TISSUE ENGINEERING AND REGENERATIVE APPROACHES TO IMPROVING THE HEALING OF LARGE BONE DEFECTS

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Abstract

Despite the high innate regenerative capacity of bone, large osseous defects fail to heal and remain a clinical challenge. Healing such defects requires the formation of large amounts of bone in an environment often rendered hostile to osteogenesis by damage to the surrounding soft tissues and vasculature. In recent years, there have been intensive research efforts directed towards tissue engineering and regenerative approaches designed to overcome this multifaceted challenge. In this paper, we describe and critically evaluate the state-of-the-art approaches to address the various components of this intricate problem. The discussion includes (i) the properties of synthetic and natural scaffolds, their use in conjunction with cell and growth factor delivery, (ii) their vascularisation, (iii) the potential of gene therapies and (iv) the role of the mechanical environment. In particular, we present a critical analysis of where the field stands, and how it can move forward in a coordinated fashion.

Keywords: Bone, vascularisation, scaffolds, gene therapy, stem cells, drug delivery, large bone defect, tissue engineering, regenerative medicine, translational and preclinical research.

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Introduction

The healing of large bone defects is a major clinical challenge. Although bone possesses remarkable repair and regenerative powers of its own, there are numerous clinical conditions in which the size, location, and/or local environment of the bone defect results in impaired healing. Large bone defects are a problem in craniomaxillofacial surgery, as well as in orthopaedics more generally. Examples of large bone defects include tumour resections, infection, fractures accompanied by substantial soft tissue trauma, congenital deformities and segmental loss. In each of these cases, the large volume of tissue that needs to be replaced makes it very challenging to achieve sufficient quantity and quality of new bone formation. In addition, the healing of larger defects is critically dependent on the presence of an appropriate vascular supply to support regeneration and remodelling of new bone tissue.

In clinical practice the standard treatment for large bone defects is the use of autogenous or allogenic bone grafting to provide an osteogenic and/or osteoconductive stimulus, and thereby promote bone regeneration and union. However, insufficient volume of available tissue, donor site morbidity (autogenous), inconsistent osteogenic activity, late biomechanical failures, and the possibility of allogenic disease transmission reduce enthusiasm for their use. While great progress has been made with the use of osteoconductive bone graft substitutes and distraction osteogenesis, it is clear that complex clinical cases where novel therapies are required still exist. Finally, the challenging wound healing environment in which large bone defect restoration often needs to take place, mandates a strategy that both fills the bone gap and promotes vascularisation and repair. Although vascularised free flaps are currently an important and successful clinical option to address these concerns, it requires a long involved and often risky operation, with attendant extended hospitalisation and high cost. These challenges have motivated the field of

musculoskeletal tissue engineering to find a solution that will aid the surgeon and the patient in tackling some of the most difficult and challenging reconstructive conundrums in both orthopaedic and craniofacial surgery. The general strategy was elegantly summarised in the Giannoudis Diamond concept, whereby the final outcome is dictated by the combination of osteogenic cells, osteoconductive scaffolds, growth factors and the mechanical environment (Giannoudis *et al.,* 2007).

This review covers the techniques and strategies that have been developed to address the multifaceted challenges posed by the complicated problem of large bone defect healing (Fig. 1). The term "large bone defect" is used here in the sense of defects that are too large to heal spontaneously *i.e.* are of critical size. The review emphasises strategies based upon tissue engineering and regenerative medicine, sometimes abbreviated collectively as TERM to emphasise their overlapping nature. Unlike fractures, critical size segmental defects have no natural healing process and thus no native biology to model. Rather, TERM for large bone defects engages a variety of approaches, including scaffold design and selection, drug and morphogen delivery, cell- and gene-based therapies, vascularisation strategies, and mechanical environments that can be used to promote regeneration of bone. The uniqueness of large bone defects is the size of the void that needs to be filled and vascularised, all in the absence of local endogenous osteogenic signals. The goal here is to give an overview of the components that have been applied to the problem to date, as well as to provide insight into how these components can be combined in future more advanced therapies.

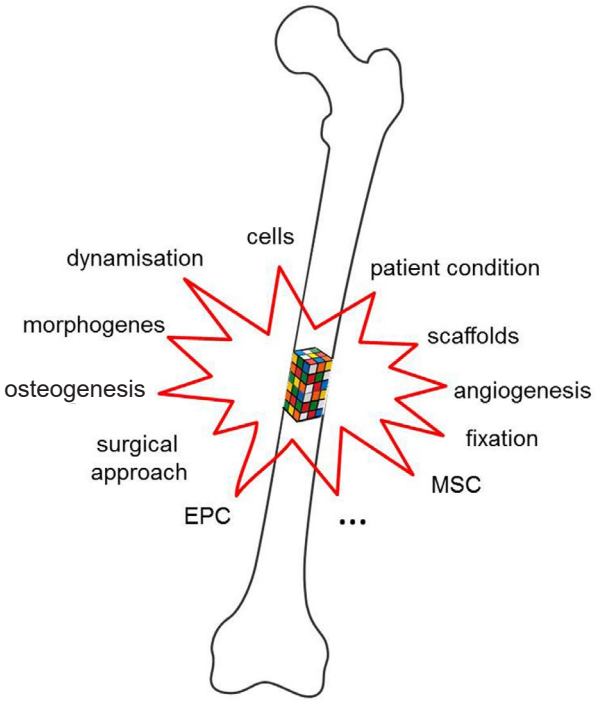

Fig. 1. Large bone defect: a multifaceted challenge. Bone healing is a complex process involving the interplay of many factors and well-orchestrated mechanisms. Tissue engineering approaches aim to resume the complexity of these events by combining scaffolds, cells, growth factors and mechanical environment. The choice of cells, their association or not with scaffolds, the local delivery of growth factors, the application mechanical stimulation of the defect (*e.g.* active dynamisation and timing), but also the patient condition and the surgical approach are as many factors influencing the healing outcome. Here, we give an overview of the techniques and strategies that have been developed in the past 10 years to address the complex situation of large bone defects.

Scaffolds

Requirements for a bone regeneration scaffold include mechanical properties (*e.g.* desired stiffness and compression resistance), degradability, macro- and micro-porosity, and nanometre-scale topography. Encompassing all of these requirements into one material or composite is challenging and limits the number of suitable base materials that would also have an expeditious route to clinical application. In the following we describe some of the materials being actively investigated for use in large bone defects. The examples used are far from being an exhaustive list.

Natural scaffolds

Many tissue engineering strategies employ scaffold materials to provide both mechanical support and biological function. A logical approach to scaffold design is to mimic the materials and architectures found in native tissues. To this end, a variety of extracellular matrix (ECM) proteins, polysaccharides and other "naturally-derived" materials have been used to create scaffolds for bone tissue engineering. ECM-derived scaffolds have the advantage that cells can recognise and bind to them by specific cell surface receptors, and thereby can receive biochemical signals directly from the scaffold (Shekaran and Garcia, 2011; Siebers *et al.,* 2005). In most cases, cells can also degrade, synthesise, and remodel these natural matrices in response to environmental cues (Ferreira *et al.,* 2012). For the repair of large bone defects, the mechanical and space-filling attributes of the scaffold are of primary importance. Pure naturally-derived materials, such as collagen scaffolds, typically have inferior mechanical properties relative to both the tissues from which they are derived and to synthetic polymer scaffolds (Gibbs *et al.,* 2014). Accordingly, their use in large bone defects without additional structural support is challenging. For this reason, there is a growing interest in decellularisation of harvested tissues for use as scaffolds, in an effort to keep the native architecture and compositional complexity intact (Cheng *et al.,* 2014). In addition, a variety of composite materials that combine the desirable features of specific protein and polysaccharide components of the ECM have been developed and used as scaffolds in bone tissue engineering (Wang and Stegemann, 2010).

The collagen superfamily of proteins consists of over 25 molecular isoforms. The most common form is type I collagen, which is a main structural constituent of many

tissues, and is the predominant non-mineral component of bone. Collagen "sponges" are typically generated by freeze-drying collagen based slurries, creating a porous architecture (O'Brien, 2011). Such sponges are used widely as haemostatic agents, and also have been used in bone repair as a delivery vehicle for bone morphogenetic proteins (BMPs) (Geiger *et al.,* 2003; Wei *et al.,* 2012) or as gene delivery platforms (Curtin *et al.,* 2012; Tierney *et al.,* 2013).. Their expanded use is being investigated in preclinical studies for a variety of orthopaedic applications (d'Aquino *et al.,* 2009; Hosseinkhani *et al.,* 2006). Type I collagen has also been reconstituted into fibrillar form by electrospinning (Huang *et al.,* 2001; Matthews *et al.,* 2002). Efforts to further mimic the composition and function of bone have led to collagen-ceramic composites (Wahl and Czernuszka, 2006). Hydroxyapatite or tricalcium phosphates are often used in this application to represent the mineral content of bone (Gleeson *et al.,* 2010; Zheng *et al.,* 2014).

