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INVITED REVIEW

Regenerative approaches in the craniofacial region: manipulating cellular progenitors for oro-facial repair

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This review aims to highlight the potential for regeneration that resides within the bony tissues of the craniofacial region. We examine the five main cues which determine osteogenic differentiation: heritage of the cell, mechanical cues, the influence of the matrix, growth factor stimulation and cell-to-cell contact. We review how successful clinical procedures, such as guided tissue regeneration and distraction osteogenesis exploit this resident ability. We explore the developmental origins of the flat bones of the skull to see how such programmes of differentiation may inform new therapies or regenerative techniques. Finally we compare and contrast existing approaches of hard tissue reconstruction with future approaches inspired by the regenerative medicine philosophy, with particular emphasis on the potential for using chondrocyte-inspired factors and replaceable scaffolds.
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Introduction

This article examines how an increased knowledge of stem cell differentiation pathways can be translated into new, or more refined methods of hard tissue regeneration in the oro-facial region; in particular, within bone and periodontium. A great deal of information now exists about the basic biology of skeletal formation and clinical procedures have advanced during this time, but the translation of laboratory findings toward more effective clinical practice has progressed at a slower rate. By including discussion of currently effective clinical protocols for hard tissue regeneration, and the biology underpinning them, we hope to provide clues as to how future treatments can be moulded from the latest advances in basic knowledge. We will review relevant data on the differentiation of osteo-progenitors and guided tissue regeneration (GTR) in the periodontium, the mechanisms of bone formation and the contrast between adult and embryonic progenitors. Moreover, the contrasts between *in vitro vs in vivo* tissue responses and the concept of soft composites for hard tissue engineering will be discussed.

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Progenitor cells

What is meant by a stem cell depends upon context; the definition of stem-ness is determined largely by *in vitro* experiments (Javazon *et al*, 2004). The haematopoietic definition emphasizes the ability to self-renew and give rise to differentiated descendants (Javazon *et al*, 2004); a developmental definition may concentrate upon the variety of tissues to which progeny can contribute (Parker *et al*, 2004, 2005). Within the adult the dichotomy surfaces as a debate around remotely located stem cells (e.g. bone marrow) and tissue-specific progenitors capable of local repair (e.g. periosteum). Such distinctions are critical to potential exploitation of the body's stem cell reservoir. There are four main categories, two require *ex vivo* manipulation of isolated autologous cells e.g. bone marrow-derived or other tissue (for instance, muscle) and two require *in vivo* stimulation of existing cells – remote activation of bone marrow-located cells or stimulation of local tissue stem cells.

In the context of this review series the *ex vivo* approaches have been explored (Miura *et al*, 2006; Maria *et al*, 2007), remote activation of bone marrow-derived cells (as occurs during cancer metastasis; e.g. Kaplan *et al*, 2005) will not be discussed; this leaves the activation of local tissue stem cells or progenitors. It is arguable that this may in fact be the best target for hard tissue regeneration strategies; the trademark plasticity of pluripotent stem cells making them difficult to control for therapeutic purposes. Equally the differences may be only semantic; the *in vitro* characteristics of cells from different sources appear indistinguishable (Aubin, 1998; see below). Whilst much of what we know regarding the biology of bone cells comes from *in vitro* studies, the diseases which afflict the skeleton reveal the dynamic processes, which are occurring at the systemic level. Bone turnover comprises bone resorption of mineral by osteoclast activity, coupled to deposition by osteoblasts (to form the encapsulated osteocytes); the bone multi-cellular unit (BMU). These two processes allow subtle remodelling of the skeleton,

but when deregulated can cause loss of bone as occurs during periodontitis or due to lack of compressive loading on alveolar bone. For repair of bone loss on this small scale, it should be possible to manipulate the existing bone anabolic cells through promotion of osteoblast function; this is currently achieved through GTR or distraction osteogenesis. It is salutary to note that these two highly successful clinical protocols have been developed independent of recent discoveries in skeletal biology and rely upon fundamental principles of bone biology: the need to exclude soft connective tissue from a bone defect and the mechano-responsiveness of bone cells respectively. Replacement of large bone segments, necessitated by trauma or surgical resection, represents a different challenge but the cells capable of forming the new element are present; the orchestration required has not yet been achieved. However, we will review the findings of basic research in this area and how these may be translated to produce enhanced treatments.

