# 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 utilises 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 imposition 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.

| Factors affecting construct efficacy *in vivo* | Factors affecting animal species selection |
|---|---|
| • Anatomic bone location | • Acquisition and treatment costs |
| • Animal species and age | • Animal breed and uniformity |
| • Animal state of health (disease states) | • Animal size relative to implant number and size |
| • Bone structure and complexity | • Availability |
| • Defect size (critical sized) and position (bone region) | • Biological characteristics analogous to humans |
| • Mechanical load and stress conditions | • Blood and biopsy sample size and number |
| • Mutational status (strain) | • Ethical considerations |
| • Nutrition | • Existing biological knowledge of the species |
| • Presence of adjacent soft tissues | • Handling and nature of the animal |
| • Presence of periosteum | • Normal activity level of the animal |
| • Post-surgical fixation | • Resistance to infection |
| • Time | • Study period and lifetime of the animal |
| • Vascularisation | • Tolerance to surgery and captivity |

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.

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

**Table 3.** Factors affecting both study animal selection and efficacy of implanted constructs within these animal models.

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

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

| Organic Substitutes for Bone | | Synthetic and Inorganic Substitutes | |
|---|---|---|---|
| **Name** | **Abbrev.** | **Name** | **Abbrev.** |
| Alginate | **ALG** | Calcium carbonate | **$CaCO_3$** |
| Allograft | *n/a* | Calcium deficient HA | **CDHA** |
| Autograft | *n/a* | Calcium phosphate | *n/a* |
| Chitosan | *n/a* | Carboxymethyl cellulose | **CMC** |
| Collagen | *n/a* | Cholesterol-bearing pullalan nanogel with acrylol residue | **CHPA** |
| Coral | *n/a* | Hydroxyapatite | **HA** |
| Cortico-cancellous human bone block | **CHBB** | Poly ethylene glycol | **PEG** |
| Demineralised bone | *n/a* | Poly ethylene glycol-diacrylate | *n/a* |
| Deproteinised bovine bone block with porcine collagen | **DBBB** | Poly lactic co-glycolic acid | **PLGA** |
| Fibrin | *n/a* | Perfluorotributylamine | **PFTBA** |
| Fibrinogen | *n/a* | Poly L lysine | **PLL** |
| Gelatin | *n/a* | Polystyrene | *n/a* |
| Hyaluronic acid | **HAA** | Poly urethane | **PUR** |
| Matrigel | *n/a* | Poly vinyl alcohol | **PVA** |
| Monoolein | *n/a* | Poly glycolic acid | **PGA** |
| Silk fibroin | *n/a* | Poly caprolactone | **PCL** |
| | | Poly propylene fumarate | **PPF** |
| | | Poly lactic acid | **PLA** |
| | | Poly-(N-isopropylacrylamide-co-acrylic acid) | **pNIPAm-co-AAc** |
| | | Silica | *n/a* |
| | | Titanium mesh | *n/a* |
| | | Tri-calcium phosphate | **TCP** |

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

**Table 5.** *In vivo* bone tissue engineering utilising growth factors including BMP-2/OP-1, BMP-7, FGF, PDGF, PTH, PTHrP, TGF-β3, VEGF and Wnt proteins. (divided into 4 parts - part 1)

