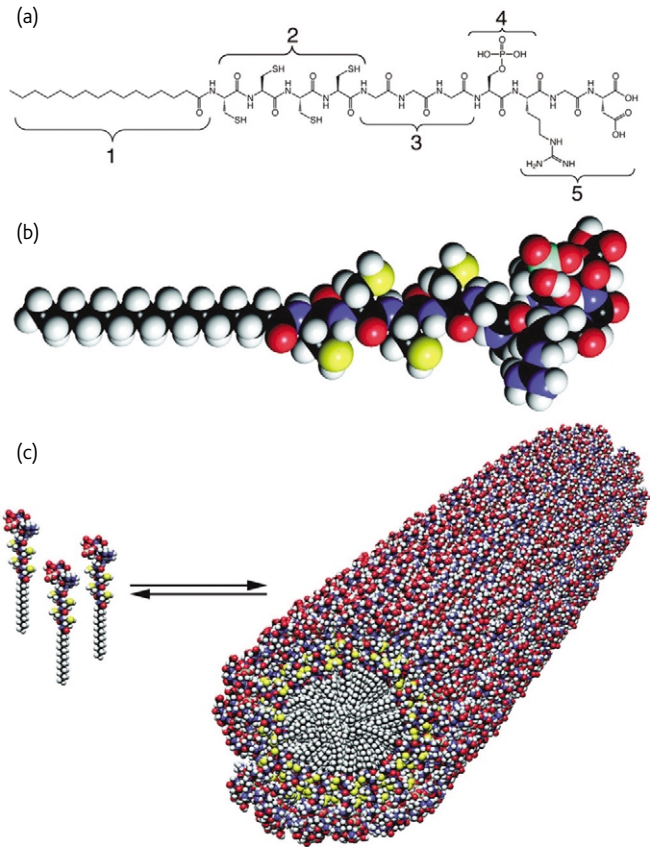

Fig. 9 Illustration of how amphiphilic peptides self-assemble to produce nanofibers. (a) The chemical structure of each peptide, including a long hydrophobic tail (1) and the three amino acid RGD motif at the head (5). (b) and (c) show how these peptides assemble to form a fiber. (Reprinted with permission from[58]. © 2001 AAAS.)

such as these have the advantage of porosity and living cells can be combined with such materials before the scaffold is fabricated. More importantly, peptides common to the extracellular matrix of the tissue under investigation can be engineered into the hydrophilic heads of such molecules, which can improve cell attachment and promote differentiation and function. Since the meshwork is so fine, the internal surface area of the scaffold is very large and cells are exposed to a high density of these ligands from all directions, which may encourage three-dimensional growth. Another fabrication method currently under investigation for making very fine scaffold networks is electrospinning, in which tiny threads of material can be fabricated to provide a porous mesh. This is accomplished by extruding a material from a fine nozzle using electrostatic force to form fibers between 3 nm and 5 μm in diameter. Again, extracellular matrix ligands can be attached to such fibers, or the fibers can be made out of biologically derived materials such as collagen[59].

Such micro- and nanopatterned scaffolds, therefore, introduce a greater level of control over the fine structure of a scaffold than conventional scaffolds and may allow control over cell patterning and cell differentiation. This level of control may be invaluable if a scaffold is to be used in combination with stem cells.

Scaffolds and stem cells

Stem cells, of course, are reliant on the extracellular environment not only to survive but also to develop into a functional tissue. So increasingly, tissue engineers are beginning to use the composition of scaffolds to persuade stem cells to differentiate. Arinzeh *et al.*[60] have shown that adjusting the ratio of hydroxyapatite to tri-calcium phosphate could influence the degree to which osteogenic differentiation of MSCs occurs, while others have begun to engineer bioactive factors into porous scaffolds. For instance, Kim *et al.*[61] have created a polyester scaffold that slowly exudes vitamin C and β-glycerophosphate and demonstrated an increase in osteogenesis from MSCs, while Yang *et al.*[62] have demonstrated increased osteogenic differentiation in a polylactic acid (PLA) scaffold spiked with a bone-specific growth factor. Alternatively, mechanical force can be used to stimulate differentiation – Altman *et al.*[63] have recently shown that applying a mechanical force to a collagen-gel scaffold can encourage MSCs to differentiate into ligament tissue.

Micro- and nanopatterned scaffolds have been investigated less well in regard to stem cells, although two recent studies highlight their attractiveness. Silva and colleagues[64] included a five amino acid, laminin-specific cell-binding domain (which binds to specific integrins on cell surfaces) at the hydrophilic head of their amphiphiles, and showed that neural stem cells could be induced to differentiate into neurons when cultured within the network. In contrast, cells grown in control scaffolds without the laminin-specific domain or on two-dimensional tissue culture plastic coated with laminin solution differentiated much less. This was hypothesized to be largely as a result of the density of the cell-binding ligands to which the cells were exposed, indicating clearly the importance of extracellular matrix in influencing cell function. In a similar study, Hosseinkhani *et al.*[65] replaced the laminin-specific domain in the amphiphilic molecule with the amino acid sequence, arginine-glycine-aspartate (RGD), a common cell-binding domain in many extracellular matrix proteins, especially collagen. They then showed that the differentiation of MSCs to osteoblasts is significantly enhanced compared with amphililic nanofibers without this sequence on to two-dimensional controls.

So far, remarkably few studies have been published on the effect of three-dimensional environments and scaffolds on ESC differentiation. In two rare examples, Levenberg and colleagues[66,67] have shown that human ESCs embedded in an extracellular matrix gel called Matrigel can be differentiated in three dimensions on conventional polyester scaffolds. In these cases, several structures that resemble primitive tissues were generated, depending on the content of the growth medium. The authors also show that tissues grown in three dimensions express higher levels of differentiation-associated proteins than those on coated two-dimensional surfaces. Interestingly, another group has

recently reported that the chondrogenic differentiation of human ESCs in a PEG hydrogel is dependent upon whether or not the hydrogel contains adhesive RGD sites, illustrating the importance of the cell matrix and microenvironment in ESC differentiation[68].

The use of more novel, patterned scaffolds should provide ESC biologists with an important new tool to stimulate and model differentiation *in vitro*. For instance, nanopatterned scaffolds, such as those using amphiphilic peptides, could be used to partition cells within a mixed population of ESC-derived cells, based on the specificity of the ligands to which different cells bind. In this way, scaffolds could play a role in directing tissue organization, not only with the aim of producing tissue for transplant but also for studying differentiation *in vitro*. Similarly, ESCs could be compartmentalized within scaffolds to study cell-cell interactions and their effect on cell differentiation and tissue formation. Such scaffolds will undoubtedly find exciting applications in the study of ESC differentiation in the future.

Conclusions

With stem cells, we are presented with a versatile material with which we may be able to rebuild many structures found in the body. But the challenge of how to construct three-dimensional tissues from them still remains. Recently, biologists and materials scientists have realized that scaffolds can and should be designed that direct and enhance cell function and differentiation. We have only scratched the surface of how these scaffolds can be used in concert with stem cells, however, and this provides us with great encouragement that they may provide us with the power to construct three-dimensional functional and intricate replicas of human tissue in the near future. mt

Acknowledgments

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