Bone stem cells

Detailed studies of bone cells demonstrate that a striking heterogeneity exists at the RNA and protein level for recognized markers of osteoblast differentiation and function. They vary in terms of proliferative potential and degree of commitment (Aubin, 1998, Owen and Friedenstein, 1988). The upshot of this complex picture is that there are very likely to be some cells at the right stage to contribute either numbers or committed osteoblasts, in response to the appropriate signal. As we accrue more evidence on the differentiation of stem cells, discrepancies in the detail are becoming apparent and these may encourage us to look more at the stimulation of local bone cells. Bone marrow-derived human mesenchymal stem cells (hMSCs) differentiated to the osteogenic lineage share only 78% of their proteins with mature human osteoblasts (Salasznyk *et al*, 2005). Global comparisons of gene expression profiles for undifferentiated mesenchymal precursors (calvarial) or hMSCs (bone marrow derived) *vs* their respective ossified progeny, are not similar. The significance is clear, if widely used bone marrow-derived MSCs do not form the same bone as tissue-specific precursors (local progenitors), results using hMSCs *in vitro* will not necessarily translate to *in vivo*; equally if delivered *in vivo*, will such cells integrate seamlessly with phenotypically distinct local cells? The clinical data on the use of limb or axial grafts to the orofacial region indicate that they may not; thus, there is a biological imperative to examine the regenerative potential of local stem cell reservoirs.

Which cells are going to respond?

Osteo-progenitors are not confined to the bone marrow, and for local restoration of lost bone these may not be the most important. The most obvious demonstration of local bone stem cell activity comes from the capability of bone to repair and regenerate unaided. However, it has been suggested that bone marrow-derived cells are responsible for this phenomenon and certainly they are recruited to sites of tissue damage and inflammation (Direkze *et al*, 2004) and circulating cells can differentiate to the osteogenic lineage (Kuznetsov *et al*, 2001). Fortunately, *in vitro* studies provide definitive evidence of the existence of progenitors within trabecular bone. The use of bone chip explants as a source of osteogenic cells is well known (Robey and Termine, 1985) and relies upon the existence of cells which are capable of migration, proliferation and differentiation. There is certainly a mixed population, with differences evident between the collagenase-released (CR) population and that which migrates from the explant (Sakaguchi *et al*, 2004). While those cells derived from the explant behave as osteoblasts (with various degrees of commitment) the fibroblast-like cells released by collagenase digestion appear to contain bone lining cells, endothelial cells and MSCs. The isolation of 100 million cells per gram of trabecular bone (Sakaguchi *et al*, 2004) or more conservative claims of 2 million cells per gram of trabecular bone (Noth *et al*, 2002; Tuli *et al*, 2003) demonstrate dramatically the potential for regeneration that exists within bone (excluding marrow). However, comparing the characterization of bone marrow-derived cells with such bone-derived cells does demonstrate comparability *in vitro* (e.g. Aubin, 1998 *vs* Sakaguchi *et al*, 2004). This further confirms the view that there is inherent regenerative capacity within the bone compartment that can be exploited for defect repair. However, it leaves unanswered the key question of which pathway of bone formation the cells will follow *in vivo*; identifying whether endochondral or intramembranous ossification is prevalent is clearly essential for schemes designed to promote regeneration.

Bone formation during embryogenesis and repair

Direct bone formation

Intramembranous ossification of foetal calvaria radiates from a central focus and progresses until it reaches the unstable joins between the plates, the future sites of the sutures (reviewed Morriss-Kay and Wilkie, 2005). The initial process, therefore, begins within an apparently uniform population of osteoprogenitor cells, bound within a membrane, following an autonomous programme of condensation and differentiation. Later growth, up until adolescence in humans, is allowed for by the maintenance of the patent suture mesenchyme. Undifferentiated mesenchymal progenitors occupy this site and are believed to contribute to the osteogenic front to accommodate growth of the underlying tissue. Such a mechanism is essential for the exoskeletal carapace of the head and is not only a feature of foetal growth, but continues into adolescence. This demonstrates that not only the cells, but also the same cellular processes that govern stem cell proliferation and differentiation may persist into adulthood. Direct ossification, therefore, appears to be a useful process to mimic for the engineering of replacement bone, but is unlikely to be capable of regenerating three dimensional shapes due to problems of vascularity. During development

and repair, such 3D structures are formed through the replacement of an avascular matrix secreted by hypertrophic chondrocytes, so-called cartilage-replacement bone formation or endochondral ossification.