| Growth Factor(s) | Animal Model | | Defect location and type | Time | Delivery system | Dose/ Conc. | Defect regeneration | Analysis methods |
|---|---|---|---|---|---|---|---|---|
| **1. Direct Delivery** | | | | | | | | |
| **BMP-2** | Large Models *#37* | Dog, Goat, Horse, Monkey, Pig and Sheep | • Drill – calvaria, cleft, condyle, patella and vertebrae<br>• Ectopic – intramuscular and subcutaneous<br>• Fusion – lumbar<br>• Segmental – femur, fibula, humerus, radius, tibia, ulna (endochondral), mandible, maxilla, periodontal ridge and zygomatic arch (intramembranous) | 3 weeks to 26 months | • Organic scaffolds – collagen, demineralised bone, gelatin and silk fibroin<br>• Inorganic scaffolds – CPC, ceramic phosphate, HA, PCL, PEG, PLA, PLGA, TCP and titanium oxide | 5 to 100,000 µg<br>1.6 to 1,500 µg/mL | 1.2 to 21 fold (bone)<br>1.7 to 2.6 fold (biomechanics) | Biochemistry, biomechanical testing, CT (micro), DXA, faxitron, histology, histomorphometry, immunohistochemistry, *in situ* hybridization, MRI, RT-PCR, radiography (micro) and SEM |
| | Small Models *#62* | Mouse, Rabbit and Rat | | 5 d to 24 weeks | • Organic scaffolds – alginate, CHBB, chitosan, collagen, coralline HA, DBBB, demineralised bone, fibrin, gelatin, hyaluronan and monoolein<br>• Inorganic scaffolds – CDHA, CHPA, CPC, PCL, PEG, PLA, PLG, PLGA, polystyrene, PUR, PVA, silica and TCP | 0.1 to 5,000 µg (other studies have used 150,000 µg)<br>2 to 4,000 µg/mL | 1.1 to 50 fold (bone)<br>1.6 to 18 fold (biomechanics) | |
| **BMP-7/ OP-1** | Large Models *#28* | Baboon, Dog, Goat, Monkey and Sheep | • Drill – calvaria, femur, humerus and condyle<br>• Ectopic – intramuscular and subcutaneous<br>• Fusion – lumbar<br>• Osteotomy – tibia and mandible<br>• OVX – osteopenia,<br>• Segmental – femur, tibia, ulna (endochondral), alveolar ridge and mandible (intramembranous) | 1 week to 1 year | • Organic scaffolds – alginate, allograft, autograft, chitosan, collagen, DBM and xenograft<br>• Inorganic scaffolds – CMC, HA, hydroxylapatite, PCL, PLGA, PLLA, polylactide, TCP and titanium | 100 to 750,000 µg<br>1,000 to 3,500 µg/mL | 1.25 to 8.3 fold (bone)<br>1.65 to 3.3 fold (biomechanics)<br>1.6 fold (osteoid) | Biomechanical testing, CT (micro), DXA, histology, histomorphometry, immunohistochemistry, MRI, radiography (micro), radioimmunoassay, and SEM |
| | Small Models *#25* | Minipig, Mouse, Rabbit and Rat | | 5 d to 12 weeks | | 0.025 to 3,500 µg<br>25 to 200 µg/mL | 1.1 to 29.5 fold (bone) – one study showed 96 fold<br>1.3 to 31 fold (biomechanics) | |
| **FGF-1/2/18** | Large Models *#5* | Dog and Primate | • Drill – calvaria, condyle, femur and tibia<br>• Ectopic – intramuscular and subcutaneous<br>• Fracture – tibia<br>• Furcation<br>• OVX – osteopenia<br>• Segmental – femur, tibia (endochondral), alveolar ridge and mandible (intramembranous) | 2 to 32 weeks | • Direct injection<br>• Organic scaffolds – collagen, gelatin and Matrigel<br>• Inorganic scaffolds – HA, hydroxypropoyl cellulose, polyethylene, polyglycolate, polylactide, TCP and titanium | 0.15 to 200 µg<br>100 to 400 µg/mL | 1.3 to 3 fold (bone) | Biochemistry, biomechanical testing, CT (micro), FTIR, histology, histomorphometry, immunohistochemsitry, radiography and SEM |
| | Small Models *#20* | Mouse, Rabbit and Rat | | 3 d to 24 weeks | | 0.01 to 200 µg<br>10 to 100 µg/mL<br>100 to 1,000 µg/kg | 1.2 to 16.4 fold (bone)<br>2.1 to 4 fold (biomechanics) | |
| **PDGF** | Small Models *#14* | Minipig, Mouse, Rabbit and Rat | • Diabetes – rat<br>• Distraction – femur<br>• Drill – calvaria, condyle and femur<br>• Ectopic – intramuscular<br>• Osteotomy – tibia<br>• Segmental – femur and mandible | 10 d to 12 weeks | • Organic scaffolds – chitosan, collagen, DBM and fibrin<br>• Inorganic scaffolds – HA, PDLLA, PGA, PLGA, PLLA and TCP | 0.01 to 750 µg<br>1 to 1,000 µg/mL | 1.45 to 10 fold (bone) | Biochemistry, biomechanical testing, histology, histomorphometry, immunohistochemistry, micro CT, radiography and SEM |
| **PTH (1-31, 1-34, 1-84, 2-34, 28-48 and 53-84)** | Large Models *#6* | Dog, Monkey and Sheep | • Drill – calvaria<br>• Fracture – femur, mandible, tibia and ulna<br>• Fusion – lumbar<br>• Diet – calcium free<br>• Marrow ablation – femur<br>• OVX/ORX/PX – osteopenia<br>• Segmental – femur, tibia, humerus (endochondral) and mandible (intramembranous) | 4 weeks to 4.5 years | • Subcutaneous injection<br>• Organic scaffolds - fibrin and RGD<br>• Inorganic scaffolds - calcium phosphate, HA, PEG and TCP | 0.75 to 7.5 µg/kg/d<br>20 to 1,000 µg/mL | 1.1 to 3.4 fold (bone)<br>1.4 to 2 fold (mechanics) | Biochemistry, biomechanical testing, DXA, finite element analysis, histology, histomorphometry, *in situ* hybridisation, immunohistochemsitry, manual palpation, micro CT, northern blot, QCT, radiography, radiolabelling, RT-PCR and SEM. |
| | Small Models *#46* | Mouse, Rabbit and Rat | | 1 h to 1 d, 2 d to 24 months | | 0.05 to 800 µg/kg/d<br>20 to 100 µg/mL | 1.1 to 13.1 fold (bone)<br>1.1 to 3.8 fold (mechanics) | |
| **PTHrP peptides (1-36 and 107-139)** | Small Models *#9* | Mouse, Rabbit and Rat | • Diabetes – mouse<br>• OVX - osteopenia (rat)<br>• Segmental – femur and ulna (endochondral) | 12 d to 6 months | • Subcutaneous injection<br>• Inorganic scaffold - silica | 10 to 320 µg/kg/d | 1.2 to 10 fold (bone) | Biomechanical testing, CT (micro), histology, histomorphometry, immunohistochemistry, RT-PCR and western blot |
| **TGF-β3** | Large Model *#3* | Baboon | • Bone-tendon transection<br>• Drill – calvaria<br>• Ectopic – intra-muscular<br>• Furcation – mandible and maxilla (intramembranous) | 30 to 90 d | • Organic scaffolds – collagen and Matrigel | 75 to 125 µg | 1.6 to 3 fold (bone) | Biomechanical testing, CT (micro), histology, histomorphometry, immunohistochemistry, RT-PCR and western blot |
| | Small Models *#2* | Mouse and Rat | | 14 to 28 d | • Organic scaffolds – calcium phosphate and collagen | 0.003 to 2.75 µg | 1.05 fold (bone)<br>1.2 fold (cartilage) | |
| **VEGF** | Small Model *#2* | Mouse | • Drill – calvaria<br>• Segmental – femur (endochondral) | 4 weeks | • Organic scaffolds – calcium phosphate<br>• Inorganic scaffolds – PLA | 1.7 µg<br>5 µg/mL | 1.65 fold (bone)<br>Enhanced vascularisation | CT (micro), histology, histomorphometry, immunohistochemistry, intravital microscopy and radiography |