Chitosan is an aminated polysaccharide that is derived from the deacetylation of chitin, a structural component of the exoskeleton of crustaceans and some fungi. Chitosan can be made water soluble, and has been used in ways similar to collagen to make sponges, meshes and scaffold materials for bone tissue engineering (Costa-Pinto *et al.,* 2011; Heinemann *et al.,* 2010). In scaffold form, pure chitosan allows cell attachment and has been suggested to be osteoinductive (Di *et al.,* 2005). Composites of chitosan, with other matrix components to improve its mechanical properties, are more commonly used for orthopaedic applications (Venkatesan *et al.,* 2012). Blends of chitosan and other materials have been electrospun into fibre meshes (Chen *et al.,* 2011; Zhang *et al.,* 2008), and composites have been used in sponge format, including combination with other polysaccharides (Park *et al.,* 2013), proteins (Wang *et al.,* 2013), and mineral (Pighinelli and Kucharska, 2013). The free amine groups on the chitosan molecule allow it to be crosslinked with the same agents as used for protein matrices (Reves *et al.,* 2013; Wang and Stegemann, 2011), which can increase its mechanical strength and resistance to degradation. In addition, the positive charge on the chitosan molecule allows the material to be used for drug and gene delivery directly from the scaffold (Cao *et al.,* 2012b; Goncalves *et al.,* 2012).

Hydrogels form another class of natural polymer scaffolds. These materials are hydrated, interconnected networks of polymer chains. An inherent advantage of such hydrogels is that they can be delivered using minimally invasive techniques, will fill defects of complex shapes and can be combined with cells and/or osteoinductive factors (Drury and Mooney, 2003). Alginate hydrogels have been used for gene (Krebs *et al.,* 2010) and growth factor delivery (Kolambkar *et al.,* 2011) and such systems have been shown to promote functional repair of critically-sized bone defects. Promising results have been obtained using natural hydrogels such as fibrin (Chung *et al.,* 2007; Woodruff *et al.,* 2007) and gelatin (Yamamoto *et al.,* 2003; Yamamoto *et al.,* 2006) as delivery vehicles for therapeutic factors for bone regeneration. One concern with certain classes of hydrogels for large bone healing is insufficient degradation of hydrogel (Rizzi *et al.,* 2006), which may impede vascularisation of the implant. Such problems can potentially be overcome by modulating the hydrogel to accelerate its rate of degradation (Alsberg *et al.,* 2001; Jeon *et al.,* 2009). Hydrogels typically do not have compression-resistant mechanical properties, but can be included within other common orthopaedic devices (*e.g.* titanium cages) and used to stimulate new bone formation.

Demineralised bone matrix (DBM) is an example of a natural biomaterial that is commonly used clinically as a bone graft substitute (Urist, 1965). Such grafts are typically produced by the acid extraction of the mineral content from allogeneic bone and contain growth factors, other non-collagenous proteins and type I collagen (Sawkins *et al.,* 2013). The rigorous processing and sterilisation that such grafts must undergo prior to implantation can negatively impact their osteoinductive properties which may at least partially explain the variable results seen with DMB (Gruskin *et al.,* 2012; *Peterson et al.,* 2004). To overcome such limitations, DBM can also be used as a delivery system for novel therapeutics (Lieberman *et al.,* 1999). Decellularised ECM derived from other mammalian tissues such as small intestine submucosa have been used as biological scaffolds for bone regeneration (Badylak *et al.,* 2009; Kim *et al.,* 2010; Moore *et al.,* 2004). It has also been demonstrated that bone-like ECM synthesised *in vitro* by osteoblastic cells can enhance osteogenesis of mesenchymal stem cells (MSCs) (Datta *et al.,* 2005), and MSC-derived ECM enhances the retention of implanted cells into the remodelling phase of healing, resulting in reproducible and complete repair of critical-sized bone defects in mice (Zeitouni *et al.,* 2012).

Synthetic scaffolds

Investigators have developed a variety of synthetic scaffolds for large bone defect healing, and a common approach involves mimicry of some aspects of the native bone ECM.

The catalogue of synthetic bone biomaterials used in critical sized defects features a wide range of biominerals, including hydroxyapatite, β-tricalcium phosphate, amorphous calcium phosphate, calcium silicate bioactive glasses, and biphasic calcium phosphates. The bone-like mineral layer formed on the surface of these materials has been shown to influence critical components of the bone formation process, including proliferation of bone-precursor cells (Chou *et al.,* 2005), osteogenic differentiation of bone-forming cells (*e.g.* marrow-derived MSCs, adipose-derived MSCs, pre-osteoblasts, and osteoblasts) (Barradas *et al.,* 2012; Chou *et al.,* 2005; Murphy *et al.,* 2005), and localised sequestering of bone growth factors (Suarez-Gonzalez *et al.,* 2012). Recent studies suggest that released mineral ions (*e.g.* calcium (Barradas *et al.,* 2012), phosphate (Shih *et al.,* 2014), magnesium (Hussain *et al.,* 2012; Schwartz and Reddi, 1979), strontium (Yang *et al.,* 2011)) may be partly responsible for the behaviour of bone precursor cells. These mineral ions have been associated with expansion of bone precursor cells, osteogenic differentiation of marrow-derived MSCs (Barradas *et al.,* 2012; Shih *et al.,* 2014), and optimised non-viral transfection of multiple bone precursors (Choi *et al.,* 2013).

Common synthetic polymer materials used to form scaffolds for bone healing include poly(alpha-hydroxy esters) (Yu *et al.,* 2010), poly(urethanes) (Guelcher, 2008), poly(propylene fumarate) (Wang *et al.,* 2006), and poly(carbonates) (Kim *et al.,* 2012; Luangphakdy *et al.,* 2013). Thermoplastics such as poly(L-lactide), poly(lactide-co-glycolide), poly(ε-caprolactone), poly(propylene fumarate, and poly(urethanes) can be readily processed to allow for interconnected macroporosity, with control over the pore size, structure, and interconnectivity. In addition, these materials can be formed *via* diverse manufacturing schemes, including casting, injection moulding, and 3-D printing. All have been applied within large bone defects, both as void filler and as an osteoconductive matrix.

Synthetic hydrogels composed of poly(ethylene glycol), poly(propylene fumarate-co-ethylene glycol), and hyaluronic acid have each been used for bone precursor cell culture *in vitro* or to enhance critical bone defect healing *in vivo*. In each case the materials can be loaded with bone precursor cells and/or pro-osteogenic molecules to stimulate bone formation (Cartmell, 2009; Salinas and Anseth, 2009). One attractive feature of these hydrogels is their ability to incorporate and deliver controllable dosages of biologically active molecules, including cell adhesion peptides, proteolytically-degradable peptides, ECM proteins, and growth factors. Another feature is the ability to form hydrogels *in situ*, which opens up new minimally-invasive clinical opportunities (Behravesh *et al.,* 2003; Kim *et al.,* 2009). Self-assembling hydrogels have been designed to gelate *in situ* and deliver environments that promote bone formation. For example, peptide amphiphiles can self-assemble into nanofibrous matrices that have been used to nucleate mineral formation (Hartgerink *et al.,* 2001), present peptides for cell adhesion (Webber *et al.,* 2010), or present peptides for growth factor binding (*e.g.* transforming growth factor (TGF)-β binding (Shah *et al.,* 2010)).

Some recent examples highlight the potential to combine distinct and complementary synthetic materials to create composite. Investigators have created composites of synthetic polymers and biominerals, taking advantage of the resultant processing benefits of the polymers and the inherent biological activity of the biominerals, resulting in enhanced scaffold compressive modulus, improved osteoconductivity, and greater osseointegration. In one example, 3D printing approaches have been used to create biomineral-coated, 70 % porous poly(ε-caprolactone) scaffolds with mechanical properties that withstand masticatory loads in the mandible, and stimulate bone regeneration as they degrade (Chanchareonsook *et al.,* 2013). Other studies demonstrated that mineral-coated hollow tubes composed of poly(ε-caprolactone) can stimulate bone regeneration in sheep tibia defects (Cipitria *et al.,* 2013) and sheep lumbar spine fusion (Yong *et al.,* 2014). These examples and others suggest that innovative manufacturing of common bone biomaterials can produce a useful toolkit for large bone defect healing.

Furthermore, while we focus this section on synthetic materials, it is noteworthy that a subset of naturally-derived polymers can also be synthetically modified to create natural/synthetic hybrids that stimulate bone formation. For example, alginate hydrogels can be modified with peptide ligands and used to deliver bone-forming stem cells or osteoinductive growth factors (Drury and Mooney, 2003; Lee and Mooney, 2012). Similarly, fibrin hydrogels can be used as a platform to covalently link pro-osteogenic (Arrighi *et al.,* 2009; Schmoekel *et al.,* 2005) or pro-angiogenic (Ehrbar *et al.,* 2004) growth factors, which are subsequently delivered during new bone formation. While these materials generally do not match the synthetic and manufacturing adaptability of synthetic materials, they open up the possibility of hybrid approaches that combine the complementary advantages of synthetic and natural components.

Synthetic scaffold design involves a series of design trade-offs, which present inherent challenges for large bone defect healing. For example, scaffolds require optimised mechanical properties for a particular clinical approach, but must also provide adequate porosity for cell infiltration and tissue formation and degradability over a timeframe that scales with the timing of new bone formation (Hollister, 2005). In addition, scaffold design parameters such as μm-scale and nm-scale geometry have become increasingly appreciated as critical regulators of osteogenesis. In particular, nm-scale pillars and fibres have been associated with enhanced osteogenic differentiation of bone-forming stem cells *in vitro* (Dalby *et al.,* 2007), as well as increased osteogenesis *in vivo* (Ingavle and Leach, 2013). The diversity of existing and emerging parameters that appear to be important for large bone defect healing will call for efficient – and perhaps high throughput – screening strategies to identify optimal scaffold materials.

