TISSUE ENGINEERED BONE USING SELECT GROWTH FACTORS: A COMPREHENSIVE REVIEW OF ANIMAL STUDIES AND CLINICAL TRANSLATION STUDIES IN MAN

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Abstract

There is a growing socio-economic need for effective strategies to repair damaged bone resulting from disease, trauma and surgical intervention. Bone tissue engineering has received substantial investment over the last few decades as a result. A multitude of studies have sought to examine the efficacy of multiple growth factors, delivery systems and biomaterials within in vivo animal models for the repair of critical-sized bone defects. Defect repair requires recapitulation of in vivo signalling cascades, including osteogenesis, chondrogenesis and angiogenesis, in an orchestrated spatiotemporal manner. Strategies to drive parallel, synergistic and consecutive signalling of factors including BMP-2, BMP-7/OP-1, FGF, PDGF, PTH, PTHrP, TGF-β3, VEGF and Wnts have demonstrated improved bone healing within animal models. Enhanced bone repair has also been demonstrated in the clinic following European Medicines Agency and Food and Drug Administration approval of BMP-2, BMP-7/OP-1, PDGF, PTH and PTHrP. The current review assesses the in vivo and clinical data surrounding the application of growth factors for bone regeneration. This review has examined data published between 1965 and 2013. All bone tissue engineering studies investigating in vivo response of the growth factors listed above, or combinations thereof, utilising animal models or human trials were included. All studies were compiled from PubMed-NCBI using search terms including ‘growth factor name’, ‘in vivo’, ‘model/animal’, ‘human’, and ‘bone tissue engineering’. Focus is drawn to the in vivo success of osteoinductive growth factors incorporated within material implants both in animals and humans and identifies the unmet challenges within the skeletal regenerative area.

Keywords: Animal model, bone tissue engineering, BMP-2, BMP-7, clinical translation, FGF, human studies, in vivo, OP-1, PDGF, PTH, PTHrP, TGF-β3, VEGF, Wnt proteins.

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Note

Tables 1-5 are included within the text of the paper. However, Supplementary Tables 1-9 can be accessed from a secondary supplementary document that is available from the eCM Journal webpage for this paper (http://www.ecmjournal.org/journal/papers/vol028/vol028a13.php)

Introduction

Tissue engineering utilizes design and construction principles to manufacture replacement tissues exhibiting competent biological function (Tabata, 2003). Regeneration or repair of critical-sized bone defects by substitution of damaged or diseased tissues requires an ability to recapitulate developmental biology processes and control tissue morphogenesis. Manipulation of tissue development and morphogenesis can be achieved through delivery of inductive signals replicating native in vivo microenvironmental cues. Utilisation of select growth factors enables controlled cell differentiation towards specified lineages (Sundelacruz and Kaplan, 2009). Spatiotemporal orchestration of growth factors in vivo is critical to successful bone tissue engineering strategies (Reddi, 2000). The aim of this review was to assess the inductive signalling aspect of current bone tissue engineering strategies, and identify individual growth factors or combinations thereof which have shown in vivo success within animal models and have been scaled to large animals prior to clinical translation within humans. It is pertinent to understand current progress to evaluate optimum strategies that can be taken forward for further study.

There is currently a range of tissue-engineered solutions advocated for bone repair and yet there remains a need for demonstrable preclinical and clinical efficacy of materials with a proven capacity to repair bone damage resulting from disease, trauma or surgical intervention. It is estimated that 3.6% of the UK population (of over 64 million) will suffer a bone fracture in their lifetime (Donaldson et al., 2008). The risk of fracture increases with age and statistics show 1 in 3 women and 1 in 5 men over 50 years of age will experience an osteoporotic fracture; a growing concern in an increasingly aged population (van Staa et al., 2001). The worldwide incidence of hip fracture is expected to increase 240% and 310% in women and men, respectively.
by 2050 (Baim and Leslie, 2012; Gullberg et al., 1997), resulting potentially in a rise from 1.6 million cases per annum to between 4.5 and 6.3 million cases (Cooper et al., 1992b). Osteoporosis is a major cause of hip fractures with equally significant financial tolls derived from both immediate medical treatment and post-treatment aid for reduced mobility, disability and increased dependency (Keene et al., 1993; Leslie et al., 2012). There are currently over 2 million osteoporosis sufferers in the UK alone whose medical treatment of related fractures (Borgstrom et al., 2010a; Borgstrom et al., 2010b; Strom et al., 2013) is predicted to cost over £2 billion by 2020 (Burge et al., 2001). Throughout the rest of Europe, osteoporosis related fractures are estimated to cost £51 billion by 2050 (Kanis and Johnell, 2005).

In addition, typically 10 % of fractures fail to repair resulting in non-union and increased socio-economic costs. There are two categories of non-union, hypertrophic (callus formation but without union) and atrophic (no callus formation). Many factors contribute to non-union fractures including avascular necrosis, bone apposition failure, poor or loss of fixation, infection, and soft tissue implantation within the defect site. A study in 2007 showed that humeral, tibial and femoral non-unions cost £15.5K, £16.3K and £17.2K, respectively, on a ‘best-case scenario’ (Kanakaris and Giannoudis, 2007). Non-union may also occur following spinal fusion surgery (posterolateral lumbar arthrodesis). Over 200,000 spinal fusion procedures are performed per annum in the US, yet non-union occurs in 10-40 % of patients undergoing single-level fusions (Boden, 2000). This is more frequent in patients undergoing multiple-level fusions. Increasing costs are expected in the future, as only a third of vertebral fractures come to clinical attention and are officially diagnosed (Cooper et al., 1992a); it is estimated that up to 29 % of vertebral fractures may go unrecognised in Europe alone (Delmas et al., 2005). Improved diagnosis of bone-related cancers is also expected to see rising costs for treatment. More than 2,000 cases are diagnosed per annum in the UK and more than 3,000 in the US.

It is thus important to understand the principles of the bone-healing cascade and manipulation with biomaterials and growth factors to aid development of successful tissue engineering strategies for effective bone regeneration and repair (Berner et al., 2012). This review examines data published between 1965 and 2013. All bone tissue engineering studies detailing an in vivo response of selected growth factors, including bone morphogenetic protein 2 (BMP-2), BMP-7/osteogenic protein 1 (OP-1), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), parathyroid hormone (PTH), parathyroid hormone-related protein (PTHrP), transforming growth factor beta 3 (TGF-β3), vascular endothelial growth factor (VEGF) and Wnt proteins, or combinations thereof, utilising animal models or human trials were included. All studies were compiled from PubMed-NCBI using search terms including ‘growth factor name’, ‘in vivo’, ‘model/animal’, ‘human’, and ‘bone tissue engineering’. Focus is drawn to the in vivo success of osteoinductive growth factors both in animals and in humans.

**Animal Model Selection**

To understand and recapitulate the healing cascade, suitable bone defects must be established in vivo through the use of appropriate animal models. Animal models allow for standardisation or elimination of variables which contribute to the success or failure of tissue engineered materials; animals may be obtained from the same source or breed, and maintained under identical environmental conditions (Khan and Lane, 2004). The bone defects must not exhibit spontaneous healing during the lifetime of the animal (Horner et al., 2010). These ‘critical-sized’ bone defects are dependent on multiple factors and remain difficult to define across anatomical location and species (Cooper et al., 2010). Previous work has defined a critical-sized defect as “a segmental bone deficiency of a length that exceeds 2 to 2.5 times the diameter of the affected bone” (Gugala et al., 2007). However, this definition is not often applied to defects within animal models, and the important parameters to report are defect size and location. Efficacy of any tissue-engineered constructs within these critical-sized defects is dependent on a number of variables detailed in Table 1 (Lindsey et al., 2006; Reichert et al., 2009; Rimondini et al., 2005). Furthermore, when choosing a

### Table 1. Factors affecting both study animal selection and efficacy of implanted constructs within these animal models.

<table>
<thead>
<tr>
<th>Factors affecting construct efficacy in vivo</th>
<th>Factors affecting animal species selection</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Anatomic bone location</td>
<td>- Acquisition and treatment costs</td>
</tr>
<tr>
<td>- Animal species and age</td>
<td>- Animal breed and uniformity</td>
</tr>
<tr>
<td>- Animal state of health (disease states)</td>
<td>- Animal size relative to implant number and size</td>
</tr>
<tr>
<td>- Bone structure and complexity</td>
<td>- Availability</td>
</tr>
<tr>
<td>- Defect size (critical sized) and position (bone region)</td>
<td>- Biological characteristics analogous to humans</td>
</tr>
<tr>
<td>- Mechanical load and stress conditions</td>
<td>- Blood and biopsy sample size and number</td>
</tr>
<tr>
<td>- Mutational status (strain)</td>
<td>- Ethical considerations</td>
</tr>
<tr>
<td>- Nutrition</td>
<td>- Existing biological knowledge of the species</td>
</tr>
<tr>
<td>- Presence of adjacent soft tissues</td>
<td>- Handling and nature of the animal</td>
</tr>
<tr>
<td>- Presence of periosteum</td>
<td>- Normal activity level of the animal</td>
</tr>
<tr>
<td>- Post-surgical fixation</td>
<td>- Resistance to infection</td>
</tr>
<tr>
<td>- Time</td>
<td>- Study period and lifetime of the animal</td>
</tr>
<tr>
<td>- Vascularisation</td>
<td>- Tolerance to surgery and captivity</td>
</tr>
</tbody>
</table>
suitable species for study, additional factors also require further consideration as detailed in Table 1 (Pearce et al., 2007; Reichert et al., 2009). A review of the literature by O’Loughlin et al. (2008) demonstrated clear preferences towards particular species for fracture related studies, including rat (38 %), rabbit (19 %), mouse (15 %), sheep (11 %), dog (9 %) and goat (4 %). The remaining 4 % comprised a wide selection of less frequently investigated animal models. Currently, no single animal model provides a representative comparison for human bone repair; rather each animal model is selected to address a particular research question. Advantages and disadvantages of current large and small animal models as human comparisons for bone tissue research are shown in Table 2. Furthermore, it is apparent that, dependent on the research question, bones of the selected animal model should exhibit significant physiological and pathophysiological analogies to human bone, regarding both macro- and micro-structure (Table 3). If animal studies are to inform clinical translation then models should be carefully selected to best recapitulate the in vivo bone environment within humans. Table 3 details the structure of bone, highlighting parameters that require consideration prior to animal model selection (Egermann et al., 2005; Liebschner, 2004). Some aspects of animal bone structure may be similar to that of humans; however, a balance should be struck with those that are different from humans. For example, preliminary investigations of bone biology and response to growth factor combinations could be assessed within small animals such as mouse or rat, as they provide a high-throughput in vivo model with similar biochemical composition to humans, and existing literature would help evaluate and interpret data. Systems could then be transferred to large animal models to assess candidate growth factors or combinations thereof in a functional setting analogous to humans. For example, defect regeneration strategies could be assessed within sheep long bone fractures as a model for large bone defects in humans, where the scale, mechanical loading and bone composition are similar to humans.

**Growth Factor Delivery Vehicles**

Following selection of an animal model and formation of a suitable critical-sized defect, a scaffold material exhibiting multifactorial properties is typically required to fill or bridge the defect site (Butler et al., 2000). Orthopaedic materials currently employed in bone regeneration studies comprise organic bone substitutes, synthetic biomaterials and/or inorganic materials (Table 4). The suitability of a selected biomaterial scaffold is governed by four factors; i) biomimicry, ii) biocompatibility, iii) biodegradability and iv) biomechanics. Successful scaffolds are thought to be those that replicate host tissue 3D architecture (porosity and microstructure enabling cell migration and vascularisation) (Bonfield, 2006; Laschke et al., 2008; Ma, 2008), and do not elicit an immunological or inflammatory response locally or systemically during either long or short-term integration. If degradation is required the material should degrade over time without production of toxic by-products, and endure mechanical and physiological stresses (Ghosh and Ingber, 2007; Howard et al., 2008; McMahon et al., 2008; Semino, 2008). Hip replacement implants are often not biodegradable, rather these implant scaffolds are selected to exhibit corrosion resistance, durability and strength sufficient enough to last the lifetime of the patient (Schauss et al., 2006). Biomechanical properties of interest include elasticity, thermostability and tensile strength of the constituent materials (El Haj et al., 2005; Guan and Davies, 2004; Lendlein and Langer, 2002).

Many researchers believe that bone scaffold material should ideally replicate/incorporate the extracellular matrix (ECM) and thus influence cell attachment, migration, proliferation, differentiation and resultant bone tissue organisation (Green et al., 2002; Karageorgiou and Kaplan, 2005; Shin et al., 2003; Yang et al., 2003). A variety of materials have been designed to address this challenge exhibiting either a bioactive osteoconductive surface (Takimoto et al., 2003), enhanced functionality as a consequence of cell-scaffold surface topography interactions (Cohen et al., 1993; Engel et al., 2008), functionalisation with a bioactive coating, or impregnation with bioactive molecules (Murphy and Mooney, 1999; Zhang et al., 2009a). Growth and development of functional engineered tissue is dependent on environmental cues, both physical and chemical (Burdick and Vunjak-Novakovic, 2009; Chan and Mooney, 2008; Quaglia, 2008). Implanted scaffolds can be designed as a delivery system for essential growth factors critical to cellular proliferation and osteogenic differentiation (Basmanav et al., 2008; Cartmell, 2009; Kanczler et al., 2008). Sustained release of encapsulated growth factors from implanted material scaffolds provides adequate localised osteoinduction at the defect site and has shown some success in vivo with respect to tissue engineered bone (Table 5) (Tabata, 2003).