Endochondral ossification via angiogenesis and perivascular cells

The entanglement of vascularization and osteogenesis has long complicated the study of bone formation *in vivo*; unlike cartilage or muscle, bone will only form within a vascularized site, even within the embryo. Details on the process of endochondral ossification are now confirming the early view (Buring, 1975; Thyberg and Moskalewski, 1979) that osteogenic precursors are perivascular cells (Colnot *et al*, 2005) and arrive with the ingress of vessels. Failure of vascularization of hypertrophic cartilage prevents ossification (St-Jacques *et al*, 1999; Hunziker and Driesang, 2003). In the absence of evidence to the contrary it seems likely that bone regeneration must follow a similar course; relying on the delivery of osteogenic cells by the vasculature.

The formation of bone *de novo* during embryogenesis has far less obstacles than that to be achieved during repair of the adult. During its formation in the foetus, blood vessels can infiltrate or perfuse the soft but future bony site as there is no (or little) mineralization; this occurs later to envelop the vessels within a bony cocoon. Angiogenesis into the cartilage is stimulated by the secretion of growth factors as the chondrocytes mature; these have the further role of stimulating osteogenic differentiation of the progenitors (Kronenberg, 2003). Repair of bone fractures in the adult is faced by the relative paucity of vessels and the presence of an insoluble mineral scaffold; these problems are obviated by the formation of a soft connective tissue callus that differentiates to cartilage before being replaced by bone. This is naturally viewed as a recapitulation of the process of endochondral ossification that occurs during embryonic development and the re-expression of developmental genes confirms this (Ferguson *et al*, 1999). The relative ease of producing bone within a soft, well vascularized tissue is readily observed in heterotopic ossification of muscle (myositis ossificans) and has been utilized for *in vivo* bone banking (e.g. Warnke *et al*, 2006; Heliotis *et al*, 2006). In this method an osteoinductive scaffold is implanted into the patient's muscle, bone formation and vascularization occur over some weeks and the graft is then removed and placed into its final location (Heliotis *et al*, 2006). The success of such approaches relies upon the inter-changeability of bone produced by precursors with different origins.

Pathways of bone formation in the clinic

Distraction osteogenesis and mechano-biology

An alternative, and very effective, method for the regeneration of bone is through distraction osteogenesis. However, while GTR and other void filling methods will certainly benefit from advances in our understanding of basic bone biology and the application of new agonists, it is likely that through studying the processes active during distraction osteogenesis (DO), we will understand more of the factors which regulate bone cell metabolism *in vivo*. That is, the benefits probably lie on the discovery side of the translation process. The mineralized skeleton is in a constant state of flux due to the opposing actions of osteoclasts, osteoblasts and osteocytes; this enables it to achieve functional adaptation by maintaining a minimal mass, but maximal structural support. The largest single determinant of bone mass is load, to which at least 40% can be directly attributed (reviewed Meyer *et al*, 2004). Osteoblasts and osteocytes are equipped (in terms of cytoskeleton and mechano-transduction pathways) to respond to mechanical cues (Klein-Nulend *et al*, 2005). Distraction osteogenesis takes advantage of this characteristic through the application of tensile strains onto the gap tissue between osteotomized bone. The procedure is classically broken into three parts: distraction, latency and contention; in so doing bone lengthening is achieved. Distraction osteogenesis is used in the craniofacial region (e.g. to rectify craniosynostosis or for lengthening of the mandible) and is recognized to follow the same sequence as that of long bones (reviewed Samchukov *et al*, 2001). As such it has much in common with fracture repair, following a sequence of osteotomy, inflammation, soft callus formation (at which point distraction is begun), hard callus formation and remodelling. The methods of bone formation include both endochondral and direct bone formation (Samchukov *et al*, 2001; Ueda *et al*, 2001). This demonstrates that through appropriate stimulation (tensile strain) local osteoprogenitors can produce a large segment of anatomically correct bone that is most reminiscent of the mechanism of regeneration that occurs in amphibia, such as the newt (Ueda *et al*, 2001). The inference, therefore, is that by reproducing the conditions which prevail during distraction osteogenesis we will be able to repair large skeletal defects; equally by understanding the pathways activated in response to tensile strain, we can identify new therapeutic targets for the maintenance of bone mass.