It is noteworthy that one reason why scaffold materials developed to date have been composed of similar base materials relates to the relatively complex regulatory path for novel bone scaffolding materials. Materials comprising new combinations of clinically established base materials typically provide a more rapid route to regulatory approval and clinical applications. In particular, while combinations of existing, FDA approved materials may only require one to demonstrate substantial equivalence to an existing "predicate device", novel scaffold materials often require substantial preclinical studies and one or more clinical studies prior to regulatory approval. The adaptable features of commonly used scaffold materials coupled with the relatively complex regulatory path of novel materials limits innovation.

Drug and growth factor delivery

A series of small molecule drugs (*e.g.* bisphosphonates) has been used to treat orthopaedic diseases such as osteoporosis, osteonecrosis, and osteolysis. However, small molecule drugs have not been widely used in large bone defect healing applications. This is perhaps not surprising, as these drug classes are not typically designed to induce formation of new bone tissue in large defects, but rather to regulate the systemic balance between bone resorption and formation. Instead, the focus of large bone defect healing studies has been on local, bone stimulating molecules known to influence natural bone development and healing, such as

growth factors, hormones, cytokines, and antibodies. The common strategy involves designing a molecule "carrier", which is then combined with an orthopaedic device (Sandor *et al.,* 2013; Warnke *et al.,* 2004). Notable examples include BMP-loaded collagen matrices combined with titanium cages (Boden *et al.,* 2000; Kanayama *et al.,* 2006) or other metallic hardware (Govender *et al.,* 2002). The now extensive clinical experience with BMPs and other bone stimulating molecules suggests critical challenges that must be addressed in the next generation of large bone defect healing strategies. Here, we focus on describing critical challenges to be addressed in next generation of drug delivery strategies, with illustrative examples included.

First, there is a significant pharmacokinetic challenge in delivery of bone stimulating molecules. Recombinant human (rh)BMP2 delivery for instance, is the most prevalent drug delivery strategy used for bone regeneration. While Medtronic's rhBMP-2-releasing device Infuse™ has achieved a great deal of clinical success in lumbar spine fusion, and widespread use in other clinical indications, it has also been associated with serious side effects (Fu *et al.,* 2013; Faundez *et al.,* 2016). The side effects result in part from the mg-scale quantity of rhBMP-2 delivered, which is multiple orders of magnitude more rhBMP-2 than one might find in a healing bone defect. These side effects could also signal that rhBMP-2 has a narrow therapeutic index, which is a measure of the difference between the clinically effective dosage and the toxic dosage of a drug. As a result, recent studies have focused on controlling the dosage and release kinetics of bone stimulating molecules in order to identify optimal pharmacokinetics for bone healing (King and Krebsbach, 2012; Seeherman and Wozney, 2005). It is not yet clear what combination of total dosage and release kinetics can stimulate bone regeneration while limiting side effects, but it is clear in pre-clinical models that sustained release can decrease the total rhBMP-2 dosage needed to stimulate bone regeneration (Jeon *et al.,* 2008; Kolambkar *et al.,* 2011). Indeed, the signalling mechanisms activated by bone stimulating molecules are typically not unique to bone formation, and molecules are often selected based on their ability to induce heterotopic bone formation. Thus, there is a general need for systematic studies on the influence of localised dosage and release kinetics.

Second, there is a substantial formulation challenge in delivery of bone stimulating molecules. Proteins with significant tertiary structure have a strong tendency to denature, degrade, and/or aggregate under standard physiological conditions, resulting in rapid loss of biological activity. For example, basic fibroblast growth factor (FGF) loses biological activity within minutes in aqueous solution in the absence of heparin (Nguyen *et al.,* 2013). These types of molecules also tend to have narrow therapeutic indices, which results in a need to deliver the molecules in a narrow dosage range. One illustrative example is vascular endothelial growth factor (VEGF), which has been shown to promote blood vessel sprouting within a relatively limited dosage range *in vivo* (Lee *et al.,* 2000). The ideal approach would be capable of stabilising bone stimulating molecules against inactivation, while also enabling controllable release kinetics from a desirable scaffold material.

In view of these challenges, most common biomaterials used for bone healing are plagued by critical limitations. Elastomeric polymer networks (*e.g.* hydrogels) allow for molecular transport, which can result in poor bioavailability of a released molecule. Thermoplastics (*e.g.* poly(alpha-hydroxy esters)) can be designed to encapsulate and release molecules with controllable dosage and release kinetics, but the biological activity of the released molecules is often significantly compromised due to aggregation, denaturation, and degradation (Zhu *et al.,* 2000). Recent studies with nano-structured materials provide promising solutions to the current challenges. Lipid nanocapsules and mineral capsules have been shown to maintain stability of proteins (Giri *et al.,* 2011). Recent studies indicate that nano-structured biomineral coatings can uniquely stabilise proteins against degradation, while also enabling controllable release kinetics by coating dissolution (Ge *et al.,* 2012; Lu *et al.,* 2009; Suarez-Gonzalez *et al.,* 2012). This direction is promising, as it may address each of the major challenges in growth factor delivery. Further, it is possible to design broadly adaptable biomineral coatings for controllable delivery of peptides, proteins, DNA, cells, and combinations thereof (Choi and Murphy, 2010; Jongpaiboonkit *et al.,* 2009; Lee *et al.,* 2010a; Lee *et al.,* 2010b; Zhang *et al.,* 2010a). In another approach, growth factors have recently been stabilised by heparin-mimetic ligands, which can be covalently linked within hydrogels (Nguyen *et al.,* 2013).

It is important to note that combining bone stimulating molecules with an appropriate scaffold while controlling stability and pharmacokinetics is just one of several inherent challenges in drug delivery. There are unique, complex dynamics in each bone defect environment that make it difficult to define a consistently desirable delivery dose and time scale. The integrity of the soft tissue envelope, status of the periosteum, and age-dependent abundance of bone-forming cell types are among the variables that are not normalised across different patient populations. These complexities make it difficult to arrive at a definitive therapeutic index for scaffold-based drug delivery. In addition, gene expression analyses have shown that over 6,500 genes are differentially regulated during bone healing (Rundle *et al.,* 2006), which suggests a molecularly complex environment in which multiple drugs may be needed to promote optimal formation of bone and other supportive tissue types (*e.g.* neural, vascular tissues), particularly in large defects. However, the substantial barriers to regulatory approval of devices that deliver a single biologic suggest that carriers for multiple biologics may not be clinically realistic in the foreseeable future. In view of this complexity, there is a clear need to develop adaptable scaffolds that can be used to gain fundamental insights into induced bone formation in a context that can then be efficiently translated to clinical applications.

Cell delivery

The rationale behind delivery of exogenous cells for bone repair is that addition of appropriate cell types may rescue or potentiate regeneration in cases where

the natural healing response is compromised or blocked. Only cells can create bone, and therefore transplantation of cells is a logical strategy to overcome recalcitrant healing. Importantly, bone healing is a highly spatially and temporally coordinated process, and therefore it is difficult to recapitulate the normal cascade of events using biomaterials or growth factors alone. Biomaterial-mediated delivery of cells is often used to enhance the engraftment, viability and function of the transplanted cells, and may also be used in conjunction with bioactive factor delivery to mimic physiological healing. Use of a scaffold or matrix typically enhances the mechanical and space-filling function of a transplant, and can provide instructive cues to guide cell function and tissue regeneration. Most cell delivery strategies focus on application of bone forming cells, such as osteoblasts or their precursors (Lee *et al.,* 2009). However, more recent approaches have also targeted concomitant modulation of other physiological processes, such as development of a nourishing vasculature (Rao *et al.,* 2015) or management of the inflammatory response (Loi *et al.,* 2016).

A variety of cell types have been used in bone regeneration strategies. For large bone defects in particular, the use of exogenously supplied cells may be necessary, due to the need for regeneration of larger tissue volumes. In these cases, scaffolds to support cell delivery promote engraftment and provide a space-filling function are often used. The choice of preferred cell type may also depend on the application and age of the patient and in some cases combinations of cells can be applied (Wise *et al.,* 2015). Osteoblasts, the cells that secrete and assemble the ECM of bone, have been delivered in hydrogel biomaterials to enhance bone formation (Alsberg *et al.,* 2001; Burdick and Anseth, 2002); however, issues of immune rejection would require an autologous source for these cells. The difficulties in isolating and expanding osteoblast cells are substantial and make their use in clinics unlikely.

A variety of progenitor cell types have also been examined as cell sources in bone tissue engineering. MSCs are multipotent progenitors that have been shown to differentiate into connective tissue cell types, including osteoblasts (Augello *et al.,* 2010; Rosenbaum *et al.,* 2008), and also have been shown to be potent sources of paracrine signalling factors (Parekkadan and Milwid, 2010) that potentiate healing. Cell surface, or CD (cluster of differentiation) markers, are commonly used to identify MSCs (reviewed in (Harichandan and Buhring, 2011)), yet they should be used with caution. There is increasing evidence that while able to distinguish between mesenchymal and haematopoietic cells, they are not able to define characteristics of stemness (Whitney *et al.,* 2009). However, CD105+ and Stro1+ cells have been proposed as clinically relevant populations. Many groups have published changes in MSC phenotype and loss of mutipotentiality with monolayer expansion (Bruder *et al.,* 1997; Banfi *et al.,* 2000; Bonab *et al.,* 2006). One factor which has been shown to be correlated with maintenance of stemness is leukaemia inhibitory factor 1 (LIF1), the expression of which decreases with monolayer expansion and during differentiation (Whitney *et al.,* 2009).