Alternative vehicles have also been utilised to deliver selected growth factors in vivo. Rather than direct delivery, the gene(s) encoding the selected growth factor(s) can be introduced to the defect site by means of viral transduction or non-viral transfection. As seen in mouse studies administering BMP-2 as the choice growth factor, either viral (Gazit et al., 1999) or plasmid vectors (Osawa et al., 2009) can be directly delivered to the defect site (Dupont et al., 2012), or pre-treated cells can be delivered (Kallai et al., 2010).

**Individual Growth Factors**

Growth factor choice in a tissue engineering approach is critical for successful bone formation. Notable growth factors known to be important for bone regeneration include BMP-2, BMP-7/OP-1, FGF, PDGF, PTH, PTHrP, TGF-β3, VEGF and Wnt proteins. These growth factors have been applied individually and in combination, through direct and indirect delivery vehicles (Table 5). In vivo paracrine and autocrine signalling cascades leading to bone formation are complex and rely on strict spatiotemporal interplay between select growth factors. Teasing apart the individual roles that each growth factor plays within bone development and healing systems is of the highest
Table 2. Advantages and disadvantages of large and small animal models for *in vivo* bone tissue engineering strategies and extrapolation for human clinical study.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Large Animal Models</strong></td>
<td><strong>Extrapolation to human studies</strong></td>
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</tr>
<tr>
<td><strong>Dog</strong></td>
<td>Tractable</td>
<td>Breed variety</td>
<td>(Aerssens et al., 1998; Kimmel and Lee, 1982; Neyt et al., 1998; Pearce et al., 2007; Skurla and James, 2005)</td>
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<tr>
<td></td>
<td>Similar trabecular bone mineral density (BMD)</td>
<td>Ethical implications</td>
<td></td>
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<tr>
<td></td>
<td>Similar biochemical composition</td>
<td>High bone remodelling</td>
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<td></td>
<td>Considerable existing literature</td>
<td>High costs</td>
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<td></td>
<td>Trained in recuperative regime</td>
<td>High mechanical strength</td>
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<td>High solid bone fusion</td>
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<td>Low non-union</td>
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<td></td>
<td></td>
<td>Quadrupedal gait</td>
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<tr>
<td><strong>Goat</strong></td>
<td>Large body size for multiple implants</td>
<td>Ethical implications</td>
<td>(Lamerigts et al., 2000; Leung et al., 2001; Pearce et al., 2007)</td>
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<tr>
<td></td>
<td>Similar BMD and biochemical composition</td>
<td>Fast revascularisation</td>
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<td></td>
<td>Similar body weight</td>
<td>High costs</td>
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<tr>
<td></td>
<td>Similar bone remodelling rate</td>
<td>Inquisitive nature</td>
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<td></td>
<td>Tolerant of ambient conditions</td>
<td>Quadrupedal gait</td>
<td></td>
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<tr>
<td><strong>Pig</strong></td>
<td>Similar anatomy, biochemical composition, BMD, bone healing and bone morphology</td>
<td>Aggressive</td>
<td>(Aerssens et al., 1998; Mosekilde et al., 1993; Pearce et al., 2007; Thorwarth et al., 2005)</td>
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<tr>
<td></td>
<td></td>
<td>Difficult to handle</td>
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<td></td>
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<td>Ethical implications</td>
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<td></td>
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<td>Excessive body weight</td>
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<td></td>
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<td>High costs</td>
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<td></td>
<td></td>
<td>High growth rate</td>
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<td></td>
<td></td>
<td>Quadrupedal gait</td>
<td></td>
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<tr>
<td><strong>Primates</strong></td>
<td>Phylogenetic proximity to humans</td>
<td>Availability</td>
<td>(Khan and Lane, 2004)</td>
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<td></td>
<td>Similar skeletal structure</td>
<td>Difficult to handle</td>
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<tr>
<td></td>
<td>Similar BMD (dependent on subspecies)</td>
<td>Ethical implications</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>High costs</td>
<td></td>
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<tr>
<td><strong>Sheep</strong></td>
<td>Age mimics human ageing in bone and osteoid volume, and mineral apposition</td>
<td>Age-dependent bone remodelling</td>
<td>(Aerssens et al., 1998; Newman et al., 1995; Pearce et al., 2007; Ravaglioli et al., 1996)</td>
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<tr>
<td></td>
<td>Considerable existing literature</td>
<td>Ethical implications</td>
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<td></td>
<td>Docile</td>
<td>Haversion remodelling at 7-9 years</td>
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<td></td>
<td>Similar body weight</td>
<td>High costs</td>
<td></td>
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<td></td>
<td>Similar long bones structure</td>
<td>High mechanical strength (adults)</td>
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<td></td>
<td>Similar biochemical and mineral composition</td>
<td>High trabecular BMD</td>
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<td></td>
<td></td>
<td>Quadrupedal gait</td>
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<tr>
<td><strong>Small Animal Models</strong></td>
<td><strong>Extrapolation to human studies</strong></td>
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<tr>
<td><strong>Minipig</strong></td>
<td>Reduced growth rate</td>
<td>Ethical implications</td>
<td>(Aerssens et al., 1998; Pearce et al., 2007)</td>
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<tr>
<td></td>
<td>Reduced body mass</td>
<td>Limited clinical translation</td>
<td></td>
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<tr>
<td></td>
<td>Similar anatomy, BMD, bone healing and bone morphology</td>
<td>Quadrupedal gait</td>
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<td></td>
<td>Similar biochemical composition</td>
<td>Size limitation for implants</td>
<td></td>
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<tr>
<td><strong>Mouse</strong></td>
<td>Availability</td>
<td>Ethical implications</td>
<td>(Gomes and Fernandes, 2010; Liebschner, 2004; O’Loughlin et al., 2008)</td>
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<tr>
<td></td>
<td>Considerable existing literature</td>
<td>High bone healing rate</td>
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<td></td>
<td>Easy to handle</td>
<td>Impractical bone fixation</td>
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<td></td>
<td>Enable disease state research</td>
<td>Limited blood and biopsy samples</td>
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<td></td>
<td>Feasibility studies prior to scale up</td>
<td>Limited clinical translation</td>
<td></td>
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<tr>
<td></td>
<td>Immunodeficient - accept xenogenic material</td>
<td>Limited long term studies</td>
<td></td>
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<td></td>
<td>Lifespan allows for age-related research</td>
<td>Limited sampling</td>
<td></td>
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<td></td>
<td></td>
<td>Quadrupedal gait</td>
<td></td>
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<td></td>
<td></td>
<td>Size limitation for implants</td>
<td></td>
</tr>
<tr>
<td><strong>Rabbit</strong></td>
<td>Availability</td>
<td>Different bone structure</td>
<td>(Castaneda et al., 2006; Liebschner, 2004; Pearce et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>Comparable long bone and lumbar structure</td>
<td>Ethical implications</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Considerable existing literature</td>
<td>High bone turnover</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Early skeletal maturity</td>
<td>Limited clinical translation</td>
<td></td>
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<tr>
<td></td>
<td>Ease of handling and size</td>
<td>Quadrupedal gait</td>
<td></td>
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<td></td>
<td>Feasibility studies prior to scale up</td>
<td>Size limitation for implants</td>
<td></td>
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<tr>
<td><strong>Rat</strong></td>
<td>Availability</td>
<td>Ethical implications</td>
<td>(Aerssens et al., 1998; Gomes and Fernandes, 2010; Liebschner, 2004; O’Loughlin et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>Considerable existing literature</td>
<td>High bone remodelling</td>
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<td></td>
<td>Easy to handle</td>
<td>Limited blood and biopsy samples</td>
<td></td>
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<tr>
<td></td>
<td>Enable disease state research</td>
<td>Limited clinical translation</td>
<td></td>
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<td></td>
<td>Feasibility studies prior to scale up</td>
<td>Limited long term studies</td>
<td></td>
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<tr>
<td></td>
<td>Immunodeficient - accept xenogenic material</td>
<td>Quadrupedal gait</td>
<td></td>
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<tr>
<td></td>
<td>Lifespan allows for age-related research</td>
<td>Size limitation for implants</td>
<td></td>
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</tbody>
</table>
**Table 3.** Factors affecting both study animal selection and efficacy of implanted constructs within these animal models.

<table>
<thead>
<tr>
<th>Physiological and pathological analogies to humans</th>
<th>Hierarchy of bone structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Macro Structure</td>
<td>• Level 1 – Whole bone</td>
</tr>
<tr>
<td>Compact and cancellous bone</td>
<td>External and internal geometry</td>
</tr>
<tr>
<td>• Micro Structure</td>
<td>• Level 2 – Architecture</td>
</tr>
<tr>
<td>Osteons</td>
<td>Internal trabecular structure</td>
</tr>
<tr>
<td>Haversian bone</td>
<td>Haversian/interstitial structure</td>
</tr>
<tr>
<td>Lamellae</td>
<td>Circumferential structure</td>
</tr>
<tr>
<td>Trabeculae</td>
<td></td>
</tr>
<tr>
<td>• Shape and curvature</td>
<td>• Level 3 – Tissue</td>
</tr>
<tr>
<td>Epiphysis</td>
<td>Individual trabeculae</td>
</tr>
<tr>
<td>Metaphysis</td>
<td>Individual osteons</td>
</tr>
<tr>
<td>Diaphysis</td>
<td>Cortical microbeam structure</td>
</tr>
<tr>
<td>• Composition</td>
<td>• Level 4 – Lamellar</td>
</tr>
<tr>
<td>Bone mineral content and density</td>
<td>Individual lamellae structure</td>
</tr>
<tr>
<td>Collagen, proteoglycans and glycoproteins</td>
<td></td>
</tr>
<tr>
<td>• Healing and Remodelling</td>
<td>• Level 5 – Ultrastructure</td>
</tr>
<tr>
<td>Callus formation</td>
<td>Molecular composition</td>
</tr>
<tr>
<td>Osteoclast resorption</td>
<td>Mineral composition</td>
</tr>
<tr>
<td>Osteoblast activity</td>
<td></td>
</tr>
</tbody>
</table>

**Table 4.** Organic, inorganic and synthetic materials for orthopaedic applications *in vivo*.

<table>
<thead>
<tr>
<th>Organic Substitutes for Bone</th>
<th>Synthetic and Inorganic Substitutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name</td>
<td>Abbrev.</td>
</tr>
<tr>
<td>Alginate</td>
<td>ALG</td>
</tr>
<tr>
<td>Allograft</td>
<td>n/a</td>
</tr>
<tr>
<td>Autograft</td>
<td>n/a</td>
</tr>
<tr>
<td>Chitosan</td>
<td>n/a</td>
</tr>
<tr>
<td>Collagen</td>
<td>n/a</td>
</tr>
<tr>
<td>Coral</td>
<td>n/a</td>
</tr>
<tr>
<td>Cortico-cancellous human bone block</td>
<td>CHBB</td>
</tr>
<tr>
<td>Demineralised bone</td>
<td>n/a</td>
</tr>
<tr>
<td>Deproteinised bovine bone block with porcine collagen</td>
<td>DBBB</td>
</tr>
<tr>
<td>Fibrin</td>
<td>n/a</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>n/a</td>
</tr>
<tr>
<td>Gelatin</td>
<td>n/a</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>HAA</td>
</tr>
<tr>
<td>Matrigel</td>
<td>n/a</td>
</tr>
<tr>
<td>Monoolein</td>
<td>n/a</td>
</tr>
<tr>
<td>Silk fibroin</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

importance to any robust and effective tissue engineering strategy. Here, the authors discuss the effect of growth factor delivery on bone formation *in vivo* following ‘direct’, ‘indirect’ and ‘combination’ administration. Within each section, the effect of administration within first of all ‘small’ animal models is discussed, followed by the effect within ‘large’ animal models. The incorporation of cells within tissue engineering strategies and their effect on bone formation is discussed case by case throughout the review.

