Stem cells in tissue engineering

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The concept of producing 'spare parts' of the body for replacement of damaged or lost organs lies at the core of the varied biotechnological practices referred to generally as tissue engineering. Use of postnatal stem cells has the potential to significantly alter the perspective of tissue engineering. Successful long-term restoration of continuously self-renewing tissues such as skin, for example, depends on the use of extensively self-renewing stem cells. The identification and isolation of stem cells from a number of tissues provides appropriate targets for prospective gene therapies.

Tissue engineering in a classical sense implies the use of organ-specific cells for seeding a scaffold *ex vivo*. The cell-based (but not necessarily stem cell-based) nature of tissue engineering served to define it, and to distinguish it from 'guided tissue regeneration' in which a scaffold is designed to encourage regeneration solely by cells residing at the site of its transplantation. Irrespective of actual clinical feasibility, the experimental design and potential use of tissue engineering approaches are endless, and range from the preclinical generation of cardiac valve substitutes, to *ex vivo* construction of nasal cartilages, to organ substitutes such as the urinary bladder (reviewed in ref. 1).

The list of tissues with the potential to be engineered is growing steadily. This is due in large part to recent progress in stem cell biology and recognition of the unique biological properties of stem cells, although not all stem cell-based therapies (for example, some that use neural stem cells, as summarized by Temple, pages 112–117) involve the construction of tissue either *ex vivo* or *in vivo*. Nonetheless, pilot studies in a variety of systems highlight great prospects for future stem cell-based tissue engineering (Table 1). But translation into clinical reality has been reached so far in only a few areas, and notably only in those areas in which there has been long-standing insight into stem cell biology.

Tissues that can now be engineered using stem cells comprise a diverse range from epithelial surfaces (skin, cornea and mucosal membranes) to skeletal tissues. These systems are inherently different in their rate of self-renewal and physical structure, two important determinants of any attempt to reconstruct tissues using stem cells (as discussed by Spradling and colleagues on pages 98–104). Appreciation of the inherent diversities of organ systems and cognate stem cells is essential for development of adequate strategies of intervention in specific areas.

Here we examine two applications of stem cells to tissue engineering. The first case is the regeneration of skin, which involves the structural formation of two-dimensional sheets. The second, more complex example is the formation of bone, which involves the reconstruction of three-dimensional shapes and internal architectures.

Engineering skin and other surfaces

Permanent restoration of tissues characterized by high and continuous self-renewal specifically requires extensively self-renewing stem cells. Haematopoiesis provides the best paradigm of a stem cell-dependent, steadily self-renewing system (see review by Weissman and colleagues, pages 105–111). Among the hierarchy of haematopoietic progenitors endowed with varying capacities to self-renew, it is only the small compartment of long-term self-renewing stem cells that is necessary for permanent and complete restoration of haematopoiesis after lethal irradiation[2]. Likewise, progenitor cells represented in a keratinocyte culture are also ranked in a hierarchy, and under clonogenic conditions form different types of colonies (holoclones, meroclones and paraclones) that vary significantly in their ability to self-renew and to generate a differentiated epidermis. Only holoclones are the product of a true stem cell, as defined by their exceptional self-replication ability (>140 doublings), which is not ascribed to either meroclones (a population of transient amplifying cells) or paraclones (a population of senescent or differentiating progenitors)[3]. In principle, a small but pure population of holoclone-generating cells would be all that is required for generating epidermal grafts. The recent identification of a keratinocyte stem cell marker may provide a new tool for this approach with respect to epidermis[4].

These theoretical predictions correlate with the clinical outcomes of skin grafting. Skin autografts are produced by culturing keratinocytes to generate an epidermal sheet, and then transplanting this sheet along with a suitable dermis-like substrate (Fig. 1a)[5]. But the success of these procedures has been variable, with graft failure resulting in many cases after a promising initial engraftment. Experimental conditions can cause depletion of the holoclone-generating compartment in cell cultures, similar to the partial depletion of the epidermal stem cell compartment that can occur *in vivo* (for example, during ageing). Retention or depletion of true stem cells in a keratinocyte population *in vitro* is significantly affected by the nature of the carrier or substrate used for culturing and grafting, and could account for instances of graft failure in clinical practice[6]. The long-term success of a skin graft thus depends on appropriate replenishment of stem cells in the graft. The specific technological challenge in restoration of epithelial surfaces in turn consists of the definition of culture conditions, and of carriers that are designed specifically to maintain an adequate stem cell compartment in the engineered graft. Dependence of epithelial surfaces on relevant stem cell compartments is further highlighted by the successful reconstruction of damaged corneas using stem cells derived from the limbus in conjunction with an amniotic membrane[7,8].