When considering clinical use of cells, the complications engendered by monolayer expansion provides a significant regulatory hurdle (Bara *et al.,* 2014). This has increasingly led to studies investigating whether freshly isolated, minimally manipulated cells can be used for bone repair. It has been shown that freshly isolated marrow cells can lead to improved bone healing if more than 1,500 colony forming units (CFU) of mesenchymal cells are applied per cm^3 of defect (Hernigou *et al.,* 2005). Combining this finding with intra-operative cell harvesting devices provides a potential mechanism by which cell therapy can be readily applied. The use of MSCs offers the possibility of using banked cells, and it has been suggested that allogeneic MSCs are hypoimmunogenic relative to other cell types (Abumaree *et al.,* 2012; Yi and Song, 2012).

MSCs from bone marrow (BMSCs) have been investigated widely in bone tissue engineering (Yousefi *et al.,* 2016). MSCs can also be isolated from adipose tissue, and these cells are often referred to adipose-derived stem cells (ASCs). Obtained through subcutaneous aspiration, adipose tissue presents advantages of easier accessibility (Strioga *et al.,* 2012) with minimal donor site morbidity (Housman *et al.,* 2002) and permits the harvest of larger numbers of MSCs compared to other sources (Fraser *et al.,* 2006). The immunophenotype and other biological characteristics of ASCs are generally similar to marrow-derived MSCs, though there are some differences (Pachon-Pena *et al.,* 2011). Indeed, according the cell isolation procedure, a mixed population of cells containing both stromal and endothelial progenitors can also be obtained intraoperatively from the stromal vascular fraction of adipose tissue. These properties make them attractive for bone regeneration (Buschmann *et al.,* 2012; Park *et al.,* 2012). Several attempts to heal large bone defects in animal models have been made using scaffolds loaded with ASCs, but with inconsistent results. Success has been reported for the healing of calvarial defects (Dudas *et al.,* 2006; Follmar *et al.,* 2007), but large segmental defects in long bones do not always heal in the absence of BMP-2 (Hao *et al.,* 2010; Li *et al.,* 2007; Peterson *et al.,* 2005). When the effectiveness of ASCs and BMSCs was compared in a large segmental defect in sheep (Niemeyer *et al.,* 2010) healing was greater with BMSCs. The latter have shown efficacy in one human study (Quarto *et al.,* 2001) and progenitor cells obtained from periosteum were able to regenerate a human phalanx when applied on a coral scaffold (Vacanti *et al.,* 2001).

Totipotent cells sources, such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), have been less commonly explored in bone tissue engineering. Culturing ESCs is technically challenging, and the embryonic source is ethically controversial. However, ESCs have recently been used to derive MSCs, which in turn have been applied to bone regeneration (Arpornmaeklong *et al.,* 2009; Kuhn *et al.,* 2014). iPSCs are a newer potential cell source that offer the possibility of generating pluripotent cells from reprogrammed adult somatic cells (Ko and Im, 2014), and recently they have been combined with scaffold materials targeted at bone regeneration (Liu *et al.,* 2013).

Lately, much work has focused on co-transplantation of multiple cell populations (*i.e.* osteogenic and angiogenic cell populations) to enhance the bone regenerative processes. Transplanting cells into large defects can create regions that are hypoxic and low in nutrients necessary for cell survival. To accelerate angiogenesis, cells capable of contributing to the formation of a new vascular supply, such as endothelial cells and endothelial progenitor cells (EPC) can be used along with osteogenic cells (Cornejo *et al.,* 2012; Duttenhoefer *et al.,* 2013; Herrmann *et al.,* 2015; Tavassol *et al.,* 2010). Osteogenic and angiogenic cells may communicate with each other to synergistically improve both of these phenotypic processes (Dariima *et al.,* 2013). EPCs are progenitor cells of haematopoietic lineage origin (Masuda and Asahara, 2003) and can be easily isolated from peripheral blood using positive surface marker selection such as CD133 and CD34 (Asahara *et al.,* 1997; Peichev *et al.,* 2000). They show a high proliferation rate compared to mature endothelial cells (Lin *et al.,* 2000).

Several EPC sub-populations can be identified in peripheral blood. Early and late EPCs (also called OEC outgrowth endothelial cells) can be identified by morphological characteristics (Hur *et al.,* 2004; Lin *et al.,* 2000). Circulating EPCs are known to be responsible for post-natal vasculogenesis, and are mobilised into the blood stream from the bone marrow niche (Asahara *et al.,* 1999). Mobilisation is promoted by ischaemia and certain cytokines such as granulocyte colony stimulating factor (G-CSF). Circulating EPCs mobilised by G-CSF have shown efficacy in healing non-unions in a rat model (Mifune *et al.,* 2008) and, when co-administered with autologous bone graft, a small human clinical trial (Kuroda *et al.,* 2014).

Interestingly, signals produced by chondrocytes may also promote the osteogenic response of stem cells (Thompson *et al.,* 2009) and may help recapitulate endochondral ossification when transplanted with osteoblasts (Alsberg *et al.,* 2002).

Several modular approaches to cell delivery are also being investigated. For example, lyophilised solid scaffolds have been developed that have shape memory properties (Thornton *et al.,* 2004). They may be delivered to a defect in a compact form using a minimally invasive approach such as through a catheter, and then a cell suspension can be subsequently delivered to rehydrate them and drive them to expand into a predetermined shape and volume. Additionally, microscale constructs have been engineered, such as hydrogel microspheres containing cells (Rao *et al.,* 2013) or self-assembling cell aggregates (Hildebrandt *et al.,* 2011; Solorio *et al.,* 2012; Dang *et al.,* 2016; Solorio *et al.,* 2015) that can similarly be injected. It is important to recognise that in some cases cell delivery may not even be necessary, if the scaffold itself can present signals capable of recruiting large enough numbers of endogenous host osteogenic cells (Schantz *et al.,* 2007) and/or anti-inflammatory or wound healing macrophages (Das *et al.,* 2013).

Vascularisation

A key challenge in the treatment of large bone defects is the establishment of sufficient vascularisation at the defect site. Because the oxygen delivery required for the survival of cells is usually limited to a diffusion distance of ~ 150-200 μm to a neighbouring microvessel (Colton, 1995), the centre of cell seeded constructs rapidly die without the establishment of a blood supply. Accordingly, various vascularisation strategies have been developed in the field of regenerative medicine and tissue engineering (Laschke and Menger, 2012), which may more successfully support the treatment of large bone defects in future clinical practice (Fig. 2). The close physical and biochemical interaction between microvessels and bone cells is essential for bone formation and repair (Carano and Filvaroff, 2003). Many angiogenic growth factors, such as VEGF or FGF, have been shown to promote the differentiation, migration and proliferation of osteoblasts (Carano and Filvaroff, 2003). On the other hand, osteogenic factors, such as BMP-2, stimulate the switch of endothelial cells from a quiescent to an angiogenic phenotype (Finkenzeller *et al.,* 2012).

An important structural determinant for adequate vascularisation is the pore size of scaffolds. It is well recognised that the ideal pore size for the ingrowth of new microvessels ranges between ~ 200-600 μm (Druecke *et al.,* 2004). In this size range, poly(lactic-co-glycolic acid) (PLGA) scaffolds for bone defect repair also display suitable oxygen diffusion, pre-osteoblast cell infiltration, proliferation and survival without losing their mechanical strength (Amini *et al.,* 2012). However, this does not necessarily require that scaffolds should be created with a homogeneous pore size. In fact, sophisticated technologies such as rapid prototyping offer the possibility to fabricate scaffolds with clearly defined porosity levels to ideally promote individual key steps of the bone healing process. Yang *et al.* developed ceramic scaffolds with sub-μm pores to improve cell/surface interactions, pores of tens of μm to support osteoconduction, and corridors of 100-600 μm to stimulate vascularisation (Yang *et al.,* 2006). Finally, the overall three-dimensional architecture of scaffolds has recently been shown to markedly affect their vascularisation.