**BMP-2**

The discovery of auto-induced bone formation in rabbits implanted with autologous demineralised, lyophilised bone segments by Marshall R. Urist in 1965 (Urist, 1965) led to the identification of osteoinductive signalling molecules named by Urist as ‘bone morphogenetic proteins’ (BMPs) (Urist and Strates, 1971). BMPs act as morphogens providing crucial signals which direct cell differentiation and tissue architecture. To date, twenty human BMP proteins have been discovered, of which eight (BMP-1 to
### Table 5. *In vivo* bone tissue engineering utilising growth factors including BMP-2, BMP-7, FGF, PDGF, PTH, PTHrP, TGF-β3, VEGF and Wnt proteins. (divided into 4 parts - part 1)

<table>
<thead>
<tr>
<th>Growth Factor(s)</th>
<th>Animal Model</th>
<th>Defect location and type</th>
<th>Time</th>
<th>Delivery system</th>
<th>Dose/ Conc.</th>
<th>Defect regeneration</th>
<th>Analysis methods</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BMP-2</strong></td>
<td>Large Models</td>
<td>Dog, Goat, Horse, Monkey, Pig and Sheep</td>
<td>3 weeks to 26 months</td>
<td>Organic scaffolds – collagen, demineralised bone, gelatin and silk fibroin</td>
<td>5 to 100,000 µg/mL</td>
<td>1.0 to 2.6 fold (biomechanics)</td>
<td>Biochemistry, biomechanical testing, CT (micro), DFA, faxitron, histology, immunohistomorphometry, immunomorphometry, in situ hybridisation, ARI, RT-PCR, radiography (micro) and SEM</td>
</tr>
<tr>
<td></td>
<td>Small Models</td>
<td>Mouse, Rabbit and Rat</td>
<td>5 to 24 weeks</td>
<td>Organic scaffolds – CPC, ceramic phosphate, HA, PCL, PEG, PLA, PLLA, TCP and titanium</td>
<td>0.1 to 5.0 µg (other studies have used 150,000 µg/kg)</td>
<td>2.0 to 4.0 µg/mL</td>
<td>1.0 to 3.0 fold (biomechanics)</td>
</tr>
<tr>
<td><strong>BMP-7/OP-1</strong></td>
<td>Large Models</td>
<td>Baboon, Dog, Goat, Monkey and Sheep</td>
<td>1 week to 1 year</td>
<td>Organic scaffolds – alginite, allalgel, autograph, chitosan, collagen, DBBB and xenograft</td>
<td>100 to 750,000 µg/kg 1.0 to 3.0 µg/mL</td>
<td>1.25 to 8.3 fold (bone)</td>
<td>Biomechanical testing, CT (micro), DFA, histology, immunohistomorphometry, immunomorphometry, MRI, radiography (micro), radioluminography, and SEM</td>
</tr>
<tr>
<td></td>
<td>Small Models</td>
<td>Minipig, Mouse, Rabbit and Rat</td>
<td>5 to 12 weeks</td>
<td>Organic scaffolds – HA, hydroxyapatite, PCL, PLGA, PLLA, polyactic acid, TCP and titanium</td>
<td>0.025 to 3.50 µg 25 to 200 µg/mL</td>
<td>1.1 to 2.5 fold (bone) – one study showed 0.8 fold (bone)</td>
<td>1.1 to 3.0 fold (biomechanics)</td>
</tr>
<tr>
<td><strong>FGF-2/1/8</strong></td>
<td>Large Models</td>
<td>Dog and Primate</td>
<td>2 to 32 weeks</td>
<td>Direct injection</td>
<td>0.15 to 200 µg 100 to 400 µg/kg</td>
<td>1.3 to 5 fold (bone)</td>
<td>Biochemistry, biomechanical testing, histology, immunohistomorphometry, immunomorphometry, micro CT, radiography and SEM</td>
</tr>
<tr>
<td></td>
<td>Small Models</td>
<td>Mouse, Rabbit and Rat</td>
<td>3 to 24 weeks</td>
<td>Organic scaffolds – collagen, gefarin and Matrigel</td>
<td>0.01 to 200 µg 10 to 100 µg/mL 100 to 1,000 µg/kg</td>
<td>1.2 to 16.4 fold (bone)</td>
<td>2.1 to 4 fold (biomechanics)</td>
</tr>
<tr>
<td><strong>PDGF</strong></td>
<td>Small Models</td>
<td>Minipig, Mouse, Rabbit and Rat</td>
<td>10 to 12 weeks</td>
<td>Organic scaffolds – chitosan, collagen, DBB and fibrin</td>
<td>0.01 to 750 µg 1 to 1,000 µg/mL</td>
<td>1.45 to 10 fold (bone)</td>
<td>Biochemistry, biomechanical testing, histology, immunohistomorphometry, immunomorphometry, micro CT, radiography and SEM</td>
</tr>
<tr>
<td><strong>PTH (1-31, 1-34, 1-84, 2-34, 24-88 and 53-86)</strong></td>
<td>Large Models</td>
<td>Dog, Monkey and Sheep</td>
<td>4 to 10 years</td>
<td>Subcutaneous injection</td>
<td>0.75 to 7.5 µg/kg 20 to 1,000 µg/mL</td>
<td>1.1 to 3 fold (bone)</td>
<td>Biochemistry, biomechanical testing, DFA, histology, immunohistomorphometry, immunomorphometry, in situ hybridisation, micro CT, northern blot, QCT, radiography, radiolabelling, RT-PCR and SEM</td>
</tr>
<tr>
<td></td>
<td>Small Models</td>
<td>Mouse, Rabbit and Rat</td>
<td>1 to 1.5, 2 to 24 months</td>
<td>Inorganic scaffolds – calcium sulfate, HA, PEG and TCP</td>
<td>0.05 to 800 µg/kg 20 to 100 µg/mL</td>
<td>1.1 to 13 fold (bone)</td>
<td>1.4 to 2.5 fold (mechanics)</td>
</tr>
<tr>
<td><strong>PTHRP peptides (1-36 and 167-199)</strong></td>
<td>Small Models</td>
<td>Mouse, Rabbit and Rat</td>
<td>12 to 6 months</td>
<td>Subcutaneous injection</td>
<td>10 to 320 µg/kg</td>
<td>1.2 to 10 fold (bone)</td>
<td>Biomechanical testing, CT (micro), histology, immunohistomorphometry, immunomorphometry, in situ hybridisation, micro CT, northern blot, QCT, radiography, radiolabelling, RT-PCR and SEM</td>
</tr>
<tr>
<td><strong>TGF-β3</strong></td>
<td>Large Models</td>
<td>Baboon</td>
<td>30 to 90 days</td>
<td>Organic scaffolds – collagen and Matrigel</td>
<td>75 to 125 µg 1.6 to 5 fold (bone)</td>
<td>Biochemistry, biomechanical testing, CT (micro), histology, immunohistomorphometry, immunomorphometry, in situ hybridisation, micro CT, northern blot, QCT, radiography, radiolabelling, RT-PCR and western blot</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Small Models</td>
<td>Mouse and Rat</td>
<td>14 to 28 days</td>
<td>Organic scaffolds – calcium phosphate and collagen</td>
<td>0.003 to 2.75 µg</td>
<td>1.05 fold (bone)</td>
<td>Biochemistry, biomechanical testing, CT (micro), histology, immunohistomorphometry, immunomorphometry, in situ hybridisation, micro CT, northern blot, QCT, radiography, radiolabelling, RT-PCR and western blot</td>
</tr>
<tr>
<td><strong>VEGF</strong></td>
<td>Small Models</td>
<td>Mouse</td>
<td>4 weeks</td>
<td>Organic scaffolds – calcium phosphate and inorganic scaffolds – PL A</td>
<td>1.7 µg 3 µg/mL</td>
<td>1.65 fold (bone)</td>
<td>Enhanced vascularity (micro CT), histology, immunohistomorphometry, immunomorphometry, in situ hybridisation, micro CT, northern blot, QCT, radiography, radiolabelling, RT-PCR and radiography</td>
</tr>
</tbody>
</table>
### Table 5. In vivo bone tissue engineering utilising growth factors including BMP-2, BMP-7, FGF, PDGF, PTH, PTHrP, TGF-β3, VEGF and Wnt proteins. (continued - part2)

<table>
<thead>
<tr>
<th>Growth Factor(s)</th>
<th>Animal Model</th>
<th>Defect location and type</th>
<th>Time</th>
<th>Delivery system</th>
<th>Dose/Conc.</th>
<th>Defect regeneration</th>
<th>Analysis methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMP-2 plus (BMP-7, BMP-7/OP-1, Epo, FGF, integrin, MSC, Fn, TGF-β2, sublimonin, VEGF or zoledronic acid)</td>
<td>Large Model Dog, Horse and Pig</td>
<td>Drill – calvaria, orbital bone (incisal) and alveolar ridge</td>
<td>4 to 9 weeks</td>
<td>Direct viral/non-viral particle injection or implant of transduced/ transfected cells with and without scaffold, or scaffold-free injections, retroviral transduction</td>
<td>5.26 to 120 µg BMP-2, 2 x 10^5 viral particles</td>
<td>1.6 fold (bone)</td>
<td>Biochemistry, biomechanical testing, CT (micro), histochemistry, immunohistochemistry, in vivo hybridisation and RT-PCR</td>
</tr>
<tr>
<td>Wnt-5A</td>
<td>Small Model Chick, Mouse and Rat</td>
<td>Drill – calvaria, SCID mouse Transfected embryo</td>
<td>4 to 12 weeks</td>
<td>Direct implantation of transduced/transfected cells with and without scaffold, or scaffold-free injections</td>
<td>0.35 to 5 x 10^5 cells</td>
<td>1.25 to 10 fold (bone) 1.0 fold (cartilage)</td>
<td>Wnt-5A reduced bone formation</td>
</tr>
<tr>
<td>PDGF</td>
<td>Large Model Mouse and Rat</td>
<td>Ectopic – subcutaneous Segmental – femur, radius and tibia (endochondral)</td>
<td>10 to 6 weeks</td>
<td>Direct delivery or non-viral particle injection or implant of transduced/ transfected cells</td>
<td>5.5 x 10^5 to 5 x 10^6 PFU/mL 1 x 10^5 cells</td>
<td>1.7 to 2 fold (bone)</td>
<td>Backscatter SEM, biochemistry, biomechanical testing, histochemistry, immunohistochemistry, micro CT, northern blot and RT-PCR</td>
</tr>
<tr>
<td>VEGF</td>
<td>Large Model Mouse and Rat</td>
<td>Ectopic – subcutaneous Segmental – femur, radius and tibia (endochondral)</td>
<td>4 to 16 weeks</td>
<td>Direct delivery of non-viral particles or implantation of transfected cells</td>
<td>20 µg 5 x 10^5 cells</td>
<td>1.6 to 2 fold (bone) 3 fold (vascularisation)</td>
<td>CT (micro), histology, immunohistochemistry, micro CT, northern blot and RT-PCR and radiography</td>
</tr>
<tr>
<td>TGF-β3</td>
<td>Large Model Mouse</td>
<td>Ectopic – subcutaneous</td>
<td>30 d</td>
<td>Direct implantation of transduced cells within scaffold</td>
<td>1 x 10^6 cells</td>
<td>3D cartilage constructs</td>
<td>Histology, immunohistochemistry and western blot</td>
</tr>
<tr>
<td>BMP-2</td>
<td>Large Model Dog, Horse and Pig</td>
<td>Drill – calvaria, orbital bone (incisal) and alveolar ridge</td>
<td>1 to 9 weeks</td>
<td>Direct delivery by injection or implant, and indirect delivery by viral/non-viral particle injection or implant of transduced/ transfected cell implants</td>
<td>5.26 to 120 µg BMP-2, 2 x 10^5 viral particles</td>
<td>1.6 fold (bone)</td>
<td>Biochemistry, biomechanical testing, CT (micro), DxA, histochemistry, immunohistochemistry, in vivo hybridisation and RT-PCR, PET (micro), radiography, rhnology and western blot</td>
</tr>
<tr>
<td>BMP-2</td>
<td>Small Model Minipig, Mouse, Rabbit and Rat</td>
<td>Drill – calvaria, orbital bone (incisal) and alveolar ridge</td>
<td>1 to 16 weeks</td>
<td>Direct implantation of transduced/transfected cells with and without scaffold, or scaffold-free injections</td>
<td>0.0025 to 200 µg BMP-2, 0.075 to 7.5 x 10^5 viral particles</td>
<td>1 to 20 fold (bone) 4 to 8.5 fold (biomechanics) 2 fold (vasculature)</td>
<td>Wnt-5A reduced bone formation</td>
</tr>
</tbody>
</table>

#### 2. Indirect Delivery

<table>
<thead>
<tr>
<th>Growth Factor(s)</th>
<th>Animal Model</th>
<th>Defect location and type</th>
<th>Time</th>
<th>Delivery system</th>
<th>Dose/Conc.</th>
<th>Defect regeneration</th>
<th>Analysis methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMP-2</td>
<td>Large Model Dog, Horse and Sheep</td>
<td>Drill – calvaria, orbital bone (incisal) and alveolar ridge</td>
<td>4 to 24 weeks</td>
<td>Direct viral/non-viral particle injection or implant of transduced/ transfected cells with and without scaffold, or scaffold-free injections</td>
<td>12 µg 0.04 to 5 x 10^5 viral particles 2 to 5 x 10^5 cells</td>
<td>1.3 to 3.2 fold (bone)</td>
<td>Biochemical testing, CT (micro), histochemistry, immunohistochemistry, in vivo hybridisation and RT-PCR</td>
</tr>
<tr>
<td>BMP-3/OPT-1</td>
<td>Large Model Dog and Goat</td>
<td>Ectopic – intramuscular Intervertebral disc transplant Segmental – femur, radius and tibia (endochondral) and mandible (intramembranous)</td>
<td>1 to 8 months</td>
<td>Direct delivery of non-viral particles or implantation of transduced/ transfected cells</td>
<td>2 x 10^5 viral particles 0.01 to 5 x 10^5 cells</td>
<td>2 to 2.5 fold (bone)</td>
<td>Biochemical testing, biochemistry, CT (micro), histochemistry, immunohistochemistry, micro CT, northern blot and RT-PCR and SEM</td>
</tr>
<tr>
<td>FGF-2</td>
<td>Large Model Dog</td>
<td>Fusion – dental root Irradiation Drill – calvaria Segmental – radius (endochondral)</td>
<td>6 weeks</td>
<td>Direct implantation of transduced cells within scaffold</td>
<td>0.0625 to 5 x 10^6 cells</td>
<td>2 to 7 fold (bone) one study showed 53.5 fold</td>
<td>Enhanced periodontal bone regeneration</td>
</tr>
</tbody>
</table>