Guided tissue regeneration

The regeneration of tooth supporting structures lost as a result of periodontal disease (Figure 1) has been a long-term goal of periodontal therapy for some years. The architecture of the periodontium is such that alveolar bone, periodontal ligament and root cementum all need to be generated to produce definitive repair. This process is

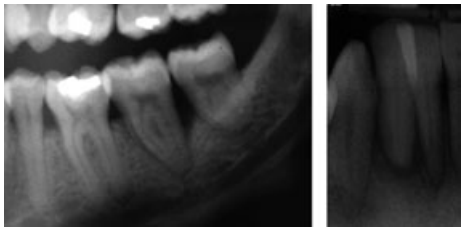

Figure 1 Periodontaldisease. Alveolar bone loss due to advanced periodontitis. The left panel shows generalized horizontal alveolar bone loss with a marked angular defect distal to the lower right second molar. The right panel shows severe loss of alveolar bone around the lower left lateral incisor

distinct from simple reattachment, where a connective tissue or epithelial layer attains reunion with the root surface. Clearly, a prerequisite for successful periodontal regeneration is the presence of a suitable population of pluripotent cells necessary for generating all the specialized hard and soft tissues of the periodontium, these cells being biologically active and in an environment that allows normal growth and development of this structure (Foster and Somerman, 2005; Taba *et al*, 2005).

Guided tissue regeneration has proved to be a useful technique in achieving regeneration of the periodontal tissues (Murphy and Gunsolley, 2003). Specifically, this technique uses barrier membranes placed into the periodontal space following surgery to prevent apical migration of epithelium or direct migration of gingival connective tissue into the periodontium coupled to the use of a packing material to preserve the volume of the wound space. This allows cells from the periodontal ligament space itself to repopulate the area and regenerate a functional periodontal attachment. While this describes the process it does not give any clues as to the mechanism, although it demonstrates that there is innate regenerative potential which requires only space, time and seclusion to restore the lost structures. For periodontal attachment at least three cell types are needed; most likely all derived from a PDL progenitor, but GTR can also be applied for the regeneration of bone alone (Donos *et al*, 2003) indicating the generality of this process. A number of materials have been used for GTR, including non-resorbable membranes composed of cellulose or expanded polytetrafluoroethylene (ePTFE) or biodegradable membranes, including collagen, polyglactic acid (PLA) and polyglycolic acid (PGA). However, the success of GTR does depend upon a plentiful supply of active progenitor cells from within the periodontal ligament space to maximize regeneration.

Supplementing GTR with exogenous growth factors

Clinical studies have demonstrated that the GTR process is most effective in healthy, non-smoking subjects who demonstrate excellent oral hygiene (Wang and MacNeil, 1998). However, the incorporation of exogenous growth factors into GTR membranes has been suggested as a method of further increasing the clinical effectiveness of this technique (Ripamonti *et al*, 1994). In theory, sustained release *in vivo* of such factors during the regenerative phase of periodontal therapy should improve cellular recruitment, activity and therefore, regeneration (Fournier and Doillon, 1996).