The vascularisation of bone defects may also be improved by the application of compounds with pro-angiogenic properties. Of interest, Holstein *et al*. reported that systemic treatment with the glycoprotein erythropoietin (EPO) is capable of stimulating bone formation, cell proliferation and angiogenesis in a femoral segmental defect model in mice (Holstein *et al.,* 2011). Compared to this systemic approach, the topical application of angiogenic growth factors at the defect site is much more common. For this purpose, the factors may be coated on the surface of solid scaffolds (Sun *et al.,* 2011) or incorporated into drug delivery systems such as microparticles or hydrogels (Geuze *et al.,* 2012; Ishida *et al.,* 2010; Ratanavaraporn *et al.,* 2011). Alternatively, platelet-rich plasma (PRP) may be applied, which represents a rich, autologous source of various growth factors and can easily be isolated from patients under clinical conditions (Lucarelli *et al.,* 2005; Jalowiec *et al.,* 2016; Lippross *et al.,* 2011). In general, it

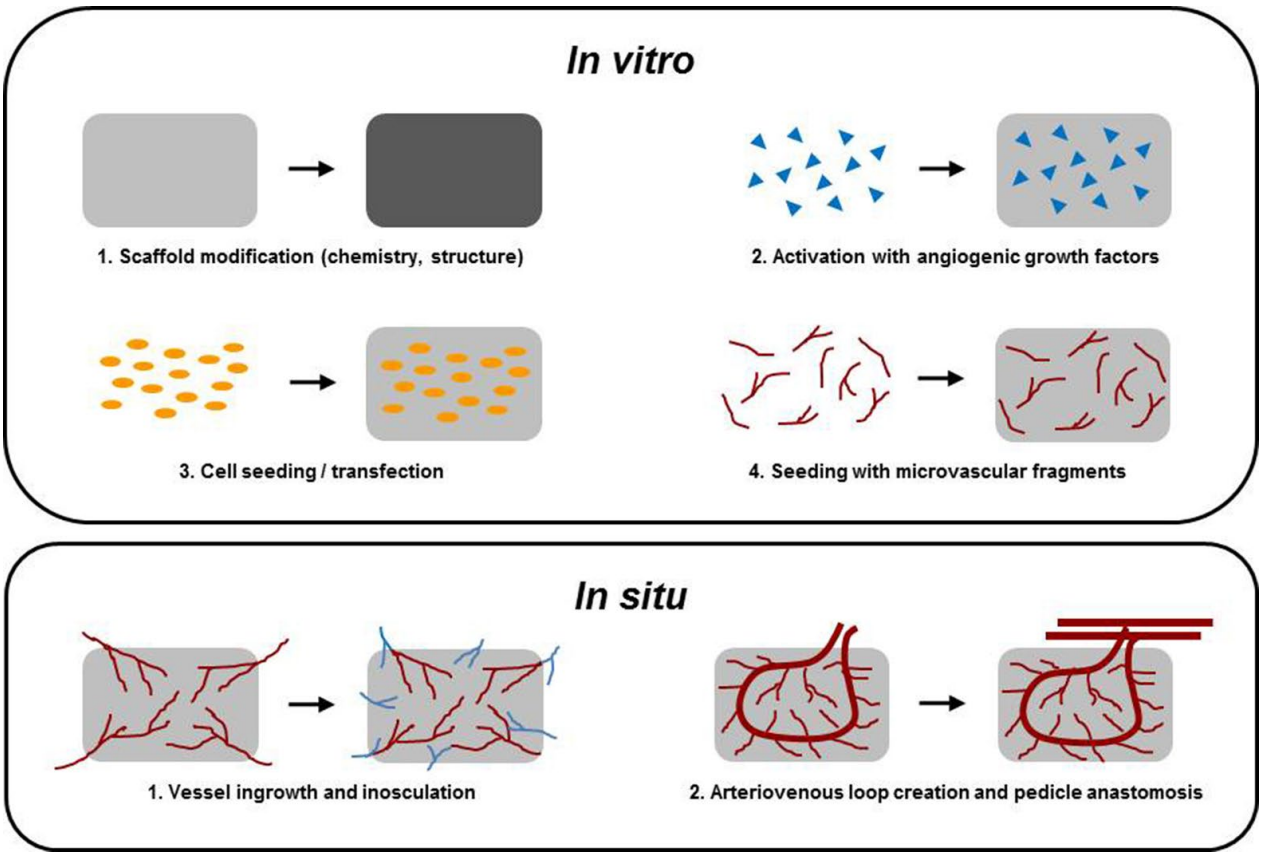

Fig. 2. Basic *in vitro* and *in situ* vascularisation strategies for tissue engineering constructs as outlined in the section "Vascularisation". *In vitro* vascularisation strategies focus on the modification of tissue constructs prior to their implantation. This can be achieved by changing the chemical and structural properties of scaffolds (**1**) or by their biological activation with growth factor delivery systems (**2**), cells (**3**) and microvascular fragments (**4**). *In situ* vascularisation strategies focus on the generation of preformed microvascular networks within scaffolds by implanting them in well-vascularised areas of the body (**1**) or by generation of an arteriovenous(AV)-loop (**2**). After this prevascularisation phase, the scaffolds are transferred to the final defect site, where they rapidly establish a blood supply by developing interconnections with the surrounding host microvasculature, *i.e.* inosculation (**1**), or by direct surgical anastomosis of a vascular pedicle (**2**).

should be noted that the efficacy of the administration of growth factors may be markedly influenced by their release rates and inactivation (Geuze *et al.,* 2012) (see section "Drug and Growth Factors delivery"). Moreover, there is no doubt that a combination of factors support individual stages of bone healing and angiogenesis (Ratanavaraporn *et al.,* 2011; Su *et al.,* 2013). Besides treatment with growth factors, local delivery of miRNA is as a novel possibility to optimise angiogenesis-osteogenesis coupling during bone defect healing. Over-expression of miR-26a in critical-size calvarial bone defect resulted in an improved vascularisation and complete defect healing (Li *et al.,* 2013).

There are several possibilities for the generation of microvascular networks within tissue constructs. These include the *in vitro* seeding and cultivation of scaffolds with vessel-forming cell types (Koike *et al.,* 2004; Wang *et al.,* 2007). However, this involves complex cell isolation, seeding, and cultivation procedures, which may not be realisable in clinical practice. Another common strategy to induce vascularisation in bone defect healing is the seeding of appropriate scaffolds with differentiated tissue-specific cells (Cornejo *et al.,* 2012; Tavassol *et al.,* 2010), EPCs (Seebach *et al.,* 2010) or multipotent stem cells (Maraldi *et al.,* 2013; Zhang *et al.,* 2010b) (see section "Cell delivery"). By this method, the formation of new blood vessels is primarily stimulated by hypoxia-driven cellular release of angiogenic growth factors during engraftment (Schumann *et al.,* 2009). The seeded cells may additionally be genetically modified to guarantee a more continuous growth factor secretion at the defect site (see section "Gene Therapy"). Promising growth factors in bone defect healing include hypoxia-inducible factor-(HIF)-1α (Zou *et al.,* 2012), VEGF (Geiger *et al.,* 2005; Li *et al.,* 2009b), FGF-2 (Guo *et al.,* 2006; Qu *et al.,* 2011), and angiopoietin-1 (Cao *et al.,* 2012a).

An interesting study by Kasper *et al.* indicates that some of the angiogenic and vasculogenic mechanisms may be additionally regulated by mechanical loading of the cells (Kasper *et al.,* 2007). Using tube formation and spheroid sprouting assays, they found a significant enhancement of angiogenesis by conditioned media from mechanically stimulated compared with unstimulated MSCs. Thus, they concluded that mechanical loading of MSCs results in a paracrine stimulation of blood vessel formation, most likely by the up-regulation of angiogenic growth

factors including VEGF and FGF. Another determinant for the vascularisation potential of MSCs is their three-dimensional arrangement. It was recently demonstrated that polyurethane scaffolds, which are seeded with multicellular MSC spheroids, exhibit a markedly improved vascularisation when compared to control scaffolds seeded with an identical number of individual MSCs (Laschke *et al.,* 2013). Immunohistochemical analyses of the implants revealed that this is due to an enhanced vessel-forming capacity of the three-dimensional MSC spheroids, making them attractive vascularisation units for future tissue engineering applications.

Taken together, all of these studies indicate that major progress has been made in recent years towards establishing novel strategies to promote angiogenesis and vasculogenesis in bone tissue engineering. However, the basic problem of all of these strategies is the fact that blood vessel formation is a time-consuming multi-step process, which cannot be accelerated limitlessly. The growth of newly developing microvessels is usually not faster than ~5 μm/h (Utzinger *et al.,* 2015). Accordingly, complete vascularisation of large bone defects by ingrowth of microvessels from the defect borders takes far too long to guarantee cell survival at the defect site. A promising concept to overcome this problem is the generation of prevascularised tissue constructs that exhibit a functional preformed microvascular network, which connects with the surrounding microvasculature, known as inosculation (Laschke and Menger, 2016).

Alternatively, it is possible to pre-vascularise scaffolds *in situ* by implanting them in well-vascularised areas of the body to promote the ingrowth of new microvessels (Laschke *et al.,* 2011). Moreover, the incorporation of an arteriovenous loop (AV)-loop, *i.e.* a ligated artery and vein (Boos *et al.,* 2013), or a vasculature bundle (VB), *i.e.* a ligated artery and vein, into scaffolds even allows the *in situ* generation of tissue constructs with a vascular pedicle, which can be surgically anastomosed to the vessels of the defect site. Of interest, Wu *et al*. found that the VB technique results in a better balance between bone regeneration and scaffold degradation than the AV-loop strategy for the prevascularisation of bone constructs consisting of β-tricalcium phosphate scaffolds and BMSCs (Wu *et al.,* 2015).

Currently, *in situ* prevascularisation represents the most promising approach to guarantee a sufficient blood supply to large bone constructs in the clinical setting. In fact, Horch *et al.* recently reported the first successful application of the AV-loop technique in two patients with large bone defects in the radius and tibia (Horch *et al.,* 2014). However, *in situ* prevascularisation strategies normally bear the disadvantage that they require repetitive surgical interventions for the implantation of scaffolds to the site of prevascularisation, and their removal for final transfer into a defect. To overcome this problem, scaffolds may be seeded in the future with adipose-derived microvascular fragments (Laschke and Menger, 2015a). These microvascular fragments are a randomised mixture of fully functional arteriolar, capillary and venular vessel segments with associated MSCs, which can be easily isolated from adipose tissue by enzymatic digestion (Laschke and Menger, 2015). After their implantation these fragments survive and exhibit a high angiogenic activity, forming new microvascular networks, which develop interconnections to the microvessels of the host tissue.