#### 3. Combinational Delivery

<table>
<thead>
<tr>
<th>Growth Factor(s)</th>
<th>Animal Model</th>
<th>Defect location and type</th>
<th>Time</th>
<th>Delivery system</th>
<th>Dose/Conc.</th>
<th>Defect regeneration</th>
<th>Analysis methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMP-2</td>
<td>Large Model Dog, Horse and Pig</td>
<td>Drill – calvaria, orbital bone (incisal) and alveolar ridge</td>
<td>1 to 9 weeks</td>
<td>Direct delivery by injection or implant, and indirect delivery by viral/non-viral particle injection or implant of transduced/ transfected cell implants</td>
<td>5.26 to 120 µg BMP-2, 2 x 10^5 viral particles</td>
<td>1.6 fold (bone)</td>
<td>Biochemistry, biomechanical testing, CT (micro), DxA, histochemistry, immunohistochemistry, in vivo hybridisation and RT-PCR, PET (micro), radiography, rhnology and western blot</td>
</tr>
<tr>
<td>BMP-2</td>
<td>Small Model Minipig, Mouse, Rabbit and Rat</td>
<td>Drill – calvaria, orbital bone (incisal) and alveolar ridge</td>
<td>1 to 16 weeks</td>
<td>Direct implantation of transduced/transfected cells with and without scaffold, or scaffold-free injections</td>
<td>0.0025 to 200 µg BMP-2, 0.075 to 7.5 x 10^5 viral particles 1 to 4 x 10^6 cells</td>
<td>1 to 20 fold (bone) 4 to 8.5 fold (biomechanics) 2 fold (vasculature)</td>
<td>Wnt-5A reduced bone formation</td>
</tr>
</tbody>
</table>
### Table 5. In vivo bone tissue engineering utilising growth factors including BMP-2, BMP-7, FGF, PDGF, PTH, PTHrP, TGF-β3, VEGF and Wnt proteins. (continued - part 3)

<table>
<thead>
<tr>
<th>Growth Factor(s)</th>
<th>Animal Model</th>
<th>Defect location and type</th>
<th>Time</th>
<th>Delivery system</th>
<th>Dose/ Conc.</th>
<th>Defect regenera- tion</th>
<th>Analysis methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMP-7/OIP-1</td>
<td>Large Models</td>
<td>Drill – calvaria and femur</td>
<td>2 to 16 weeks</td>
<td>Direct delivery by injection or implant, and indirect delivery by viral non-viral particle injection or transduced transduced cell implant</td>
<td>5 to 5,000 µg</td>
<td>5 fold (bone)</td>
<td>Biochemistry, biomechanical testing, CT (micro), osteohistology, histomorphometry, immunohistochemis- try, X-ray, micro-CT and SEM</td>
</tr>
<tr>
<td>BMP-2</td>
<td>Human</td>
<td>Bone augmentation</td>
<td>6 weeks</td>
<td>Direct delivery by injection or implant, and indirect delivery by viral non-viral particle injection or transduced transduced cell implant</td>
<td>0.9 to 100 mg</td>
<td>Enhanced bone healing was observed in the majority of patients</td>
<td></td>
</tr>
</tbody>
</table>

### Table 5. In vivo bone tissue engineering utilising growth factors including BMP-2, BMP-7, FGF, PDGF, PTH, PTHrP, TGF-β3, VEGF and Wnt proteins. (continued - part 4)

<table>
<thead>
<tr>
<th>Growth Factor(s)</th>
<th>Animal Model</th>
<th>Defect location and type</th>
<th>Time</th>
<th>Delivery system</th>
<th>Dose/ Conc.</th>
<th>Defect regenera- tion</th>
<th>Analysis methods</th>
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</thead>
<tbody>
<tr>
<td>BMP-7/OIP-1</td>
<td>Large Models</td>
<td>Drill – calvaria and femur</td>
<td>2 to 16 weeks</td>
<td>Direct delivery by injection or implant, and indirect delivery by viral non-viral particle injection or transduced transduced cell implant</td>
<td>5 to 5,000 µg</td>
<td>5 fold (bone)</td>
<td>Biochemistry, biomechanical testing, CT (micro), osteohistology, histomorphometry, immunohistochemis- try, X-ray, micro-CT and SEM</td>
</tr>
<tr>
<td>BMP-2</td>
<td>Human</td>
<td>Bone augmentation</td>
<td>6 weeks</td>
<td>Direct delivery by injection or implant, and indirect delivery by viral non-viral particle injection or transduced transduced cell implant</td>
<td>0.9 to 100 mg</td>
<td>Enhanced bone healing was observed in the majority of patients</td>
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</table>
Table 5. In vivo bone tissue engineering utilising growth factors including BMP-2, BMP-7, FGF, PDGF, PTH, PTHrP, TGF-β3, VEGF and Wnt proteins. (continued - part 4)

<table>
<thead>
<tr>
<th>Growth Factor(s)</th>
<th>Animal Model</th>
<th>Defect location and type</th>
<th>Time</th>
<th>Delivery system</th>
<th>Dose/Conc.</th>
<th>Defect regeneration</th>
<th>Analysis methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMP-7/OP-1</td>
<td>Human</td>
<td>• Mandible reconstruction Non-union fracture Long bone osteotomy Lumbar fusion Pseudarthrosis</td>
<td>2 weeks to 60 months</td>
<td>Organic scaffolds – allograft, autograft, collagen and xenograft Inorganic scaffolds – CMC, TCP and titanium</td>
<td>2.5 to 17.5 μg – one study; 2.000 μg (5.5mg) average</td>
<td>Accelerated bone healing and increased bone tissue and mechanical strength was observed</td>
<td>Clinical assessment, CT, histology, histomorphometry, physical examination, radiography and scintigraphy</td>
</tr>
<tr>
<td>PDGF</td>
<td>Human</td>
<td>• Periodontitis</td>
<td>9 months</td>
<td>Organic scaffolds – allograft</td>
<td>0.5 to 5 mg/mL</td>
<td>1.12 to 1.17 fold (bone)</td>
<td>Clinical assessment, histology and radiography</td>
</tr>
<tr>
<td>PTH</td>
<td>Human</td>
<td>• Healthy adults</td>
<td>2 months to 2 years (one study - 7 d)</td>
<td>Subcutaneous injection</td>
<td>20 to 100 μg</td>
<td>Accelerated bone healing through upregulation of bone markers and resultant bone tissue was observed</td>
<td>Biochemistry, clinical assessment, CT, DXA, histology, histomorphometry, QCT, other imaging, such as MRI, bone turnover markers, clinical and radiographic assessment</td>
</tr>
<tr>
<td>PTHrP/PTH peptides (1-34 and 1-36)</td>
<td>Human</td>
<td>• Healthy adults</td>
<td>2.4 to 2 weeks</td>
<td>Subcutaneous injection</td>
<td>2 to 80 pmol/kg/h</td>
<td>Bone formation was activated in postmenopausal females, but inhibited in healthy adults</td>
<td>Biochemistry</td>
</tr>
</tbody>
</table>

Abbreviations: 1,25(OH₂)D3 (1,25-dihydroxyvitamin D3), 2MD (2-methylene-19-nor-(20S)-calcitriol), BMP (Bone morphogenetic protein), BMP-2/7 (Bone morphogenetic protein 2/7), CDHA (calcium deficient hydroxyapatite), CHBB (chiro-cancellous human bone block), CHPA (chitosan-agarose Binder with parathyroid hormone), CMC (carboxymethylcellulose), CPC (calcium phosphate cement), CT (computerized tomography), DBBM (demineralised bone matrix), DXA (dual energy x-ray absorptiometry), Epa (erspinase protein), FGF (fibroblast growth factor), FTIR (Fourier transform infrared spectroscopy), HA (hydroxyapatite), HAA-PV (Alkaline acid and poly-vinyl alcohol functionalized with hydroxy acid groups), ID2 (Intra-hydroxyvivitamin D2), ID3 (Intra-hydroxyvivitamin D3), IBM (macrophage myeloperoxidase), IDH (inosine deoxyribonucleic), TGFβ (transforming growth factor beta), TGFβ3 (transforming growth factor beta 3), VEGF (vascular endothelial growth factor), Wnt (wingless-type MMTV integration site family), BMP-8a (bone morphogenetic protein 8a), BMP-2 (bone morphogenetic protein 2), BMP-7 (bone morphogenetic protein 7), FGF (fibroblast growth factor), PDGF (Platelet derived growth factor), PTH (parathyroid hormone), PTHrP (parathyroid hormone receptor-related protein), PPARγ (peroxisome proliferator-activated receptor γ), PPARα (peroxisome proliferator-activated receptor α), PPARδ (peroxisome proliferator-activated receptor δ), PPARβ/δ (peroxisome proliferator-activated receptor β/delta), BMP binding protein, OP-1 (osteogenic protein-1), ORX (orchitectonic), OXV (ovariectomized), PCL (poly-caprolactone), PLA (poly-D, lactic acid), PEG (poly ethylene glycol), PLLA (poly-l-lactide), PIP (polyamide-amidcompact), PPL (poly-D-L-lactic acid), PPTA (polyetheramide), PPF (poly(propylene fumarate), PPF (poly(propylene fumarate), PPF (polyethylene terephthalate), PPF (poly(propylene fumarate), TGFβ (transforming growth factor beta), BMSC (Bone marrow mesenchymal stem cells), MSC (mesenchymal stem cells), PBS (phosphate-buffered saline), TEM (transmission electron microscopy), SEM (scanning electron microscopy), FTIR (Fourier transform infrared spectroscopy), MRI (magnetic resonance imaging), QCT (quantitative computed tomography), PCT (corticosteroid), PHTBA (polymethyl methacrylate), PFA (polycarbonate), PX (polyethylene glycol), PVA (polyvinyl alcohol), OP-1 (osteogenic protein-1), BMP-2/7 (Bone morphogenetic protein 2/7), RT-PCR (real time polymerase chain reaction), SEM (scanning electron microscopy), TRAP (tartrate resistant acid phosphatase), TRAP (tartrate resistant acid phosphatase), TEM (transmission electron microscopy), TGFβ (transforming growth factor beta), VEGF (vascular endothelial growth factor), Wnt (wingless-type MMTV integration site family), and XTM (X-ray tomographic microscopy) • number of publications.

BMP-8a has a known osteochondral function (Even et al., 2012). BMP-2 specifically is a disulfide-linked homodimer with a known role in osteoblast differentiation. Abundant use of recombinant human BMP-2 (rhBMP-2) within animal models has demonstrated successful in vivo bone regeneration and repair, and has been extensively examined as an osteoinductive growth factor for tissue engineering (Supplementary Table 1).

Direct administration of BMP-2

A review of the literature revealed variable increased bone formation and defect regeneration ranging between 1.2 and 2.5 μg/kg/h for large animals (Itoh et al., 1998; Sheehan et al., 1999; Woo et al., 2010b), and 2 to 80 pmol/kg/h for small animals (Itoh et al., 1998; Sheehan et al., 2003) and 2 μg/mL to 4 mg/mL for small animals (Bax et al., 1999; Woo et al., 2001). There does not appear to be any correlation between dosages and fold increase in bone formation or time to healing. Therefore, reporting both defect volume and implant volume would help comparison of studies. It is important to clarify distinctions between studies and that ideal comparisons would be made between identical animals and anatomic defect locations, of which to date there are not enough publications for statistical comparison.

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Indirect administration of BMP-2
Further studies have utilised indirect delivery by viral transduction or non-viral transfection of rhBMP-2 to the bone defect site with a view to enabling sustained localised growth factor delivery. The two main avenues for application are either virus particles/plasmid vectors (Dupont et al., 2012; Ishihara et al., 2008), or transduced/transfected cells (Lazard et al., 2011; Lin et al., 2008). The former influences non-specific host cell-terminated tissue regeneration, whilst the latter enables exogenous cell-derived tissue repair. Indirect delivery studies showed a maximum 9-fold increase in bone formation (Wang et al., 2009), demonstrating reduced tissue repair in comparison to direct delivery. This may be due to under-dosing at the defect site due to inadequate uptake by endogenous cells or production by exogenous cells. Continuous production of rhBMP-2 at the defect site may also have had negative or limiting effects on bone formation, compared to a single dose in most direct delivery studies. Spatiotemporal delivery at the defect site is therefore of paramount importance for augmentation of defect regeneration.