Bone morphogenetic proteins (BMPs) are candidate molecules for mediating periodontal attachment (Ripamonti and Reddi, 1997). These molecules can induce the formation of bone (Wang *et al*, 1990) and play an important role in mediating early interactions during tooth development, including initiation and morphogenesis of the tooth germ (Vainio *et al*, 1993; Aberg *et al*, 1997); but an obvious role for BMPs during early root development is less clear. Although *Bmp4* is expressed in apical mesenchyme of the early root and *Bmp3* in cementoblasts at later stages, other members are not expressed (Yamashiro *et al*, 2003). However, some periodontal regeneration, including alveolar bone development, has been achieved in enclosed tooth roots using implantation of partially purified human BMP extracts in combination with demineralized bone matrix (Bowers *et al*, 1991). In addition, several animal models provide evidence for enhanced periodontal regeneration in the presence of BMPs; bovine BMP fractions combined with a collagenous matrix in the Baboon (Ripamonti *et al*, 1994), recombinant human BMP2 in both rat and beagle dog models (King *et al*, 1997; Kinoshita *et al*, 1997) and BMP7 in primates (Ripamonti *et al*, 1996), whilst investigations using BMP12 have been more equivocal (Sorensen *et al*, 2004; Wikesjo *et al*, 2004). What has also become clear from these animal studies is that there can be an increased risk of ankylosis in teeth treated with exogenous BMPs, particularly BMP2 (Ripamonti *et al*, 1994; Giannobile *et al*, 1998) and, therefore, the quantity and method of delivery of these proteins is likely to be an important determinant of their future success (Wang *et al*, 2005). Interestingly, fibroblast growth factor-2 (FGF2), a member of another large family of signalling molecules, is also able to stimulate periodontal regeneration in animal models without associated ankylosis (Takayama *et al*, 2001; Murakami *et al*, 2003). Certainly, knowledge regarding the individual and combined roles of these complex proteins during periodontal development and importantly, their possible therapeutic significance, needs to be further refined (Ripamonti and Reddi, 1997).

Progenitor cells of the periodontium reside in mesenchyme of the dental follicle and investigators have now begun to focus on the cellular mechanisms required to induce differentiation of a particular lineage. Interestingly, BMP2 is more effective in the induction of a cementoblast/osteoblast phenotype within cells of the dental follicle in comparison to those of the periodontal ligament itself (Zhao *et al*, 2002), but somewhat surprisingly, this molecule has an inhibitory effect upon the differentiation and mineralization of mature cementoblasts *in vitro* (Zhao *et al*, 2003). If effective and reproducible clinical regeneration of the periodontium is to be achieved, the combinatorial and diverse response of individual cell populations within the periodontium will need to be fully understood.

A number of other cytokines have also been implicated as having a potential role in periodontal ligament regeneration. Platelet-derived growth factor (PDGF) and insulin-like growth factor-1 (IGF1) are both active in bone (Hock *et al*, 1988; Canalis *et al*, 1991) and in combination are able to stimulate formation of new bone and cementum in periodontal lesions (Lynch *et al*, 1989; Rutherford *et al*, 1992; Howell *et al*, 1997). The successful delivery of PDGF to sites of periodontal destruction via recombinant adenoviral vectors has further increased the clinical application of these proteins (Giannobile *et al*, 2001), as have the positive outcome of human studies using recombinant PDGF-BB (Nevins *et al*, 2003). In addition, nerve growth factor (NGF) has recently been shown to induce expression of bone and cementum-specific markers in human periodontal ligament cells and vascular endothelial growth factors (VEGFs) in human

microvascular endothelial cells (Xu *et al*, 2006). It is clear from the activity in this area that there is room for progress in the strategies used for the regeneration of periodontal bone, some developments which may be translated to the clinic are discussed below.