Mechanical factors

Mechanical stability is known to be an important factor for bone healing outcome. Indeed, beside the quality of the implant, a large amount of experimental and clinical evidence confirms that the course of fracture repair can be influenced by mechanical stimuli, and that controlled instability at the fracture site (dynamisation) can deeply affect bone regeneration. However, optimal loading parameters to enhance fracture healing have not yet been entirely defined. There are still many uncertainties concerning the magnitude of the load, the loading timing after fracture, but also the type of loading (*e.g.* axial, bending). Despite the considerable attention paid to fracture healing and, to some degree, sub-critical size osteotomies, there is very little literature on the effects of the mechanical environment on the healing of large bone defects.

Mechanical stimulation of bone can be classified according to the type of motion applied. Since Goodship and Kenwright (Goodship and Kenwright, 1985), several groups have shown that a cyclic, axial, compressive displacement applied to a diaphysal fracture or osteotomy induces higher healing by the formation of a stronger cartilaginous callus leading to earlier bone bridging (Claes *et al.,* 1998; Wolf *et al.,* 1998; Yamaji *et al.,* 2001).

In certain studies, strains in the range of 5 % and 15 % were shown to be beneficial (Claes and Heigele, 1999; Wolf *et al.,* 1998; Yamaji *et al.,* 2001). In other studies, however, maximum strains of 7 % have been described to be beneficial to the gap bridging (Augat *et al.,* 1998; Claes *et al.,* 1997; Claes *et al.,* 1998), and larger displacement was described as resulting in more fibrous tissue leading to delayed bone union. In a nice experimental set up, Hente (Hente R *et al.,* 1990) looked at the effect of defined strain on new bone formation (Fig. 3). A strain gradient from 0 % to more than 100 % was applied along a fracture gap. Results showed that 0 % strain did not promote callus formation, while strain from 30 % and higher induced massive callus formation but without any evidence of bridging. However, a strain of 5 % in this system was found to be the most efficient to induce solid bridging of the gap.

Another parameter, which is still a point of discussion, is the optimal initiation of stimulation. At the cellular level, it has been shown that mesenchymal cells differentiate toward osteogenic or chondrogenic lineages at early stages of the healing process, depending on the mechanical environment (Le *et al.,* 2001; Thompson *et al.,* 2002). Studies comparing timing of initiation of axial loading in a rat osteotomy model, showed a positive effect of direct post-surgery stimulation (Klein *et al.,* 2003; Weaver *et al.,* 2010). In addition, Weaver also reported a positive effect of a later starting point (10 d post-surgery), while an intermediate time point (3 d) was not as beneficial. A beneficial effect of a later starting point was also described

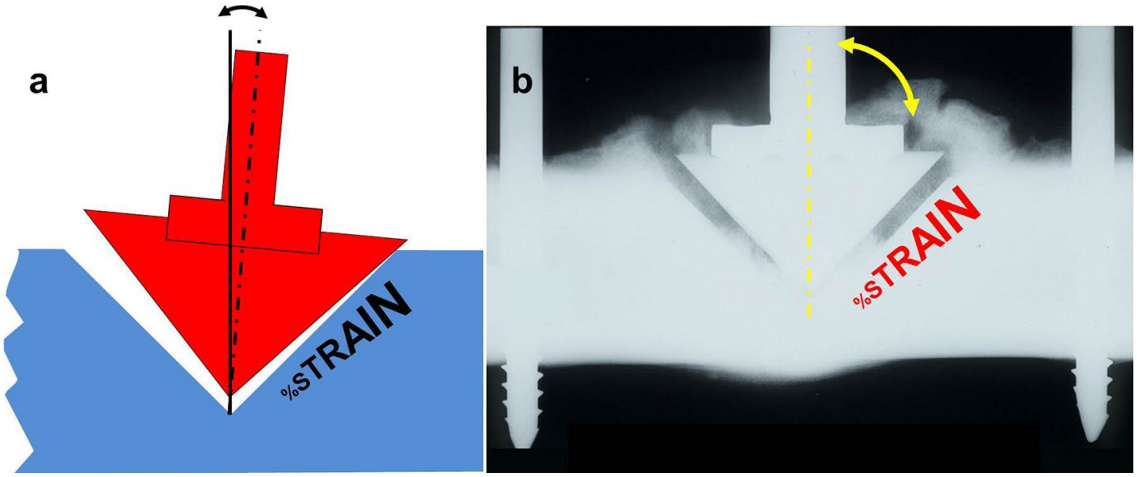

Fig. 3. Effect of strain on callus formation (adapted from (Hente *et al.,* 1990)). (**a**): schematic representation of the experimental set up. A portion of bone is cyclically tilted along its gap of origin, creating a gradient of strain from 0 (tip of the fragment) to 100 % (top of the fragment). (**b**): X-ray imaging showing the presence of bone bridging in the lower strain area compared to the higher strain where larger callus formation without bridging was observed.

by others and was explained by the fact that this delay might be favourable to the initiation of neo-vascularisation (Claes *et al.,* 2002; Wallace *et al.,* 1994).

However, while several groups have studied the effect of mechanical stimulation on small defects (1 to 2 mm osteotomy), only few studies have reported the effect of mechanical loading on a large bone defect. In a critical size goat femur bone defect (filled with demineralised bone and MSCs), treated with either a dynamic intramedullary rod or a static intramedullary rod, an overall better neovascularisation of the implants was seen in the dynamised cases (10 % strain) compared to the rigid fixations (Hou *et al.,* 2010). Callus formation and bone healing was compared in a 5 mm defect treated with BMP-2, when the defects were stabilised with interchangeable external fixators creating low, medium or high axial stiffness (Glatt *et al.,* 2012). Under constant stiffness, the low stiffness group showed an increased healing rate when compared to the medium or high stiffness groups. While switching at day 14 from low stiffness to a high stiffness fixator (reverse dynamisation), showed by far improved bone healing compared to all other groups. Epari *et al.* subsequently provided a theoretical basis for these observations (Epari *et al.,* 2013).

Thus a systematic comparison of the three above cited parameters (timing, amplitude and loading type) is still missing. Additionally, studies investigating osteosynthesis devices designed specifically for critical sized defects are lacking. Such studies are needed to provide a clearer view about the effects of mechanical stimulation on the healing of large bone defects.

Additional approaches

Endochondral bone tissue engineering

In vitro bone tissue engineering strategies commonly focus on promoting direct osteoblastic differentiation within cell-seeded constructs, mimicking the process of intramembranous ossification. Such engineered tissues often fail to promote bone regeneration following implantation (Lyons *et al.,* 2010), leading to increased interest in endochondral bone tissue engineering strategies (Thompson *et al.,* 2014). This involves the implantation of tissue engineered cartilage in an attempt to recapitulate the normal long bone development process whereby a cartilaginous template becomes hypertrophic, is vascularised, and is ultimately replaced with bone. The logic of this approach is that chondrocytes are better equipped to survive within the nutrient and oxygen deprived environments that exist within a large bone defect (Farrell *et al.,* 2009; Gawlitta *et al.,* 2010). Furthermore, hypertrophic chondrocytes progressing along the endochondral pathway are known to release factors such as VEGF to promote vascularisation of the implanted tissue (Farrell *et al.,* 2009; Gawlitta *et al.,* 2010).

Chondrogenically primed BMSCs also have an inherent tendency to become hypertrophic and undergo endochondral ossification (Farrell *et al.,* 2009; Pelttari *et al.,* 2006; Scotti *et al.,* 2010; Vinardell *et al.,* 2012b). This has motivated the use of cartilaginous constructs engineered using BMSC-seeded scaffolds for bone regeneration. One of the earliest demonstrations of this concept was reported by Huang and colleagues (Huang *et al.,* 2006), who found that cartilage tissue engineered *in vitro* using BMSCs could be used for carpal bone reconstruction in a rabbit model. More recent studies have provided greater insight into the mechanisms by which chondrogenically differentiated BMSCs promote bone formation *in vivo*. TGF-β typically used to promote chondrogenic differentiation of BMSCs, has been shown to promote the expression not only of genes associated with chondrogenesis and hypertrophy, but also the production of factors critical to vascularisation such as VEGF and matrix metalloproteinases (Farrell *et al.,* 2009; Pelttari *et al.,* 2006). Following implantation, chondrogenically primed

MSCs have been shown to directly contribute to new bone tissue formation, and also to facilitate the recruitment of host cells capable of further driving osteogenesis (Farrell *et al.,* 2011; Pelttari *et al.,* 2006; Scotti *et al.,* 2010). MSC-seeded scaffolds have also been shown to promote greater vascularisation than osteoblast-seeded scaffolds *in vivo* by activating an endochondral ossification process and recruiting host-derived CD31+ endothelial cells, followed by a second wave of host derived CD146+ cells that display characteristics of MSCs (Tortelli *et al.,* 2010). Chondrogenically primed MSCs can accelerate the regeneration of critically sized bone defects in small animal models. Cartilage grafts were used to promote regeneration using a murine segmental tibial defect model (Bahney *et al.,* 2014). This study also used lineage tracing experiments to show the regenerate was graft-derived, suggesting the direct transformation of chondrocytes into bone forming cells (Bahney *et al.,* 2014). Finally, it has recently been demonstrated that chondrogenically-primed MSC-laden scaffolds support greater repair of critical-sized cranial defects than osteogenically stimulated constructs (Thompson *et al.,* 2016). Taken together, these studies demonstrate the potential of endochondral tissue engineering strategies for orchestrating bone regeneration.