Combination administration of BMP-2
The bone-healing cascade is a complex process whose effective recapitulation is dependent on an exquisite interplay between multiple growth factors (Grimes et al., 2011). A number of in vivo studies have thus investigated the use of growth factors in combination rather than single factor application (Table 5). Wang et al. (2012) delivered 5 µg of BMP-2 and 5 µg of BMP-7 via an implanted collagen sponge within a minipig calvarial defect, and demonstrated a 1.5 fold increase in bone formation compared to either growth factor alone. Koh et al. (2008) also investigated BMP-2 and BMP-7 indirect delivery via implantation of transduced cells within a mouse calvarial defect. The study showed a maximum 2-fold increase in bone formation compared to individual growth factor administration. Combination with other factors also demonstrated increased bone formation; Fujimura et al. (2002) showed a maximum 3.5-fold increase with BMP-7 and FGF-2 treatment, compared to 1.7 fold with BMP-7 alone. Clearly, there are benefits to dual combinations over single factors. Other factors and compounds used in combination with rhBMP-2, whether directly or indirectly, included BMP binding protein (BBP) (Lee et al., 2011), erythropoietin (Sun et al., 2012a), FGF (Springer et al., 2008), a4-integrin (Kumar et al., 2010a), Runx2 (Lee et al., 2010), TGF-β2 (Thorey et al., 2011), tobramycin (Glatt et al., 2009) and zoledronic acid (Doi et al., 2011; Schindeler et al., 2011). A number of studies have incorporated mesenchymal stem cells (MSCs) (Hou et al., 2007; Kim et al., 2009) providing a healthy inducible cell source within the defect site (Dawson et al., 2014). Taken together these studies demonstrated between 1.1 and 4 fold (Hou et al., 2007; Thorey et al., 2011) increased bone formation and defect regeneration (Supplement 1). Interestingly, some studies have shown that combined growth factor delivery does not enhance bone formation in comparison to single growth factor application. Terella et al. (2010) and Springer et al. (2008) demonstrated no further enhancement of bone regeneration above controls with BMP-2 treatment in combination with MSCs and FGF-2, respectively. Indeed, Egermann et al. (2006) revealed a significant systemic retardation of bone formation within sheep injected with BMP-2 expressing adenovirus. This negative effect may be due to i) the combination of growth factors chosen or, ii) inhibitory or competitive effects between the combinations selected. The growth factor most often used successfully in combination with BMP-2 remains VEGF, where studies have reported a 1.4 to 20 fold (Xiao et al., 2011; Zhang et al., 2011a) increase in bone formation. Co-administration with VEGF induced vessel ingrowth bringing endogenous cells to the defect site, which could be triggered by BMP-2 to differentiative towards the osteogenic lineage and deposit new bone matrix. Ultimately, the aim of combination treatment is to support and augment native healing processes, and to do so requires a specific spatiotemporal approach with select growth factors.

BMP-7/OP-1
BMP-7, also known as OP-1, constitutes another BMP family member routinely used in bone tissue engineering strategies (Supplementary Table 2). Many in vivo studies have utilised BMP-7 on the basis that its osteoinductive potential can drive enhanced bone defect regeneration (Kidder et al., 2009; Lee et al., 2013).

Direct administration of BMP-7
Analogous to BMP-2, BMP-7 has been employed in many large (baboon (Ripamonti et al., 2001a), dog (Fukuroku et al., 2007), goat (den Boer et al., 2000), monkey (Cook et al., 2009)) and small (minipig (Warnke et al., 2006), mouse (Lee et al., 2013), rabbit (Haidar et al., 2010b) and rat (Haidar et al., 2010a)) animals with variable increased bone formation and defect regeneration ranging between 1.25 and 8.3 fold (Blokhuys et al., 2001; Salkeld et al., 2001) and 1.1 and 29.5 fold (Hamdy et al., 2003; Kidder et al., 2009), respectively. One interesting study by Chen et al. (2006) demonstrated a staggering 96 fold increase in mineralised callus formation after just 2 weeks with high dose OP-1 (200 µg). The bone defect model used however was complicated by Staph. Aureus infection and results should be carefully interpreted. Success variability may be dosage dependent since direct administration of BMP-7 has ranged from 100 µg to 3.5 mg (Reichert et al., 2012; Ripamonti et al., 2000) in large animals (some studies employed 65 mg to 750 mg (Lind et al., 2001; Salkeld et al., 2001)) and 0.025 µg to 3.5 mg (Sampath et al., 1992; Warnke et al., 2006) in small animals. However, it remains to be ascertained whether this suggested correlation is positive (higher dose results in higher bone formation (Chen et al., 2006; Haidat et al., 2010b; Ripamonti et al., 2000)), negative (higher dose results in lower bone formation (Cook et al., 2005; Soballe et al., 2004)) or whether it indeed exists (bone formation remains unaffected by dosage (Hamdy et al., 2003; Leknes et al., 2008)). Standardisation of a species dependent bone defect model of specific dimensions and anatomic location, rather than a simply stated ‘critical sized’ defect, would inevitably aid interpretation of in vivo data. In turn, this would also help comparison between studies regarding the efficacy of individual growth factors such as BMP-7.
to drive osteogenic bone formation. The variety of defects currently investigated include segmental (Chen et al., 2002; Forriol et al., 2009; Reichert et al., 2012), drill (Lee et al., 2013; Zhang et al., 2004), fusion (Blattert et al., 2002; Grauer et al., 2004; Magin and Delling, 2001) and ectopic implantation intramuscularly (Haidar et al., 2010a; Spiro et al., 2010) and subcutaneously (Sampath et al., 1992; Wei et al., 2007), which together emphasise the lack of and need for standardisation. Indeed, Takigami et al. (2007) showed that anatomic location of the defect affected the efficacy of implanted BMP-7 on bone regeneration, where treatment of 10 mm femoral defects at the proximal and distal ends demonstrated 1.5-fold increased and 1.4-fold decreased bone formation. Other interesting observations which warrant further investigation include non-augmentation of bone formation following BMP-7 treatment and altered structure of new bone. Mont et al. (2001) showed that bone formation was the same with or without BMP-7 administration on allograft. It is important therefore to choose the scaffold material carefully as endogenous factors within the graft matrix may have masked the effects of BMP-7. Lammen et al. (2009) also reported a lack of bone augmentation following BMP-7 administration on bone filler. Spiro et al. (2010) showed that diclofenac treatment altered BMP-7-induced bone structure, decreasing trabeculae number and increasing spacing. Encouragingly, alternative ways of controlling inflammation at the defect site include the addition of BMP-binding peptide (BBP), which demonstrated a 1.5-fold reduction (Lee et al., 2011).

Indirect administration of BMP-7
An alternative delivery method for growth factor delivery, as previously discussed, is viral transduction or non-viral transfection. BMP-7 has been indirectly delivered through the use of viral particles (2 x 10^6 in large animals (Zhang et al., 2007), and 1.8 to 2.5 x 10^5 in small animals (Dunn et al., 2005; Zhang et al., 2012b)) and transduced/transfected cells (1 x 10^6 to 5 x 10^6 in large animals (Chaoefong et al., 2013; Zhu et al., 2010), and 1 x 10^6 to 2 x 10^6 in small animals (Li et al., 2010a; Zhang et al., 2010c)). Zhang et al. (2007) demonstrated a 2-fold increase in bone formation after implantation of viral particles, whilst Zhu et al. (2010) demonstrated a 2.5-fold increase after implantation of transduced bone marrow stromal cells (BMSCs) into dog and goat, respectively. Interestingly, the same study by Zhu et al. showed that implantation of non-transduced BMSCs also increased bone formation, but to a lesser degree (1.5 fold). Clearly, addition of cells alone without modification or growth factor loading can enhance bone defect regeneration. Typically, small animal studies demonstrated a 1.03 to 5 fold (Li et al., 2010a; Zhang et al., 2012b) increase in bone formation, although one study by Hidaka et al. (2003) reported 21 fold increased bone formation. However, this study investigated spinal fusion compared to segmental defects investigated in lower fold increase studies.

Combination administration of BMP-7
Additional factors have been successfully utilised in combination with BMP-7 to aid bone regeneration. Combination of BMP-7 with BMP-2 constitutes an additive approach where two osteogenic factors are hypothesised to further enhance the osteogenic outcome, whilst keeping individual dosages low (Koh et al., 2008; Wang et al., 2012). Alternatively, combinations with VEGF (Roldan et al., 2010) or TGF-β3 (Ripamonti et al., 2010) constitute mutualistic approaches where the angiogenic factor induces vessel ingrowth into the defect (2 fold (Zhang et al., 2010a)), the chondrogenic factor induces cartilaginous matrix production to fill the defect void, and the osteogenic factor induces resultant callus mineralisation and eventual bone formation (three lineages important for recapitulation of the in vivo bone healing cascade). Other factors and compounds used in combination with BMP-7 include BBP (Lee et al., 2011), FGF-2 (Ma et al., 2007), insulin-like growth factor 1 (IGF-1) (Yang et al., 2010), pamidronate (Yu et al., 2010a), PDGF (Zhang et al., 2012a), PTH (1-34) (Morgan et al., 2008), TGF-β1 (Ripamonti et al., 2001b) and thrombospondin 1 (TSP-1) (Gelse et al., 2011). Taken together these combinations have demonstrated increased defect regeneration from 1.4 to 5.3 fold in large animals (Ripamonti et al., 2010; Zhang et al., 2009b), and 1.2 to 15 fold in small animals (Yang et al., 2010; Zhang et al., 2012a). Although enhanced bone formation was observed in most studies, fold increases were not superior to those investigating BMP-7 alone. This may be due to under or over-dosing of one or both of the delivered factors. Consequently, balance between combination choice and dosage should be carefully considered as one study demonstrated a 2-fold decrease in bone formation following high dose pamidronate (2 mg) compared to low dose (20 μg) pamidronate (Yu et al., 2010a). A number of studies have also investigated BMP-7 combinations with cells including BMSCs (Zhang et al., 2011b), MSCs (Tsiridis et al., 2007a) or osteoblasts (Reichert et al., 2011). Rather than flood the defect site with copious exogenous growth factors, these approaches endeavoured to augment the effect of BMP-7 through addition of an inducible cell source, and demonstrated a 1.5- to 8.8-fold increase in bone formation (Reichert et al., 2011; Takigami et al., 2007). Again, fold increases were not superior to those of BMP-7 alone, which may be due to non-optimal balance between dosage and cell number, or even non-optimal spatiotemporal delivery of BMP-7.

It is important to note that the different outcomes observed within all the collated studies described here, with the administration of BMP-2 and BMP-7 in vivo, may not solely be dependent on dosage but also on receptor expression. Inter-species receptor expression can vary considerably and may ultimately govern the response to BMP dosage.

FGF
FGFs constitute a large growth factor family with over 20 members and are involved in many biological processes from embryonic development regulating cell proliferation, migration and differentiation, to homeostasis orchestrating tissue maintenance and repair (Ornitz and Itoh, 2001). FGF-1 to FGF-10 all bind FGF receptors (FGFR) and have characterised functions in bone development and healing (Ornitz and Marie, 2002). A review of the literature
revealed the most abundant member utilised within bone tissue engineering strategies in vivo was FGF-2, also known as basic FGF (Hirata et al., 2013; Hong et al., 2010; Maehara et al., 2009; Shirakata et al., 2010).

Direct administration of FGF

FGF-2 has been administered to large animals including dog (Murakami et al., 2003) and primate (Takayama et al., 2001) at dosages from 0.15 to 200 µg (Hosokawa et al., 2000; Nakamura et al., 1998), and small animals including mouse (Kodama et al., 2009), rabbit (Nakasa et al., 2008) and rat (Tsurushima et al., 2010) at dosages from 0.01 to 200 µg (Komaki et al., 2006; Zellin and Linde, 2000) (Supplementary Table 3). Considering related fold increases in bone formation within large (1.3 to 3 fold (Nakamura et al., 1998; Shirakata et al., 2010)) and small animals (1.1 to 16.4 fold (Goodman et al., 2003; Hong et al., 2010)), it is interesting to note that higher dosages correlated with greater fold increases (defects with highest fold increase included tibial fracture and calvarial defect respective to large and small animals). Evidently, data suggest positive correlation between FGF-2 treatment and bone formation, potentially due to induced vessel ingrowth and ossification at the defect site (Guo et al., 2006; Maehara et al., 2009). However, administering the correct dose relative to defect size and location is paramount, since Nakasa et al. (2008) demonstrated a 1.5 fold decrease in lamellar bone formation following administration of 100 µg FGF-2 to a 5 mm full thickness femoral condyle defect. Although lamellar bone tissue was reduced, vascularisation and osseointegration were elevated, indicating accelerated maturation of extant bone. Indeed Bland et al. (1995) also demonstrated callus maturation without augmentation of bone tissue formation. Interaction of FGF-2 and condylar tissue may, in this instance, have had predominant effects on chondrogenesis, rather than osteogenesis. Nakasa et al. (2005) also investigated ectopic delivery of FGF-2 (subcutaneous implantation in rabbit) and observed extensive osteoid deposition, suggesting that interactions between the delivered growth factor and surrounding tissues dictate outcome.