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Engineering the correct shape and physical structure of a graft is relatively simple in epidermis and other surface epithelial tissues, and indeed can be achieved *ex vivo*. This reflects the essentially two-dimensional organization of skin and other epidermal surfaces, and is in sharp contrast with systems such as the skeleton, where effective engineering involves the reconstruction of complex shapes and architectures. These directly affect proper function, are normally generated over a long period of time, and cannot be achieved *ex vivo*.

Engineering the skeleton

Skeletal stem cells (SSCs; also known as bone-marrow stromal stem cells, or mesenchymal stem cells) are found in the subset of clonogenic adherent marrow-derived cells, and are able to undergo extensive replication in culture. Upon ectopic *in vivo* transplantation in model systems, all of the main tissues found in bone as an organ (bone, cartilage, adipocytes and haematopoiesis-supporting stroma) are formed (Figs 1b and 2a,f) (ref. 9, and reviewed in refs 10,11). SSCs obtained in culture must be combined with appropriate carriers before transplantation. These provide a three-dimensional scaffold in which a vascular bed can be established, and transplanted progenitor cells can differentiate and form a bone/marrow organ. Many suitable materials are being generated and perfected, including synthetic hydroxyapatite/tricalcium phosphates (Figs 1b and 2a), and polyglycolic and polylactic acids[12,13]. Most are effective in supporting bone regeneration, either alone or in conjunction with growth factors such as bone morphogenetic proteins. However, so far, little attention has been devoted to their compatibility with long-term maintenance of stem cell properties, or to the ultimate fate of the bone–biomaterial composite being generated at the site of transplantation.

In the simplest procedure for bone reconstruction, SSCs isolated in culture from a limited volume of bone marrow are expanded *ex vivo*, loaded onto an appropriate carrier, and locally transplanted (Fig. 1b). This procedure results in the effective repair of critical size defects, which are larger than what can be repaired by resident cells, either spontaneously or as guided by an osteoconductive device (Figs 1b and 2b,c)[14–17]. Convincing preclinical studies adopting this approach have led to preliminary observational studies in humans[18], and clinical trials are about to start in a number of centres. An alternative approach has led to the generation of fully vascularized bone flaps of the desired shape *in vivo*, prior to their use for local transplantation (Fig. 2d, e). In these experiments, SSCs loaded into appropriate carriers and transplanted into a non-skeletal site surrounding an artery and vein; here they generate a vascularized segment of bone, the size and shape of which are dictated by the carrier geometry[19].

All of these applications can be categorized as surgical intervention — through transplantation of a 'biological' prosthetic device — for the cure of physical defects in an otherwise normal skeleton. But the existence of SSCs raises additional hopes and challenges for treatment of more complex and generalized diseases, such as crippling genetic diseases. Here, the concept of using SSCs to replace misfunctioning bone cells with normal ones meets another set of important obstacles, which further illustrate the diversity of the skeleton and its stem cells compared with other stem cell-based systems. One is the route of systemic delivery of the number of progenitors needed to produce a biological effect. Some

Table 1 Current approaches to tissue engineering

Stem cell-based tissue engineering		Non stem cell-based tissue engineering[1]	
Blood vessels[23,29]	Liver[31]	Bladder	Meniscus
Bone[14–17]*	Pancreas [41]	Cartilage (ear, nose and joints)*	Oral mucosa
Cartilage[39]	Nervous tissue[42]*	Heart valves	Salivary gland
Cornea[7,8]*	Skeletal muscle[24,27]	Intestine	Trachea
Dentin[40]	Skin[5,6]*	Kidney	Ureter
Heart muscle[28,29]			Urethra

*In clinical trials or clinical observational studies.