A number of questions still have to be addressed to fully realise the potential of engineered cartilage for large bone defect healing. These include determining the optimal duration of chondrogenic pre-culture for MSCs (Yang *et al.,* 2015), as well as the identification of factors that both promote hypertrophy *in vitro* and accelerate endochondral bone formation *in vivo*. MSCs implanted subcutaneously into nude mice have been shown to form bone trabeculae only if they have generated supporting hypertrophic tissue structures prior to implantation (Scotti *et al.,* 2010). More advanced hypertrophic maturation of MSCs *in vitro* was also found to promote the formation of larger bony tissues *in vivo* (Scotti *et al.,* 2010). Another challenge involves the identification of suitable biomaterials to support endochondral bone regeneration (Cunniffe *et al.,* 2015), as well as engineering *in vitro* cultures that facilitate the development of hypertrophic cartilage of sufficient scale to treat large bone defects. MSCs seeded onto collagen-based scaffolds and directed along an endochondral pathway *in vitro* have been used to generate a scaled-up bone organ *in vivo* which was found to contain a fully functional haematopoietic compartment (Scotti *et al.,* 2013). Synthetic and natural polymeric scaffolds (Yang *et al.,* 2013; Yang *et al.,* 2015) and various hydrogels (Dickhut *et al.,* 2008) can also potentially be used for engineering scaled-up endochondral bone tissue. An improved understanding of how environmental cues (specific to a bone defect) will regulate endochondral bone regeneration is also required. For example, it has been shown that factors such as a low oxygen environment (Sheehy *et al.,* 2013) as well as certain mechanical cues like compression (Thorpe *et al.,* 2013) and hydrostatic pressure (Vinardell *et al.,* 2012a) can suppress markers of hypertrophy in MSCs. This highlights the need to consider the many factors that contribute to poor outcomes in complex bone fractures and segmental defects when designing novel endochondral bone regeneration strategies.

Gene Therapy

Although several different osteogenic growth factors show promise as agents of bone healing, their clinical deployment is constrained by delivery problems. In particular, it is not possible to deliver these proteins locally at physiological concentrations in a sustained fashion. With BMPs -2 and -7, this problem has been addressed clinically by their implantation at very high doses on simple scaffolds. This provides modest clinical efficacy and, at least in the case of BMP-2, provokes a number of adverse events, some serious (Carragee *et al.,* 2011; Faundez *et al.,* 2016) (section "Drug and Growth Factor delivery"). Gene transfer technologies offer to solve these problems. They also remove the concern that preparations of recombinant proteins may contain denatured, and possibly immunogenic, molecules. Moreover, there is evidence that cells respond better to endogenously synthesised growth factors than their recombinant equivalents.

The basic concept is quite simple. A gene, or more usually a cDNA, encoding a protein of interest is delivered by a vector to the site of an osseous defect. This protein is synthesised locally in an endogenous, authentic fashion for as long as the cDNA is present and expressed. By incorporating regulatory elements, it is possible to control both the level and duration of transgene expression. Although most pre-clinical development has focused on delivering cDNAs that encode secreted growth factors, gene transfer is particularly well suited to delivering intracellular proteins, such as transcription factors (Tu *et al.,* 2007), the Lim mineralisation proteins (Lattanzi *et al.,* 2008) and non-coding species of RNA (Levi *et al.,* 2012).

Gene delivery can be accomplished with non-viral and viral vectors. Although non-viral methods are less expensive and generally considered to be safer than viral vectors, they are also much less efficient. Transgene expression is usually low and transient. However, the literature contains examples demonstrating the ability to heal osseous defects in animal models using non-viral gene transfer methods (Kimelman-Bleich *et al.,* 2011; Li *et al.,* 2009a). Nevertheless, most research involves the use of viral vectors which, although more difficult and expensive to prepare, are much more efficient.

Viral vectors that have been explored in the context of bone healing are retrovirus (Rundle *et al.*, 2008), lentivirus (Virk *et al.,* 2011), adenovirus (Baltzer *et al.,* 2000), adeno-associated virus (AAV) (Ito *et al.,* 2005) and baculovirus (Lin *et al.,* 2012). Each has advantages and disadvantages in terms of ease of preparation and use, persistence in the host, immunogenicity, carrying capacity, serotype and so forth. Much effort has been devoted to engineering novel and improved versions of many of these viral vectors, so simple descriptors are increasingly difficult.

Use of viral vectors raises issues of safety, which is a key issue for non-lethal indications such as bone healing. The major safety concern with retroviruses, including lentiviruses, is insertional mutagenesis, which has led to the development of leukaemia in human subjects in a clinical trial for Severe Combined Immunodeficiency Disease (Hacein-Bey-Abina *et al.,* 2003). The major safety issue with adenovirus is the strong immune responses that it generates; these led to gene therapy's first death, in 1999

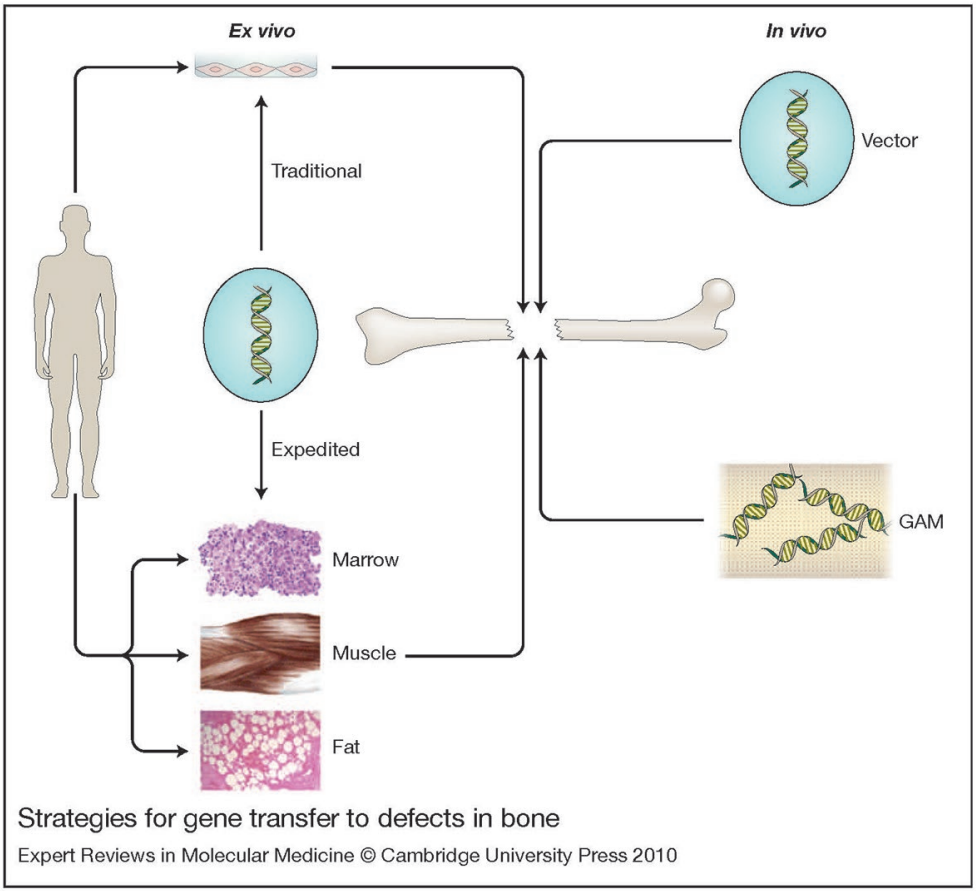

Fig. 4. Current approaches for gene delivery to osseous lesions. There are two general strategies: *in vivo* (right hand side) and *ex vivo* (left-hand side). For *in vivo* gene delivery, the vector is introduced directly into the site of the osseous lesion, either as a free suspension (top right) or incorporated into a gene-activated matrix (GAM) (bottom right). For *ex vivo* delivery, vectors are not introduced directly into the defect. Instead they are used for the genetic modification of cells, which are subsequently implanted. Traditional *ex vivo* methods (top left) usually involve the establishment of cell cultures, which are genetically modified *in vitro*. The modified cells are then introduced into the lesion, often after seeding onto an appropriate scaffold. Expedited *ex vivo* methods (bottom left) avoid the need for cell culture and scaffolds by genetically modifying tissues such as marrow, muscle and fat, intraoperatively and inserting them into the defect during a single operative session (Evans, 2010).

(Raper *et al.,* 2003). That said, the incidence of severe adverse events in gene therapy trials has been remarkably low and the safety issue is as much one of psychology as biology. Ironically, given recent disclosures concerning the severe adverse events generated by the large amounts of BMP-2 present in Infuse®, USA (InductOs, Europe) (Carragee *et al.,* 2011), it is possible to argue that, in this particular application, gene therapy may well be safer than protein therapy.