Compared to FGF-2, a handful of studies utilised FGF-1, also known as acidic FGF (Bland et al., 1995; Dunstan et al., 1999; Kelpke et al., 2004), and one study utilised FGF-18 (Carli et al., 2012) which has been shown to promote chondrogenesis amongst many other functions. FGF-1 was administered between 3 and 7 µg in small animals including mouse (Dunstan et al., 1999), rabbit (Bland et al., 1995) and rat (Kelpke et al., 2004). Dunstan et al. (1999) demonstrated 8 to 10 fold increased bone formation. However, the animal model was ovariecotomised to create a state of osteoporosis and therefore resultant data require careful interpretation prior to comparison with that of other animal models. Fold increase would be expected to drop in a normal animal model as baseline bone regeneration levels would be higher than those in osteoporotic models. The same study also investigated injection of FGF-1 adjacent to mouse calvaria, which demonstrated a 3-fold increase in bone thickness. However, injection with FGF-2 exhibited a 7-fold increase in bone thickness, suggesting FGF-2 is a more potent osteoinductor compared to FGF-1. Kelpke et al. (2004) demonstrated increased osteogenesis assessed by alkaline phosphatase, osteocalcin and osteopontin expression; however, bone tissue formation was not reported. Increased blood vessel ingrowth was observed and reported to range between 2 and 2.6 fold. Augmentation of vasculature can be beneficial for bone regeneration as it supplies an endogenous inducible cell source to populate and repair the defect site. Carli et al. (2012) delivered 0.5 µg FGF-18 to a 5 mm segmental femoral defect and demonstrated a 5-fold increase in percentage bone volume. On first observation, this would suggest that FGF-18 is a potent osteoinductor; however, this is only one study and was tested within a mutated mouse model which showed impaired bone formation. Further study is required to draw conclusions regarding in vivo bone tissue formation efficacy of FGF-1 and FGF-18.

Indirect administration of FGF

FGF-2 has been indirectly delivered to both large (dog (Tan et al., 2009)) and small animals (mouse (Meng et al., 2012), rabbit (Guo et al., 2006) and rat (Qu et al., 2011)) through implantation of transplanted cells (6.25 x 10⁴ and 5 x 10⁴ cells (Guo et al., 2006; Meng et al., 2012)). Bone formation was modestly increased 2 to 2.7 fold (Kwan et al., 2011; Meng et al., 2012). However, one study by Hall et al. (2007) reported a 53.5 fold increase in percentage cancellous bone area (0.4 % in the control group increased to 21.4 % in the FGF-2 treated group). As previously discussed, care should be taken when comparing this with other data as the animal model used was haematopoietic deficient resulting in decreased baseline healing and therefore elevated fold increases in comparison to normal animal models. Most direct administration studies deliver FGF-2 in a single dose direct to the defect site and may be encapsulated within a carrier for controlled release over time, whereas indirect administration through endogenous or exogenous cell expression leads to continuous FGF-2 delivery. Lower bone tissue augmentation observed within indirect administration studies may be a consequence of constant exposure to FGF-2 stimulation. A spatiotemporal release profile would be more suitable to bone tissue-engineering strategies eligible for clinical translation.

Combination administration of FGF

Investigation of other tissues would aid understanding of whether lower fold increase in bone tissue was due to preference or induction of pre-bone tissue formation, such as osteoid deposition or cartilage production. Indeed, Iwaniec et al. (2003) demonstrated 8 fold increased osteoid deposition compared to 1.8 fold increased bone formation rate. Combinations should be carefully selected, as Behr et al. (2012) demonstrated that combination of FGF-2 with either BMP-2 or VEGF resulted in less bone formation compared to BMP-2 and VEGF in combination. However, selection according to the task at hand, whereby combination treatment with FGF-2 may be required to induce void filling callus formation and osteoid production prior to combination treatment with BMP-2 for mineralisation. A staged approach may be necessary for efficient and effective bone healing requiring multiple growth factors delivered spatiotemporally in
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growth factor investigations would ultimately provide fast and efficient cross-evaluation. PDGF appears to enhance bone regeneration through angiogenic induction and augmentation of surrounding vasculature. However, where increased bone formation is reported following high dose PDGF by Nash et al. (1994) reduced mechanical strength within newly formed bone was also reported. Quality alongside quantity of newly formed bone should therefore be factored into any analysis of bone defect regeneration. Although many studies have reported a positive correlation between PDGF administration and enhanced bone healing, these observations were not shared by all. Kaipel et al. (2012) demonstrated the failure of PDGF treatment to increase bone healing within a femoral segmental defect in rat. The same study also demonstrated failed healing following administration of another angiogenic factor VEGF. Interestingly, administration of BMP-2 within this study enhanced bone healing, suggesting that osteogenic factors are either a prerequisite for bone augmentation, or that they are required to drive progression of endogenous endochondral ossification.

Indirect administration of PDGF
Anusaksathien et al. (2004) reported similar negative findings with continuous PDGF exposure where treatment resulted in reduced mineralisation at the defect site. Delivery was indirect, through implantation of transduced cells. However, reduction was observed after 3 weeks then reversed and increased after 6 weeks. This suggests that temporal exposure within a larger network of bone healing processes dictates the effect of implanted PDGF. Addition of an angiogenic factor may not necessarily correlate with an angiogenic response, and is dependent on spatiotemporal delivery. Indeed, indirect administration of PDGF has been shown to modestly increase bone volume within the defect site between 1.7 (Chang et al., 2010) and 2 fold (Zhang et al., 2012a) following delivery of 5.5 x 10^8 to 5.5 x 10^9 PFU/mL within rats. Clearly, there is interplay between the growth factor delivered and endogenous processes at the defect site, which ultimately control the response observed. It is therefore valuable to successful tissue engineering strategies, to investigate and compare these interactions.

Combination administration of PDGF
A number of studies have investigated combination treatment with PDGF and several other growth factors including bFGF (Meraw et al., 2000), BMP-2 (Martino et al., 2011), BMP-7 (Zhang et al., 2009b), IGF-1 (Nociti Junior et al., 2000), osteogenin (Marden et al., 1993), TGF-β1 (Reyes et al., 2012) and VEGF (El Backly et al., 2013). Bone formation was reported to increase between 1.4 and 2.3 fold (Zhang et al., 2009b) within large animals (dog (Zhang et al., 2009b)) following PDGF dosages around 5 μg/mL for direct administration (Nociti Junior et al., 2000), or 2 x 10^{10} viral particles for indirect administration (Zhang et al., 2009b). Combination treatments within small animal (mouse (El Backly et al., 2013), rabbit (Reyes et al., 2012) and rat (Park et al., 2013)) studies reported increased bone formation between 2.5 (Xu...
et al., 2012) and 10 fold (Reyes et al., 2012) following PDGF dosages ranging between 0.05 (Martino et al., 2011) and 200 µg (Marden et al., 1993).

**PTH**

PTH is an 84 amino acid polypeptide secreted by chief cells of the parathyroid gland and is an essential regulator of both calcium and phosphate metabolism which has important ramifications for bone. Regarding mineral homeostasis, PTH acts to increase serum calcium through gastrointestinal absorption, renal reabsorption and liberation from bone reserves (Alkhiary et al., 2005; Podbesek et al., 1983). Continuous PTH treatment results in bone resorption, functioning indirectly through osteoblasts rather than directly via osteoclasts. However, intermittent PTH treatment has been shown to result in osteoblast stimulation and increased bone formation (Hock and Gera, 1992).

**Direct administration of PTH**

Many truncated forms of PTH have been directly administered in vivo most often by subcutaneous injection within large (dog (Daugaard et al., 2012), monkey (Vahle et al., 2008) and sheep (Arrighi et al., 2009)) and small animals (mouse (Takahata et al., 2012), rabbit (Lehman et al., 2007) and rat (Qiu et al., 2013)) at variable dosages from 0.75 to 7.5 µg/kg/day (Manabe et al., 2007) (20 to 1,000 µg/mL (Arrighi et al., 2009; Jung et al., 2007a), and 0.05 to 800 µg/kg/day (Mohan et al., 2000; Rihani-Bisharat et al., 1998) (20 to 100 µg/mL (Jung et al., 2007b)), respectively (Supplementary Table 5). Treatment resulted in enhanced bone formation between 1.1 and 3.4 fold (Arrighi et al., 2009; Vahle et al., 2008) within large animals, and between 1.1 and 13.1 fold (Komatsu et al., 2009; Li et al., 2001) in small animals. Respective bone mechanical strength was also increased ranging from 1.4 to 2 fold (Daugaard et al., 2011; Vahle et al., 2008) for large animals and 1.1 to 3.8 fold (Reynolds et al., 2011; Sloan et al., 2010) for small animals. Thus teriparadine (PTH 1-34), the truncated PTH molecule, is a successful osteoporosis molecule with clear anabolic bone formation activity. In brief, PTH administration leads to increased bone formation and mechanical strength over time, possibly through a reduction in osteoclast number (Manabe et al., 2007). Indeed, Nozaka et al. (2008) reported a 5.3-fold reduction in osteoclast number. However, other studies by O’Loughlin et al. (2009) and Takahata et al. (2012) reported contrasting results with a 2.5- to 4-fold increase in osteoclast number. Markers of bone formation such as osteocalcin and alkaline phosphatase were also shown to be increased between 1.2 and 3.1 fold (Komrakova et al., 2011; Qiu et al., 2013), indicating upregulation of osteoblast activity. PTH-enhanced osteoblast activity has been shown to reduce periodontal disease-induced bone loss by as much as 2.3 fold (Marques et al., 2005). Conversely, continuous PTH infusion, investigated by Ma et al. (2001), demonstrated a significant drop in bone formation markers (3 to 7.5-fold drop in osteoprotegrin which binds RANKL blocking RANK-induced osteoclastogenesis) and increase in RANKL expression (5.5 to 27 fold) leading to a 3 fold increase in osteoclast number. Consequently, the adopted administration regimen has significant implications for bone formation. Vahle et al. (2004; 2008) showed treatment withdrawal reversed bone enhancement after 3 years in sheep and 24 months in rat. Caution should therefore be taken when striking a balance between treatment period and, importantly, dosage, as Vahle et al. (2004) also demonstrated bone neoplasia with high dose PTH over prolonged periods in rats. However, the delivery vehicle may aid beneficial outcomes from continuous PTH administration, since Arrighi et al. (2009) demonstrated a maximum 3.4-fold increase in bone formation within sheep femoral and humeral defects following PTH fusion protein within fibrin glue. One interesting observation which may need future consideration for comparative purposes is the source of PTH under investigation. Li et al. (2001) reported a significant difference in the potency of two differently sourced PTH peptides, where bovine PTH was 4 to 6 fold more potent than rat PTH. As previously mentioned, some studies have investigated cartilage formation as a precursor to bone tissue generation. Following PTH treatment chondrogenesis/cartilage formation was increased 3 to 9.9 fold (Kakar et al., 2007; O’Loughlin et al., 2009) leading to enhanced trabeculated callus formation (Reynolds et al., 2011). Bone architecture and structure are important quality indicators, yet many studies report only simple measurements of bone quantity.

**Combination administration of PTH**

PTH has been used in combination treatment of bone defects with growth factors including BMP-2 (Kempen et al., 2010), BMP-7 (Morgan et al., 2008), FGF-2 (Lane et al., 2003b), IL-6 (Rozen et al., 2007) and PTHrP (Xue et al., 2005), and bisphosphonates including alendronate (Campbell et al., 2011), ibandronate (Yang et al., 2013), pamidronate (Aspenberg et al., 2008), tiludronate (Deltmas et al., 1995) and zoledronic acid (Li et al., 2013). Some studies have delivered PTH with cells, including periodontal ligament cells (Wolf et al., 2012), BMSCs (Pettway et al., 2005) and MSCs (Yu et al., 2012b). Together, these studies have demonstrated increased bone formation between 1.3 (Morgan et al., 2008) and 4.1 fold (Kempen et al., 2010) in small animals following dosages from 10 to 90 µg/kg/day. One large animal study demonstrated between 2 and 4 fold following dosages of 0.015 µmol/kg (Delmas et al., 1995). Whilst bone formation was enhanced, it is important to note here that the same selection of studies together demonstrated enhanced bone formation between 1.2 (Niziolek et al., 2009) and 3.1 fold (Pettway et al., 2008) following treatment with PTH alone. Combination treatment also augmented mechanical parameters of new bone tissue including strength and stiffness between 1.2 (Morgan et al., 2008) and 3.1 fold (Rozen et al., 2007). Wolf et al. (2012) demonstrated 1.2 to 3 fold increased bone marker expression. One interesting study by Niziolek et al. (2009) delivered PTH with the antibiotic rapamycin and demonstrated reduced bone mineral density (BMD). This study highlights the need to standardise drug regimen between animal models as antibiotics are often administered following surgery and defect preparation. Drug selection should be carefully considered so as not to hinder effective defect regeneration.
**PTHRP**
As a regulator of endochondral bone development, PTHrP maintains growth plate width and structure through balanced inhibition of chondrocyte maturation (Kobayashi et al., 2002). In adults, PTHrP interferes with osteocytic-mediated sclerostin inhibition of bone formation (Robling et al., 2008), and binds receptors of the osteoblast lineage inducing enhanced bone formation (Karaplis, 2001).