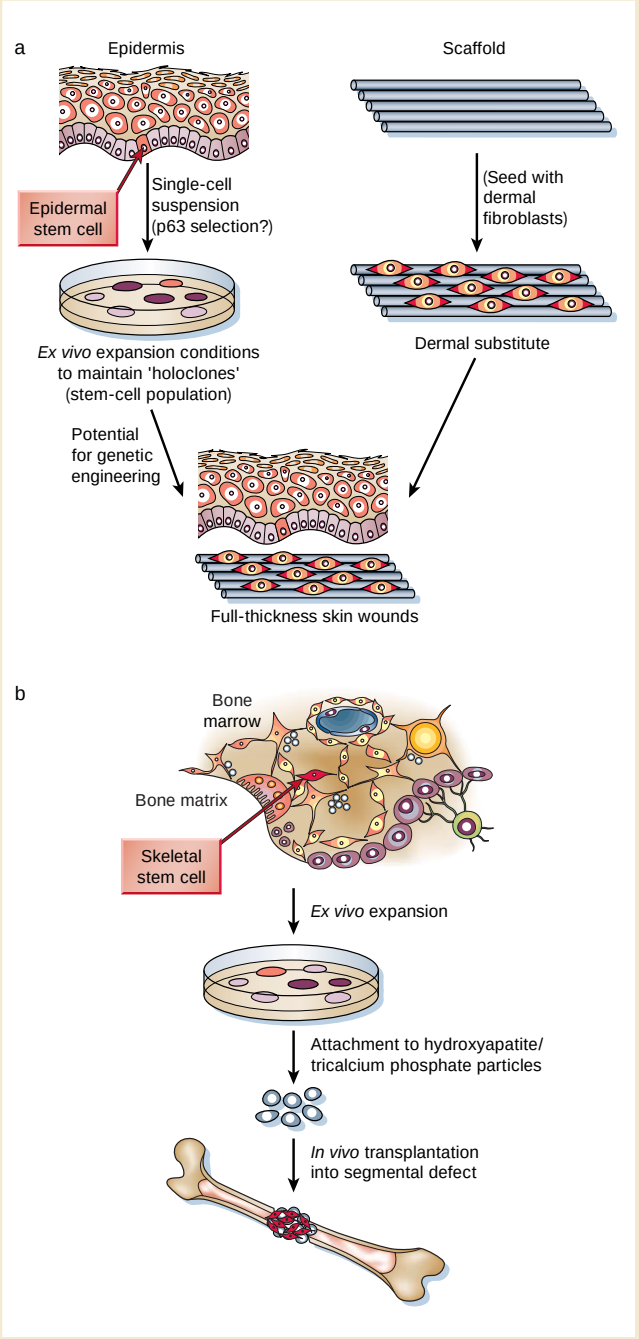

Figure 1 Regeneration of two-dimensional (skin) and three-dimensional (bone) tissues using stem cells. **a**, Skin autografts are produced by culturing keratinocytes (which may be sorted for p63, the recently described, epidermal stem cell marker) under appropriate conditions not only to generate an epidermal sheet, but also to maintain the stem cell population (holoclones). The epidermal sheet is then placed on top of a dermal substitute comprising devitalized dermis or bioengineered dermal substitutes seeded with dermal fibroblasts. Such two-dimensional composites, generated *ex vivo*, completely regenerate full-thickness wounds. **b**, Bone regeneration requires *ex vivo* expansion of marrow-derived skeletal stem cells and their attachment to three-dimensional scaffolds, such as particles of a hydroxyapatite/tricalcium phosphate ceramic. This composite can be transplanted into segmental defects and will subsequently regenerate an appropriate three-dimensional structure *in vivo*.