Tissue engineering approaches

There are a number of excellent reviews covering hard tissue reconstruction in the oro-facial region (e.g. Caplan, 2005; Eppley *et al*, 2005) and as one could predict, many of the proposed and deployed tissue engineering approaches use a solid material (reviewed Yoshikawa and Myoui, 2005), whereas this is rarely, if ever, the biological solution. This emphasizes the somewhat surprising fact that it is easier to induce bone growth around muscle, than it is at the site of the defect. However, it should also be noted that ectopic bone is not permanent, being resorbed over time (Urist and Lietze, 1980). It is interesting that bone regenerative strategies have frequently sought to mimic precisely that part of the bone which is refractory to angiogenesis and thus bone repair; the mineral. This is also borne out by the fact that intact bone is not osteo-inductive but demineralized bone is (Urist, 1965). Of course the reason for this is that there are competing demands upon the material, it must meet mechanical standards and these override the cellular demands. Strategies devised to date have relied almost exclusively upon the deployment of BMP-related proteins within a scaffold, often based on calcium phosphate (Seeherman and Wozney, 2005). This approach has a solid background in oro-facial reconstruction (Lee, 1997) and more recently in combination with free flap grafting this approach has achieved short-term success in patients (Heliotis *et al*, 2006; Warnke *et al*, 2006). However, the use of BMPs comes from the discovery of their osteo-inductive properties in adult muscle, where it is well recognized that this induction occurs via a cartilage intermediate (Urist, 1965; Wozney *et al*, 1988). Consistent with this, where BMPs have been successfully used in patients (Heliotis *et al*, 2006; Warnke *et al*, 2006) the site of bone formation was intramuscular not osseous. It has been possible to isolate and study cells from several niches within the bone compartment, from both adult and embryonic forms; their differentiation to the osteoblastic fate *in vitro* is routine. Equally, well-characterized models of bone induction exist, in particular the formation of ectopic bone in muscle made famous by Urist (1965). The bone anabolic effects of BMPs are not doubted and they do play myriad roles in development (Pizette and Niswander, 2000). BMPs 2 and 6 play a distinct but limited role in the development of bone as indicated by their expression in hypertrophic chondrocytes, double knockout of these factors in mice causes reduced bone mass and an inability to repair fractures via endochondral ossification (Kugimiya *et al*, 2005). The potential of BMP6 in the induction of bone has been further enhanced by the demonstration of its activity on hMSCs *in vitro* (Friedman *et al*, 2006); this data has overturned the earlier evidence, which indicated that BMPs could not induce osteogenic differentiation in these assays (Leboy and colleagues – Diefenderfer *et al*, 2003; Osyczka *et al*, 2003). The discrepancy suggests that the BMP ligands (2, 4, 6 and 7) are not interchangeable as had been supposed.

We should, therefore, be well stocked with knowledge to translate to the clinic; many trials of BMPs have been carried out, but the results have not been as good as promised. There must, therefore, be some dislocation between the basic biological findings and their application to bone regeneration. A key area that may have contributed to the failure of translation is that the production of ectopic bone in muscle or dermis occurs via a cartilage intermediate (Reddi and Huggins, 1972), it is not the same as stimulating cells to undergo osteoblastic differentiation directly. This results in the potential use of factors which ectopically induce bone and cartilage, but fail to enhance bone regeneration. An example of this is the ability of TGFβ to stimulate bone formation in the abdominal muscles, while being unable to increase bone formation in the calvaria (Duneas *et al*, 1998). It is still somewhat paradoxical, however, that although it is connective tissue cells that respond to osteo-induction in heterotopic ossification, this is the tissue that we must exclude using GTR-based strategies. This work has been extended through the use of a wider range of protein growth factors to enhance (periodontal) bone regeneration including VEGF, PDGF, IGF-1, FGF-2, etc. (Schliephake, 2002; Taba *et al*, 2005), but none of these have surpassed the *in vivo* effects of BMPs (Schliephake, 2002). All have been shown to promote osteogenic differentiation of mesenchymal cells but details of how they act, on which cells, for how long, are lacking; what is promising about these molecules is that they can promote angiogenesis.

The regenerative approach to bone augmentation and repair

If regenerative medicine differs from conventional medicine it is in the pathways that one seeks to stimulate (or antagonize) and in the local or targeted nature of the stimulus (Figure 2). In more general terms its aim is to recapitulate the developmental processes used in the initial genesis of that tissue to effect perfect repair. It requires the activation of progenitor cells capable of proliferation and differentiation to the desired end-point. This is contrasted with wound repair, which produces scar tissue to heal (Ferguson *et al*, 1996). In some instances, such as long bone fracture repair, the recapitulation of an embryonic process occurs resulting in perfect repair: this is also a regenerative event. Bone regeneration *in vivo* is preceded by angiogenesis, the osteo-progenitors are perivascular cells; an agent may stimulate osteoblastic differentiation *in vitro*, but if the vessels have not delivered the progenitors this activity will be of no use when deployed *in vivo*. The shift in emphasis from the properties of materials to the potential of the cells has led to new approaches cognizant of the capacity and the needs of the cells – in terms of factors and extra-cellular matrix (Figure 3).