**Direct administration of PTHrP**
PTHRP analogues and truncated peptides have been utilised directly in small animal models, including mouse (Lozano et al., 2010), rabbit (Trejo et al., 2010), and rat (Stewart et al., 2000) (Supplementary Table 6). Bostrom et al. (2000) injected the PTHrP analogue RS-66271 within a rabbit ulna segmental defect (1 mm), whilst Trejo et al. (2010) implanted the C-terminal PTHrP (107-111) epitope within a rabbit femur epiphyseal defect (5 mm). These two studies demonstrated a 2 to 10 fold increase in bone volume (Table 5). Analogues and truncated peptides have also been assessed in disease models. The C-terminal PTHrP (107-139) peptide (Lozano et al., 2010) and N-terminal PTHrP (1-36) peptide (Lozano et al., 2009) were examined within diabetic mice and found to reverse diabetic-induced bone loss when administered at 100 µg/kg every other day. Interestingly, the N-terminal PTHrP (1-36) peptide (40 µg/kg/day) and PTHrP analogue RS-66271 (80 µg/kg/day) were investigated in ovariectomised rats (osteoapenia model) and found to reverse bone loss and enhance new bone formation exhibiting increased (3 fold) biomechanical strength (Stewart et al., 2000; Vickery et al., 1996).

**Combination administration of PTHrP**
Porto-Nunez et al. (2010) and de Castro et al. (2011) both used the N-terminal PTHrP (1-36) and the C-terminal PTHrP (107-139) peptides in combination in the ovariecomitised and diabetic mice, respectively. Following injections at 80 and 100 µg/kg, bone loss was reversed and bone volume increased 1.5 fold. Although, different dosage regimes were implemented between these two studies, both observed an increase in BMD; 1.1 and 2.2 fold. Evidently, PTHrP plays an important role in bone formation and use of active analogues and peptides will not only augment bone healing, but can also reverse bone loss due to disease.

**TGF-β3**
A central component of the healing cascade in any bone defect is the formation of cartilage tissue, a precursor to immature bone, which subsequently becomes mineralised (Dimitriou et al., 2005). TGF-β3 is a potent chondrogenic growth factor enhancing hyaline cartilage formation in vivo (Ripamonti et al., 2009a; Tang et al., 2009).

**Direct administration of TGF-β3**
Ripamonti et al. (2009b; 2008) and Teare et al. (2008) investigated the direct delivery of TGF-β3 (5 to 125 µg) within adult Chacma baboons and demonstrated a 1.75- to 3-fold increase in bone formation (Supplementary Table 7). Direct TGF-β3 delivery (3 ng to 2.75 µg) within small animal models, including mouse (Kovacevic et al., 2011) and rat (Opperman et al., 2002), only showed a 1.05 fold increase in bone volume. However, cartilage formation showed a more robust augmentation of 1.23 fold (Table 5). Low levels of bone formation was also observed by Rizk and Rabie (2013), following investigation of TGF-β3 transduced cells within a mouse ectopic subcutaneous implant model; considerable cartilage constructs were generated without significant bone formation. Release of the chondrogenic factor TGF-β3 in vivo would be anticipated to induce cartilage formation. An appropriate osteogenic signal would then be required to drive mineralisation of this induced cartilage. Indeed, addition of osteogenic protein 1 (OP-1) (Ripamonti et al., 2010) or MSCs (Mrugala et al., 2008) within large animal models have been shown to increase bone formation 5.3 fold; a vast improvement over TGF-β3 alone.

**Combination administration of TGF-β3**
Small animal studies have investigated TGF-β3 delivery in combination with BMP-2 (Oest et al., 2007), chondrocytes (Park et al., 2010b), MSCs (Park et al., 2010a), Sox9 (Park et al., 2012), and TGF-β1 (Kim et al., 2010a). Between 10 and 100 ng/mL TGF-β3 was used within these studies and TGF-β3 in combination with BMP-2 induced 12.8 to 13 fold more bone (Oest et al., 2007; Simmons et al., 2004) where combination with chondrocytes induced 1.6 to 22 fold increased collagen (Na et al., 2006; Park et al., 2009). These studies confirm the combination of chondrogenic and osteogenic factors in a defined spatiotemporal pattern can lead to more enhanced bone tissue formation than application of TGF-β3 alone.

**VEGF**
VEGF constitutes a sub-family comprised of 5 members (VEGF-A to VEGF-D, and placental growth factor). VEGF-A is the most important of these members with a significant role in both vasculogenesis (de novo vasculature formation) and angiogenesis (vessel formation sprouting from existing vasculature) (Byrne et al., 2005). Hypoxia and necrosis are major concerns at sites of bone damage and contribute to healing failure. Formation of healthy vasculature through the use of VEGF-A to supply oxygen and nutrients at these sites is of paramount importance to efficient bone defect regeneration (Geiger et al., 2007).

**Direct administration of VEGF**
Currently only a limited number of studies have utilised VEGF for in vivo bone defect regeneration (Supplementary Table 8). Kanczler et al. (2008) implanted 1.7 µg rhVEGF165 on poly(lactic acid) (PLA) scaffold within a mouse femur 5 mm segmental defect. After 4 weeks, augmentation of blood vessel formation was observed alongside a 1.65-fold increase in bone volume (Table 5). The same study implanted human BMSCs in combination with rhVEGF165 but observed no further enhancement. A study by Wernike et al. (2010) observed enhanced vascularisation but negligible impact on bone regeneration within a mouse calvarial 4 mm drill defect.

**Indirect administration of VEGF**
A number of studies have investigated indirect delivery of VEGF165 through implantation of plasmid DNA within...
a mouse femur 8 mm defect (Keeney et al., 2010), or transfected cells within rabbit long bone 10 mm and 15 mm segmental defects (Geiger et al., 2007; Li et al., 2009b). Together these studies demonstrated 1.6- to 2-fold increase in bone formation with augmented vascularisation.

Combination administration of VEGF

A number of studies have explored the application of angiogenic VEGF (0.2 to 20 μg) and osteogenic BMP-2 (0.5 to 120 μg) in combination within large animals, including dog (Geuze et al., 2012) and pig (Ramazanoglu et al., 2011), and small animals, including mouse (Behr et al., 2012; Samee et al., 2008), rabbit (Hernandez et al., 2012), and rat (Kempen et al., 2009). Roldan et al. (2010) and Zhang et al. (2010a) combined VEGF with BMP-7 and observed neovascularisation in the absence of any significant increase in bone regeneration. Li et al. (2009a) investigated the use of VEGF with BMP-4 and observed impaired ectopic bone formation using a high VEGF ratio. Interestingly, when VEGF release was slow and sustained, impairment was no longer observed. Recruitment of blood vessels into the defect site, instructed by VEGF, typically complicates bone formation due to the increased localised bone remodelling and callus formation by osteoblasts. Zhang et al. (2011a) found that VEGF delivery using a hydrogel resulted in faster degradation, which ultimately has repercussions for controlled dual growth factor release profiles. It is thus self-evident that spatiotemporal control of the select growth factor release for induction of angiogenesis, chondrogenesis, and osteogenesis is central for successful bone tissue repair.

Wnt Proteins

A diverse family of signalling glycoproteins (19 members; Wnt1 to Wnt16), Wnt proteins are involved in a myriad of cellular processes, including cell proliferation, migration and differentiation (De Boer et al., 2004).

Direct administration of Wnt proteins

Zhou et al. (2009) injected 100 ng Wnt3A into a mouse model of delayed skeletal development and observed both increased parietal bone volume and a 1.4 fold reduction in suture area (Supplementary Table 9). Only one other study, at the time of writing this review, had investigated Wnt3A utilisation, using liposomal vesicle injection for direct delivery of Wnt3A to 1 mm tibial mouse fracture model (Minear et al., 2010).

Indirect administration of Wnt proteins

Given the cost of Wnt proteins, focus has centred on indirect delivery using transduced and transfected cells (Table 5). Nalesso et al. (2011) and Qiang et al. (2008) both injected Wnt3A-transfected cells within severe combined immuno deficient (SCID) mice and observed a 1.5 fold increased cartilage formation and a 1.12 fold increased BMD. Liu et al. (2009) injected Wnt1 transduced cells within SCID mice and observed a dose dependent enhancement of bone formation (1.25 fold). Implantation of Wnt4 transduced MSCs in SCID mice with a 5 mm calvarial defect resulted in extensive integrated enhanced mineralised bone tissue (Chang et al., 2007). The same study implanted Wnt1 transduced cells within an alveolar defect in SCID rats and observed a 3- to 5-fold increase in bone formation. 1.75 fold increased bone formation was also observed by Bennett et al. (2005; 2007) within transgenic mice following Wnt10B plasmid injection into mouse embryos. These different studies indicate that Wnt proteins can augment in vivo bone formation, although success in bone tissue engineering will be dependent on Wnt protein selection. Injection of Wnt6 transfected cells within the chick limb bud inhibited chondrogenesis and promoted myogenesis (Geetha-Loganathan et al., 2010). Wnt5A plasmid injection within mouse embryos generated transgenic mice exhibiting a variety of developmental defects, including reduced endochondral and intramembranous bone formation (van Amerongen et al., 2012), although control of spatiotemporal expression exhibited increased calvarial ossification.

In summary, select exogenous factors can be successfully applied as part of a tissue-engineering regimen for in vivo bone regeneration. It is the opinion of the authors that BMP-2 provides the greatest bone regeneration in vivo, and that careful spatiotemporal release with additional factors may provide synergistic or additional signalling leading to further augmentation. Supporting literature discussed here details a maximum 50-fold increase in bone formation following BMP-2 administration (Table 5). BMP-7 may provide a suitable alternative to BMP-2 with similar osteogenic potency. However, many studies failed to quantify enhanced tissue formation or failed to record any changes therein and it may be that the recorded fold increases may indeed be higher. Nevertheless, these animal studies have informed clinical translation resulting in BMP-2, BMP-7/OP-1, PDGF, PTH and PTHrP transition from animals to humans. The current prohibitive protein production costs or minimal supporting in vivo literature may explain the lack of FGF, TGF-β3, VEGF and Wnt protein clinical translation to date.

Human Trials

In contrast to animal models, human patients display unpredictable idiopathic variations in their ability to form bone, inter and intra-family genetic variations, and systemic multifactorial inconsistencies derived from age, sex, weight, diet, disease, health status, lifestyle, medication, drug abuse/addiction, and numerous environmental factors (Sandhu et al., 1995). Consequently, large subject numbers are required in any clinical trial before examination can yield comparative data of value (Khan and Lane, 2004). That said, tissue engineering-based approaches to bone regeneration in humans have already been successfully translated.

BMP-2

Approval for the use of rhBMP-2 in humans was granted by the European Medicines Agency (EMA) in 2002, and by the U.S. Food and Drug Administration (FDA) in 2004 (McKay et al., 2007), following a pivotal study by the BESTT (BMP-2 evaluation in surgery for tibial trauma) study group (Govender et al., 2002). The study
reported both a reduced need for secondary intervention, and enhanced fracture healing following treatment with rhBMP-2 on absorbable collagen sponge (ACS). BMP-2 has subsequently become the subject of intense examination in vivo (Supplementary Table 1). Clinical studies have included facial reconstruction (cleft and mandible defects) (Cicciu et al., 2012; Dickinson et al., 2008; Herford and Boyne, 2008), maxillary sinus floor augmentation (Triplett et al., 2009), long bone non-unions (Tressler et al., 2011), tibial fractures (Jones et al., 2006; Swiontkowski et al., 2006), and lumbar fusions (Mladenov et al., 2010; Taghavi et al., 2010). Facial reconstruction and bone augmentation studies all delivered rhBMP-2 on ACS at a concentration of 0.75 to 1.5 mg/mL. Boyne et al. (2005) demonstrated increased bone formation suitable for dental implants. Fiorellini et al. (2005) and Tripplet et al. (2009) performed similar dental studies revealing 2-fold increase in bone formation for dental implants and functional longevity, respectively. Dickinson et al. (2008) also demonstrated the efficacy of rhBMP-2/ACS for bone regeneration in vivo through improved healing and reduced morbidity in cleft defects. Treated patients exhibited 95 % closure compared to 63 % in non-treated patients. New bone formation and closure of non-union fractures was observed by Tressler et al. (2011) and Johnson et al. (1988b). Additional advantages of utilising rhBMP-2 on ACS over iliac crest autograft included a 1.35 fold reduced operative time and 1.4 fold reduced intraoperative blood loss, both of which aided effective surgery (Tressler et al., 2011). 92.3 % to 98 % of treated patients (Burkus et al., 2009; Haid et al., 2004) compared with 70 % to 89 % of control patients (Dawson et al., 2009; Dimar et al., 2009) exhibited successful fusions of lumbar vertebræe demonstrating enhancement of rhBMP-2-induced bone formation (Supplementary Table 1). Other rhBMP-2 studies reported reduced back and leg pain (Burkus et al., 2003a) and reduced arm and neck pain (Baskin et al., 2003). The major carrier utilised for rhBMP-2 was ACS, however alternative carriers were utilised including autograft and allograft bone (Buttermann, 2008; Taghavi et al., 2010), gelatin (Johnson et al., 1988a), hydroxyapatite-tricalcium phosphate (HA-TCP) particles (Dawson et al., 2009), polyetheretherketone (PEEK) (Klimo and Peelle, 2009), and poly(lactic co-glycolic acid) (PLGA) (Johnson et al., 1988b; Katayama et al., 2009).