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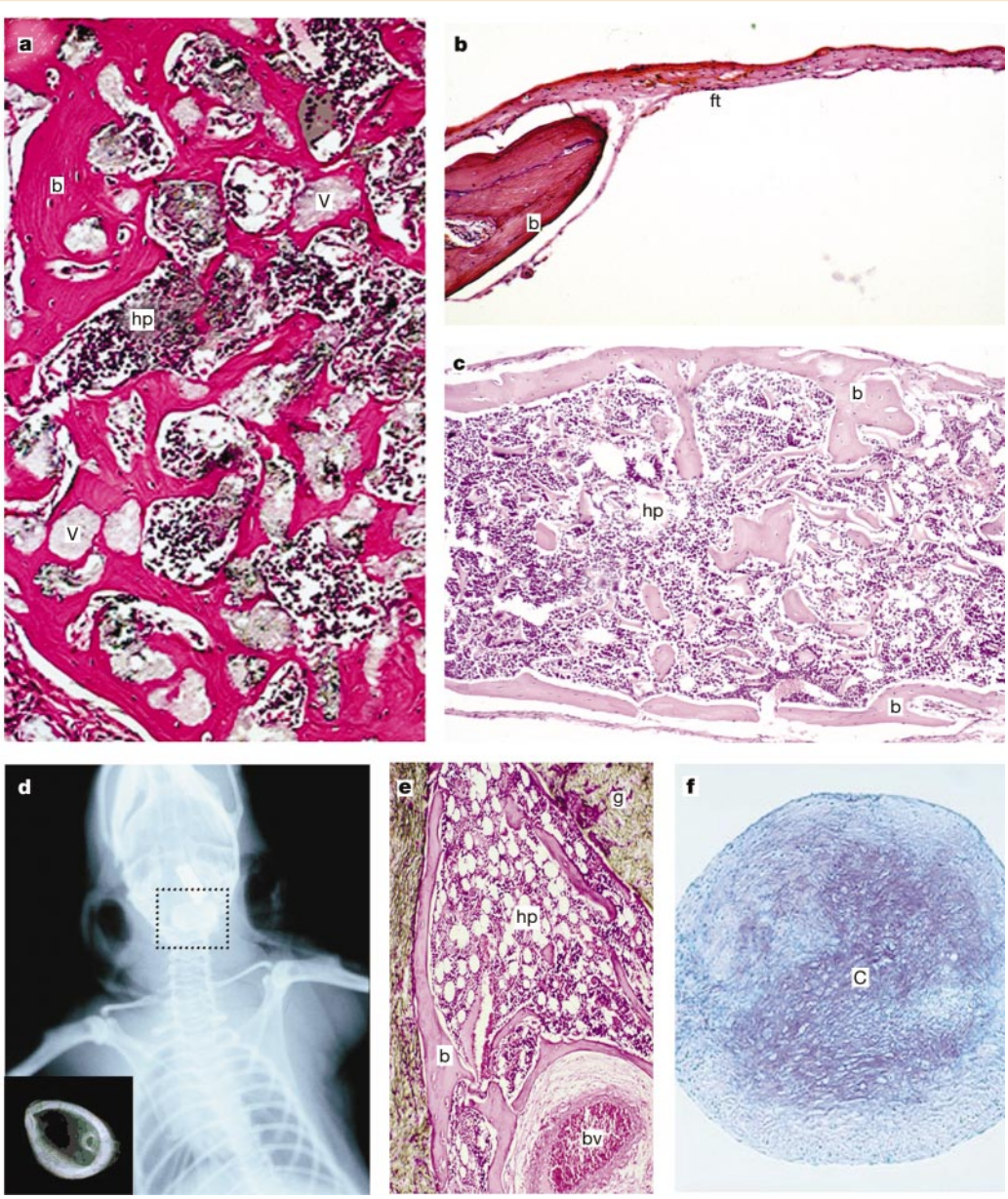

Figure2 Bone regeneration by marrow-derived skeletal stem cells (SSCs). **a**, SSCs transplanted in conjunction with appropriate vehicles (v) such as hydroxyapatite/tricalcium phosphate generate an network of bone (b) and stroma composed of adipocytes and reticular cells that support haematopoiesis (hp). **b**, Critical size defects that do not heal spontaneously, such as those as in the calvaria, can be completely regenerated by SSC–vehicle constructs (**c**); ft, fibrous tissue. **d**,**e**, In addition, by wrapping SSC–vehicle constructs around an artery and vein (bv) in conjunction with Gore-Tex (g) to prevent collateral vascularization (dotted box in **d**), fully vascularized bone flaps of desired shape can be constructed. **f**, Under appropriate conditions, SSCs can also form cartilage (c) as demonstrated by alcian blue staining and type II collagen immunohistochemistry (not shown).

experimental evidence is available for limited engraftment of osteogenic progenitors that are infused (as is done with haematopoietic stem cells or HSCs) through the systemic circulation[20]. But evidence for a biologically significant effect of the systemic infusion of SSCs is not available, and cure of systemic skeletal diseases using approaches borrowed simplistically from haematology is not in sight. Definitive preclinical work in animal models, compliant with strict criteria for assessment of engraftment, must be conducted before these types of procedures reach clinical trials[11], although preliminary clinical studies are underway[21].