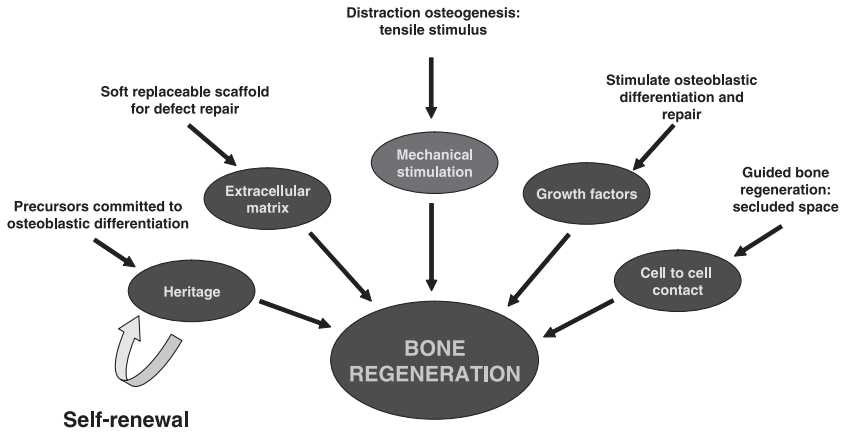

Figure 2 Multiple effector pathways can be, or need to be, activated to achieve bone and tissue regeneration. These underpin current strategies, such as distraction osteogenesis and guided bone regeneration as well as informing the design of new therapies based on growth factor delivery or resorbable scaffolds

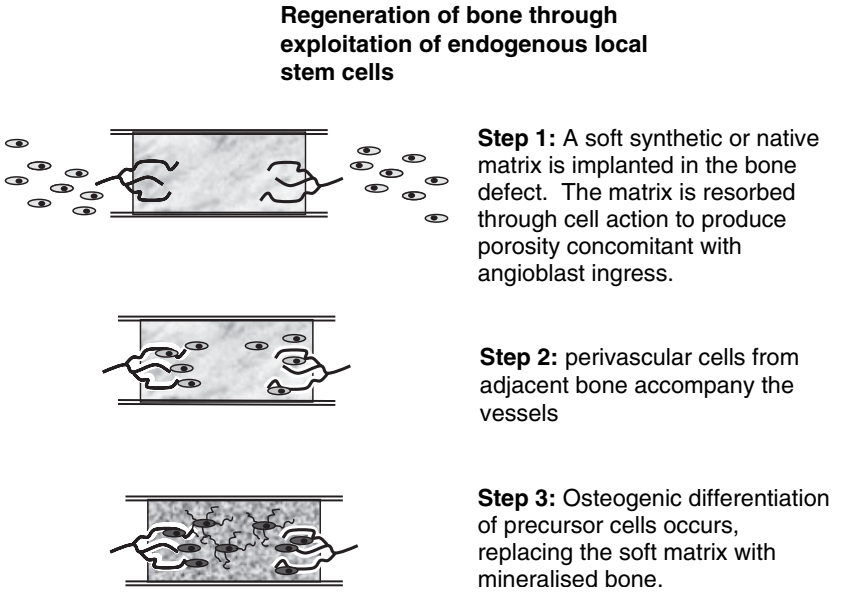

Figure 3 Regeneration of bone through exploitation of endogenous local stem cells

Hubbell *et al* have pursued this theme using a synthetic but degradable scaffold (Lutolf *et al*, 2003) to regenerate bone successfully in a non-load bearing model (critical size defect); this demonstrates the potential of a more biomimetic approach (see Figure 3). This represents an important breakthrough in terms of recognizing the importance of the cellular contribution to tissue regeneration; matching the properties of the construct to the needs of the neighbouring cells. This more closely matches the mechanism of endochondral ossification that occurs during development, growth of the long bones and repair (Kronenberg, 2003). When coupled to similarly inspired osteogenic stimulants such an approach may truly herald an advance in the clinical treatment of defect repair. The greater challenge will then be to retain the eucellular qualities of the scaffold, while enhancing the mechanical properties for load-bearing applications.