However, in the last few years a number of studies have cast a long shadow on the clinical efficacy (successful adverse-free outcomes) of rhBMP-2 for spinal fusion. Off label use of rhBMP-2, reported within several studies, has shown significant rhBMP-2-related side effects including urogenital complications, wound complications, increased inflammation and increased cancer risk. Furthermore, a number of reviews indicate an apparent failure of early journal publications to report, or under report, associated complications and selective individual patient reporting. These reviews also indicate misrepresentation of efficacy, together with duplicate publications (Carreon et al., 2008; Fu et al., 2013; Mesfin et al., 2013; Moshel et al., 2008).

**BMP-7/OP-1**

Early studies utilising BMP-7 in humans were first reported between 1999 and 2001 (Friedlaender et al., 2001; Geesink et al., 1999; Laursen et al., 1999; van den Bergh et al., 2000). These studies investigated BMP-7 delivery on ACS to long bone osteotomy and non-union, lumbar interbody fusion and maxillary sinus augmentation (Supplementary Table 2). Van den Bergh et al. (2000) and Groeneveld et al. (1999) reported 1.2 to 9.7 fold increased osteoid formation following treatment with 2.5 mg BMP-7 for maxillary sinus augmentation. Less successful outcomes were reported by Laursen et al. (1999) and Jeppsson et al. (1999) regarding lumbar fusions, where enhanced bone resorption was observed and only 1 of 4 patients exhibited successful bone bridging. Conversely, Geesink et al. (1999) demonstrated new bone formation within tibial osteotomies as early as 6 weeks following treatment with 2.5 mg BMP-7 in all but one patient. Friedlaender et al. (2001) demonstrated safe application of BMP-7 in vivo with non-union healing comparable to autograft controls. Together, these studies pioneered BMP-7 use in vivo and led to FDA approval for use in long bone non-unions in 2001 and posterolateral lumbar fusions in 2004 (Ong et al., 2010). Regarding lumbar fusion surgery, BMP-7 was delivered at 3.5 mg per vertebral side (7 mg in total). Vaccaro et al. (2003; 2004; 2005) reported improved Oswestry scores measure low back pain, radiographic fusion in 50 % to 55 % of patients, and bone bridging in 70 % to 91 % of patients. BMP-7 was repeatedly shown to increase bone formation similar to autograft (Johnson et al., 2002; Kanayama et al., 2006; Vaccaro et al., 2004). However, over-zealous application of BMP-7 can have side effects, as Kim et al. (2010b) demonstrated significant ectopic bone formation along the surgical track following delivery of 17.5 mg. It is important to note that dosage is relative to defect site and that where high dose in one anatomic location may be excessive, in another location within a different size defect the same dose may be more appropriate. Indeed, 17.5 mg appeared excessive in lumbar fusion, but Hernandez-Alfaro et al. (2012) demonstrated safe administration of 2 g BMP-7 within a 60 mm mandibular defect and reported stable osseointegration of titanium mesh implant after 1 year. Numerous pseudarthrosis and non-union fracture studies standardised BMP-7 dosage to 3.5 mg. Complete defect healing was observed in patients treated for pseudarthrosis, where treated bones were reportedly pain free and load bearing (Anticevic et al., 2006; Fabek et al., 2006). However, Lee et al. (2006) did not observe new bone formation in any of the 5 patients that received treatment. A study by Ekrol et al. (2008) also demonstrated a lack of BMP-7 induced bone healing within radial osteotomies reporting decreased healing rates and reduced stability compared to autograft. Non-union fracture studies reported better results following administration of 3.5 mg BMP-7. 75 % (Friedlaender et al., 2001) to 100 % (Giannoudis et al., 2009) of treated patients exhibited healed defects within 3 (Kanakaris et al., 2008) to 16 months (Giannoudis et al., 2009). This compared with only 68.3 % (Calori et al., 2008)
to 85% (Friedlaender et al., 2001) of patients who received autograft. These studies also reported decreased healing time (1.5 fold), hospital stay (3.4 fold) and treatment cost (1.9 fold) (Dahabreh et al., 2007; Ristiniemi et al., 2007). Taken together, current literature supports the application of BMP-7 within tissue engineering strategies for localised bone defect repair and regeneration. However, dosage should be carefully considered with respect to the defect site to reduce unwanted side effects.

**PDGF**

Only one study, at the time of writing, was found to utilise PDGF for in vivo bone regeneration in humans. Nevins et al. (2003) administered between 0.5 and 5 mg/mL PDGF-BB to patients with advanced periodontitis and interproximal intrabony and/or molar class II furcation defects (Supplementary Table 4). As with all human studies, investigation of new bone is limited and analysis of specific bone parameters is restricted to non-invasive techniques. The study assessed defect regeneration through vertical probing depth and found a 1.12 fold reduction following PDGF treatment compared to xenograft bone in collagen. Clearly, PDGF treatment provides a functional alternative to xenograft for effective defect regeneration.

**PTH**

PTH has been administered in humans for many years investigating its efficacy for bone formation within healthy adults (Horwitz et al., 2011), adults with low BMD (Ryder et al., 2010), postmenopausal women (Schafer et al., 2013), mandibular defects (Kwon et al., 2012) and vertebral fractures (Nakamura et al., 2012) (Supplementary Table 5). Continuous delivery has been shown to reduce bone formation markers and increase bone resorption. Horwitz et al. (2011) indeed demonstrated extensive bone resorption with high dose PTH (4 pmol/kg/h) delivered by continuous infusion pump, resulting in hypercalcemia. Intermittent delivery increased bone turnover within low BMD patients who exhibited 2.1 fold increased bone formation, and 2.7 fold increased bone resorption (Ryder et al., 2010). Nakamura et al. (2012) also demonstrated increased BMD (2.3 to 6 fold) within vertebral fracture patients following intermittent administration (56.5 μg/week). Standard delivery of PTH at 20 μg/d to patients with mandibular defects resulted in 5.4 to 5.7 fold increased bone marker expression (Kwon et al., 2012), and 1.5 (Kuchler et al., 2011) to 11.6 fold (Bashutski et al., 2010) increased bone formation with augmented implant integration (1.2 to 4.7 fold). Most studies reviewed investigated PTH administration within postmenopausal women at dosages ranging between 20 and 100 μg/d. These studies reported reduced healing time (1.3 (Aspenberg et al., 2010) to 1.6 fold (Peichl et al., 2011)) and fracture incidence (2.8 to 7 fold) (Neer et al., 2001), whilst BMD and mechanical strength were reportedly increased 1.02 to 1.05 fold (Keaveny et al., 2012), and 4.2 to 7.7 fold (Keaveny et al., 2008), respectively. Clearly, PTH can augment bone defect repair and increase innate BMD.

**PThRP**

PThRP has been used clinically via subcutaneous or intravenous injection. Horwitz et al. (2011; 2005) published data detailing systemic delivery of N-terminal PThRP (1-36) peptide within healthy adults at dosages between 2 and 28 pmol/kg/h led to profound suppression of bone formation (Supplementary Table 6). Another study revealed that 1.3- to 1.4-fold suppression in bone formation could be reversed following PThRP analogue cessation. Consequently, continuous infusion can enhance bone resorption and decrease bone formation, whilst intermittent infusion can lead to a net increase in bone formation. Fraher et al. (1992) demonstrated increased serum calcium and urinary phosphate when healthy individuals were injected with N-terminal PThRP (1-34) peptide at 8 or 80 pmol/kg/h. Thus, PThRP translation from animal to human studies has, to date, not yielded similar responses, indeed the use of PThRP analogues has had the opposite effect with increased bone resorption observed in the clinic. However, a study by Plotkin et al. (1998) using the N-terminal PThRP (1-36) peptide delivered by subcutaneous injection within post-menopausal oestrogen deficient women observed activation of bone formation and a 1.3- to 1.45-fold reduction in bone resorption. The function of these analogues may be modified by the hosts hormonal status; pre or post-menopause. Minimal literature on the in vivo use of PThRP analogues highlights the need for further investigation before definitive conclusions can be drawn.

**Future directions**

It is clear the use of select growth factors in vivo can augment bone formation and potentially repair defects. Utilisation of animal models has proven informative for clinical translation of bone tissue engineering strategies. However, complications associated with spatiotemporal release of growth factors regarding longevity, bioactivity and carrier release kinetics have impeded progress. Parallel, synergistic and consecutive delivery of multiple growth factors appears key to successful bone regeneration. The authors envisage coordinated spatiotemporal release of select growth factors recapitulating in vivo signalling cascades leading to bone tissue formation. The importance of understanding the developmental processes underpinning bone tissue formation, and their importance in contextualising signalling cascades and the growth factors involved in regenerative medicine is gaining prominence, as understanding these processes is vital to informed clinical bone therapies (Smith et al., 2013; Turner et al., 2013). The data presented here demonstrate the complex and convoluted interplay between administered growth factors with variable success for bone tissue formation dependent on species, dosage and combination. Thus, before robust bone tissue engineering can be achieved (and more importantly interpreted), it will be important to understand the functional interplay between
growth factors and how this leads to bone formation under different conditions. For example, the chick model provides an ideal system for investigating bone development biology (Smith et al., 2013). Organotypic culture of embryonic chick femora ex vivo enables investigation and elucidation of processes involved in skeletal development and bone repair (Kanczler et al., 2012; Smith et al., 2014a; Smith et al., 2014b; Smith et al., 2012). Models such as the chick may indeed fulfill the requirement for a simple, relatively high throughput and cost effective research tool with which to inform, create and optimise bone tissue engineering strategies.

Conclusions

Evaluation of the osteotropic factors presented here confirms the potential of these factors to augment bone formation in vivo cementing their selection for bone reparation. Current reports indicate that BMP-2 and BMP-7 have significant potential to augment bone formation (up to 50 fold and 96 fold, respectively) through induced osteogenesis and osseointegration of tissue-engineered implants. However, lessons from off-label complications and issues surrounding potential adverse events associated with rhBMP-2 in spinal fusion need to be carefully considered. In order, FGF, PTH, Wnt proteins, PTHrP analogues, PDGF, TGF-β3 and VEGF have demonstrated up to 16.4, 13.1, 12, 10, 10, 3 and 2 fold increased bone formation following direct and indirect delivery. Although not as potent as BMPs, these growth factors clearly have important benefits in any tissue engineering strategy. Sequential release of these angiogenic, chondrogenic, and osteogenic factors recapitulating native environmental cues is undoubtedly critical to successful bone augmentation. Many studies have therefore investigated combinational growth factor delivery to further enhance bone regeneration. However, most combination treatments to date appeared to enhance bone formation to a lesser degree. In order, BMP-2, BMP-7, PTH, FGF and PTHrP demonstrated 20, 15, 4.1, 3.3 and 3 fold increased bone formation, respectively. TGF-β3 and VEGF combination treatments conversely showed further enhancement with 13 and 20 fold increased bone formation. Important to note here is that both TGF-β3 and VEGF were combined with BMP-2, which on the one hand improved their osteo-inductive potential and yet, apparently, diminished the osteo-inductive potential of BMP-2. Combinational PDGF treatments demonstrated similar augmentation to PDGF treatments alone. Wnt proteins were not found to have been used for combination treatment. Considering reported bone formation following combination treatment, it is evident that suboptimal spatiotemporal delivery and complicated in vivo interplay is hindering further enhancement. Further understanding the complex spatiotemporal interactions between growth factors in vivo, through use of appropriate animal models, will aid generation of clinically transferable and effective bone tissue engineering strategies. A number of studies have demonstrated successful bone tissue engineering in humans using the growth factors discussed here. However, a lack of bone tissue quantification and adequate controls limits correlation between growth factor efficacy in animals and that in humans. This further highlights the need for standardised investigation with specified measurable parameters in vivo. Connecting observations in animal models to those in humans will, ultimately, further our understanding of growth factor induced bone formation. Controlled orchestration of clinically relevant and functional in vivo bone formation may finally deliver on the long heralded promise of bone regeneration for an increasingly aged population.

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Discussion with Reviewers


through which molecular mechanisms (biomechanics/activation of particular signalling cascades)?

**Authors:** Biomaterials are discussed briefly within the introductory section, to equip the reader with a basic understanding of current tissue engineering strategies and thereby position them better to understand the tables. Biomaterials were not the aim of this review, and further discussion of the biomaterials may be misleading as the cohort of studies are not representative of the field as they were negated from the original search parameters. References to mesenchymal stem cells are only made when specifically discussing individual publications that utilised them and other cells. However, we have added our recent review to provide a reference overview for the reader.

Again, further discussion would not be representative of the entire field.

**Reviewer 1:** Many of the biomaterials listed are used to deliver and release growth factors both in bone and articular cartilage. Did anyone study whether a specific biomaterial can influence in different ways the activity of the same growth factor in these tissues?

**Authors:** To cross compare all parameters within the data tables is a significant undertaking and would increase the overall review size extensively. However, the data are present in all supplementary tables for the reader to assess as required. Comments have been made throughout the text, highlighting these differences between studies and that interpretation should be carefully considered.