Most investigators have used cDNAs encoding proteins that promote the osteogenic differentiation of mesenchymal cells. BMP-2 or BMP-7 are popular choices, as the recombinant proteins are already in clinical use. There is also enthusiasm for using cDNAs encoding angiogenic factors such as VEGF (Li *et al.,* 2009b), because osteogenesis is known to have an absolute requirement for angiogenesis (see section "Vascularisation"). Other transgenes of experimental interest include cycloxygenase, which promotes osteogenesis *via* prostaglandin synthesis (Rundle *et al.,* 2008), and parathyroid hormone 1-34 (Bonadio *et al.,* 1999), among others. Although the choice of osteogenic genes is understandable, long bone fractures mainly heal through the initial phases of cartilaginous callus formation and subsequent endochondral ossification. In recognition of this, there is increasing interest in promoting the endochondral route to healing large bone defects (see section "Endochondral bone tissue engineering"). BMP-2 could be a useful transgene in this regard, because it promotes both chondrogenesis (Palmer *et al.,* 2005) and the endochondral differentiation of MSCs (Steinert *et al.,* 2009).

Regardless of the vectors and transgenes that are used, there are two major strategies for their deployment: *in vivo* and *ex vivo.* During *in vivo* delivery the vector is introduced directly into the osseous defect. This has advantages of simplicity, but raises safety concerns. *Ex vivo* delivery is more cumbersome and expensive, but does not introduce vector into the body and provides the opportunity to deliver both osteoprogenitor cells and osteogenic genes concomitantly to the defect. The considerable cost and complexity of *ex vivo* gene delivery with autologous cells can be mitigated with allogeneic, universal donor cells,

or by developing expedited protocols where autologous cells or tissues are removed, genetically modified, and reimplanted in a single operative session (Evans *et al.,* 2007).

Based upon these principles, four main approaches have emerged (Fig. 4) for delivery of genes to osseous lesions: *in vivo* gene delivery by direct injection or with gene-activated matrices (GAMs), and *ex vivo* delivery using expanded autologous cells or expedited approaches accomplished intra-operatively.

The direct injection of adenovirus vectors encoding BMP-2 (Baltzer *et al.,* 2000; Betz *et al.,* 2006) or BMP-6 (Bertone *et al.,* 2004; Ishihara *et al.,* 2008) can heal critical size femoral defects in rats, rabbits and horses. However, it is not reliably effective in all animals and generates a strong neutralising immune reaction to the vector. These immune reactions were sufficiently strong to prevent efficacy in large bone defects in sheep (Egermann *et al.,* 2006b), unless the sheep had been treated previously with cortisone, an immunosuppressant (Egermann *et al.,* 2006a). Of concern, immune reactions to human BMP-2 were generated in the sheep model, possibly reflecting the strong adjuvant properties of adenovirus.

GAMs provide an alternative approach. The original GAM comprised a collagenous scaffold impregnated with plasmid DNA encoding BMP-4 (Fang *et al.,* 1996) or the first 34 amino acids of parathyroid hormone (PTH 1-34) (Bonadio *et al.,* 1999), presently used as the drug teriparatide (Forteo®) to treat osteoporosis. Impressive data were reported in rat and canine models, but further development was hindered by the low levels of transgene expression. GAMs incorporating improved non-viral (Tierney *et al.,* 2012) or viral vectors show more efficient gene transfer and expression in animal models.

Allograft revitalisation is an extension of the GAM principle in which AAV vectors are coated onto allograft bone (Ito *et al.,* 2005). After implantation, host progenitor cells encounter the vector and express transgenes, leading to resorption of the allograft and its replacement with host bone. Success has also been reported when AAV is coated onto poly(epsilon-caprolactone) (Dupont *et al.,* 2012).

Lieberman's group pioneered the *ex vivo* approach, successfully using an adenovirus vector encoding BMP-2 in conjunction with BMSCs (Lieberman *et al.,* 1999). To expedite matters, they now use buffy coat cells obtained intra-operatively from bone marrow, in conjunction with a lentivirus vector that gives higher and more persistent transgene expression (Virk *et al.,* 2011). Because of concerns about insertional mutagenesis with lentivirus, the inclusion of a suicide gene, to be activated in the event of malignant transformation or other severe adverse event, is being explored (Alaee *et al.,* 2014).

An alternative expedited, *ex vivo* approach makes use of the remarkable osteogenic properties of muscle, reflected in the high incidence of heterotopic ossification of muscle after blast injuries and joint replacement surgery, as well as in the disease *fibrodysplasia ossificans progressiva.* The latter occurs as a result of an activating mutation in a BMP receptor, suggesting that a sustained BMP signal efficiently induces bone in muscle. Use of an adenovirus encoding BMP-2 (Ad.BMP-2) provides such as signal. Musgrave *et al.* showed that the intra-muscular injection of Ad.BMP-2 induced bone in muscle (Musgrave *et al.,* 1999). This has been adapted in a strategy where biopsies of autologous muscle are transduced with Ad.BMP-2 and implanted into critical sized defects in rats (Evans *et al.,* 2009). Autologous fat is also effective, but less reliably than muscle. Of note, this abbreviated *ex vivo* procedure eliminated the humoral response to adenovirus (Evans *et al.,* 2009).

Despite a considerable literature, reviewed in references (Evans, 2010; Evans, 2012; Pensak and Lieberman, 2013), reporting successes in healing large bone defects in animal models by gene therapy, it is not used clinically. There are a number of reasons for this, including the need for studies in large animals, which are costly and take a long time. Often, insufficient attention is paid to pharmacology, toxicology and other important matters of this nature. Furthermore, the scientists who undertake the pre-clinical research are often naïve when it comes to the process of research translation, which involves a wide spectrum of expertise, ranging from regulatory issues, to clinical trial design, ethics, and so forth. It is wise to involve individuals with the necessary expertise early in the research programme to forestall subsequent barriers to translation (Madry *et al.,* 2014).

Another constraint to clinical application lies in the simple fact that we do not know how much of a given gene product is needed at which time during the healing process and for how long. Such information would greatly advance the field.

Conclusions

At a minimum, the regeneration of bone requires the balanced contributions of scaffolds, cells, morphogenetic signals, vascularisation and mechanics. Each of these elements is being studied intensively, and considerable advances have been made in developing new understandings, concepts and information. Because each of these components has many facets, and therefore an even greater number of permutations, there are innumerable theoretical combinations that frustrate any straightforward development of new, osteogenic technologies.

But it is also noteworthy that, despite decades of research in this area using different combinations of the components discussed in this review, we still lack an approved engineered product that gives robust, reliable results in the clinic. It is possible that, given the impossibly large number of permutations of the base components studied for the healing of large, osseous, segmental defects, researchers have not yet arrived at the optimal combination. However, it is also possible that we are missing something. Bone, after all, normally heals by itself, whereas large segmental defects do not. Perhaps we need to go back to the beginning and discover why large segmental defects in otherwise healthy individuals do not heal? Perhaps formulating strategies based upon the way fractures heal naturally is inappropriate?

Regardless of the technologies that actually work reliably in advanced pre-clinical models, the clinical

development of such technologies is constrained by the regulatory environment that governs their deployment, as well as the financial realities of health care economics. The reality may be that without adequate stratification and identification of patients, complex therapies may not provide the economic benefit to make them viable. As described in this critical review, progress is occurring on several fronts and it should be only a matter of time before patients can benefit from better ways to heal large segmental defects. Achieving this will require interactive, well-funded, sustained consortia including biologists, physical scientists, clinicians, translational scientists, and industrial partners.

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Discussion with Reviewers

P. Habibovic: In the Conclusion section, the authors state: "At a minimum, the regeneration of bone requires the balanced contributions of scaffolds, cells, morphogenetic signals, vascularisation and mechanics". I would like to challenge this statement by stating that a therapy encompassing all these component will never reach the clinic, because of the high cost and complex regulations. Could the authors respond to this statement?
Authors: The reviewer identifies an important issue that is touched upon in the paper, but not explored in detail: how to make TERM (Tissue Engineering and Regenerative Medicine) affordable. This will probably require expedited approaches that do not use expanded, autologous cells but harness intrinsic, biological processes. Greater investigation of simple rehabilitation techniques could also pay dividends. Investigators need to bear in mind cost, as well as science, when developing technologies.

I. Martin: In the conclusions, it is mentioned that "perhaps, we need to go back to the beginning and discover why large segmental defects in otherwise healthy individuals do not heal". Could you further elaborate your recommendation on the approaches to be followed or nature of the parameters to be investigated to bring forward our fundamental knowledge of the biological processes which need to be better controlled?
Authors: The early biological responses to an osseous injury seem important determinants of whether and, if so, how, a defect will heal (Glatt *et al.,* eCM 2012; Kolar al, Tissue Engineering Part B. 2010). This suggests that study of the early biology of a segmental defect would be profitable. Useful comparisons could be made between a critical sized defect in the presence or absence of an osteogenic growth factor, or between a large osseous in a bone that does not heal (*e.g.* femur) and one where a similar sized defect spontaneously heals (*e.g.* rib). Pre-paradigmatic observations in such systems promise to generate experimentally testable hypotheses.

Editor's note: The Scientific Editor responsible for this paper was Joost de Bruijn.