New candidate pathways

We have described how the gene expression profile of foetal and adult bone precursors and their differentiated descendants varies, what then is the likelihood of adult bone stem cells responding to such factors? The evidence that exists indicates that they will: *Indian hedgehog* is re-expressed during long bone fracture repair in the adult mammal (Ferguson *et al*, 1999; Le *et al*, 2001). Preliminary studies on adult osteo-progenitors also confirm the view that stimulation of the Hedgehog pathway has regenerative potential; calvarial regeneration is enhanced by gene-mediated delivery of *Sonic hedgehog* (Edwards *et al*, 2005). Mouse calvarial osteoblasts and human bone marrow-derived MSCs respond to Hedgehog agonists *in vitro* by up-regulating alkaline phosphatase and depositing mineral (P.G.B., unpublished observations). During the development of the skeleton there is interplay between Hedgehog and both Wnt and BMP signalling pathways (Spinella-Jaegle *et al*, 2001; Rawadi *et al*, 2003; Hu *et al*, 2005). Co-stimulation of the Wnt pathway and BMP pathway produces pronounced osteogenic differentiation of multipotent cells *in vitro*, and promotes new calvarial bone formation *in vivo* (Mbalaviele *et al*, 2005). While causative sites of *Wnt* ligand expression are still sought,

overwhelming genetic evidence has demonstrated their importance. Different mutations in the Wnt co-receptor *Lrp5* have been shown to cause high bone mass disorders or, alternatively, osteoporosis in humans (reviewed Westendorf *et al*, 2004). Osteoblast genesis from precursors in the foetus is dependent upon active Wnt signalling; in its absence chondrogenesis ensues, either in calvarial-derived osteoblasts or in the long bone rudiments (Day *et al*, 2005; Hill *et al*, 2005). Application of Wnt ligand itself is hampered by the nature of the protein; it is hydrophobic, lipid modified and consequently insoluble. However, small molecule screens are identifying novel compounds capable of Wnt pathway interference or stimulation (Liu *et al*, 2005; McMillan and Kahn, 2005). Again, as our knowledge of the optimal stimulation of osteogenic differentiation improves we are finding, perhaps unsurprisingly, that it matches well the growth factors present in hypertrophic cartilage; Hedgehog, Wnt, Bmp, Fgf and VEGF ligands are all expressed in this location (Kronenberg, 2003).

Our own efforts in this direction have focussed on the use of dense collagen, produced from hydrated native collagen through the elimination of excess water. This yields a dense collagen matrix with a high protein content and increased mechanical properties (Brown *et al*, 2005). Collagen is well recognized as an ideal cellular environment, especially for the culture and differentiation of osteoblasts (e.g. Casser-Bette *et al*, 1990), but has very weak mechanical properties, due to its hyper-hydrated state when reconstituted. We have examined cellular differentiation in this milieu and found it to be an excellent conduit for osteogenic differentiation of bone marrow-derived mesenchymal stem cells and primary osteoblasts. Collagen has also been recommended as a drug delivery device (Freiss, 1998; Wallace and Rosenblatt, 2003) and for the release of growth factors (Kanematsu *et al*, 2004). We hope that this combination of traits will at the very least provide a test-bed for agonist delivery and ideally act as a blueprint for the characteristics of a synthetic scaffold.

Conclusion

There are myriad factors, which complicate the translation of breakthroughs in the basic biology of osteoprogenitor differentiation to regeneration of oro-facial tissues. Almost all mechanistic data on human-specific responses can only be derived from *in vitro* experiments; the source of the cells used for these studies will greatly influence the outcome and conclusions. Fortunately research into the fundamental nature of the source cells is elucidating the basis of such differences. These descriptive studies can, therefore, complement the functional experiments carried out through transgenesis, within model organisms and *in vitro*. By combining this data we will be best placed to develop the necessary technologies for clinical implementation; this can ultimately prove that we understand how to motivate bone stem cells.

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