Another pertinent point is that renewal in a postnatal skeleton is markedly slower than in skin or blood (the whole skeleton being completely replaced only about three times in a lifetime, whereas the skin is replaced once a month). Consequently, we would expect replacement of skeletal tissue with infused SSCs to occur over longer timescales compared to rapidly self-renewing tissues, even if issues related to efficient cell delivery and systemic engraftment were resolved. Alternative means for local intervention in genetic diseases are conceivable. For example, engineered SSCs could be transplanted locally at the site of a clinical event (such as a fracture or deformity) in genetic diseases of osteogenic cells, such as osteogenesis imperfecta (caused by mutations in genes encoding collagen type I) or fibrous dysplasia of bone (caused by activating missense mutations of the GNAS1 gene). But hopes for a systemic cure of genetic disorders of the skeleton will rely on more imaginative avenues. In some cases these might include *in utero* transplantation, whose feasibility with SSCs has been shown in preliminary studies[22]. As with the previously described techniques, caveats related to efficiency of engraftment and biological effects remain to be addressed.

Heterotopic stem cells for tissue engineering

The marrow is at centre stage for future technological developments in tissue engineering, not only as the only organ in which at least two types of stem cells (HSCs and SSCs) reside, but also as the organ in which progenitors for a number of distant tissues can be found. Recent studies indicate that the traditional wall separating the haematopoietic and mesodermal tissue systems and lineages is being demolished. Cells capable of regenerating blood vessels, skeletal muscle and cardiac muscle are found in the marrow[23–25]. The unexpected potential for myogenesis and cardiomyogenesis has been

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ascribed to both HSCs and stromal (mesenchymal) stem cells in the marrow[26–29]. SSCs can perhaps give rise to neurons or glia[30], purified mouse HSCs can regenerate liver cells[31], and cells able to regenerate bone are also found in blood[32]. Perhaps what we have referred to as the HSC is in itself much more — a true multipotent stem cell with transgermal potentials[2,31], normally devoted to haematopoiesis as a result of local cues.

Marrow cells offer the advantage of being easily harvested and cultured from an adult organism, and the HSC can be isolated and purified *ex vivo*. Although most of these applications are a long way from any immediate clinical use, these studies raise basic scientific issues that are far from being settled or rationalized, similar, for example, to the issue of 'plasticity' of postnatal stem cells[10,11]. They provide insight on how different the scene of tissue engineering could be in the relatively near future. Beyond theoretical considerations, and pending further experimental proof where needed, the existence of heterotopic and pleiotropic stem cells in the bone marrow has obvious practical implications for the future of stem cell therapy that should not be missed.

Reconstruction versus correction

Delivery of factors that would stimulate stem cells *in situ* to initiate a process leading to regeneration rather than scar formation has long been pursued. But success has been limited owing to problems of dosage, lack of full activity of recombinant factors, and inability to sustain a factor's presence for an appropriate length of time. To overcome these problems, 'gene-activated matrices' are being investigated that comprise plasmids coding for factors in a variety of delivery vehicles. These would transduce cells *in vivo* to bring about appropriate regeneration, and trials aimed at osteoporotic fractures are being planned[33]. In these attempts, the traditional principles governing the use of carriers merge imaginatively with the frontier of genetic engineering. No doubt, if reconstruction was the initial frontier of tissue engineering, genetic correction is the next. As stem cells are the critical ingredient in tissue regeneration, they are also the critical targets of any strategy aimed at correcting a genetic defect.

Advances in our ability to genetically manipulate cells *ex vivo* using viral and non-viral transducing agents has circumvented many of the potential hazards of direct gene transfer in humans[34]. Successful stable transduction of holoclone-generating epidermal cells with retroviral vectors has paved the way to the first trial of gene therapy for a devastating skin disease, junctional epidermolysis bullosa, a recessive disease caused by a misfunctioning laminin molecule[35]. However, in dominant-negative diseases, a mutant gene must be silenced, and the techniques for doing this are in their infancy. Homologous recombination and site-directed mutagenesis of stem cells *ex vivo* would be the ultimate goal[36,37], but antisense RNA, RNA interference and hammerhead and hairpin RNAses offer intermediate strategies to at least prevent expression of a mutant protein[38].

With the prospect of stem cell-mediated gene therapy, the very definition of tissue engineering evolves into engineering of tissue function. Today, stem cell-based approaches to tissue reconstruction open unpredicted applicative (and market) opportunities. Although this discussion has been limited to systems where extensive preclinical and clinical studies have already been conducted, more exciting avenues are in sight in many areas. But enthusiasm over what unquestionably represents a markedly innovative technique with huge therapeutic potential must be balanced against stringent standards of scientific and clinical investigation. In addition, we must remain aware of the wide range of basic and applied issues associated with each system, with the targeted problems, and with the predicted solutions. □

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