**Table 5.** *In vivo* bone tissue engineering utilising growth factors including BMP-2/OP-1, BMP-7, FGF, PDGF, PTH, PTHrP, TGF-β3, VEGF and Wnt proteins. (contined - part2)

| Growth Factor(s) | Animal Model | | Defect location and type | Time | Delivery system | Dose/ Conc. | Defect regeneration | Analysis methods |
|---|---|---|---|---|---|---|---|---|
| **1. Direct Delivery (continued)** | | | | | | | | |
| **Wnt 3A** | Small Model *#2* | Mouse | • Delayed skeletal development – suture closure<br>• Segmental – tibia (endochondral) | 4 weeks | • Injection – protein suspension and liposomal vesicles | 0.5 μg/mL | Enhanced bone healing and reduced suture area approx. 1.4 fold | Biochemical testing, CT (micro), histology, immunohistochemistry, *in situ* hybridisation and RT-PCR |
| **2. Indirect Delivery** | | | | | | | | |
| **BMP-2** | Large Models *#6* | Dog, Horse, Pig and Sheep | • Drill – calvaria, iliac crest, orbital bone (lacrimal) and patella<br>• Ectopic – intra-muscular and subcutaneous<br>• Fusion – lumbar<br>• Segmental – femur, fibula, metacarpal, metatarsal, radius (endochondral) and mandible, maxilla (intramembranous) | 4 to 24 weeks | Direct viral/non-viral particle injection or implant of transduced/ transfected cells with and without scaffolds<br>• Viral transduction – adenovirus, retrovirus, lentivirus<br>• Non-viral transfection – plasmids and vectors<br>• Organic scaffolds – alginate, allograft, autograft, collagen, demineralised bone matrix, fibrin, fibrinogen and Matrigel<br>• Inorganic scaffolds – PEG-diacrylate, PFTBA and titanium mesh | 12 μg<br>0.04 to 5 x $10^{11}$ viral particles<br>2 to 5 x $10^{7}$ cells | 1.3 to 3.2 fold (bone) | Biomechanical testing, CT (micro), DXA, histology, histomorphometry, immunohistochemistry, *in vivo* imaging, RT-PCR, and radiography (micro) |
| | Small Models *#32* | Minipig, Mouse, Rabbit and Rat | | 1 to 35 weeks | | 3 to 75,000 μg<br>0.001 to 7 x $10^{10}$ viral particles<br>0.005 to 5 x $10^{7}$ cells | 1.3 to 9 fold (bone)<br>2.7 to 10.9 fold (biomechanics) | |
| **BMP-7/ OP-1** | Large Models *#3* | Dog and Goat | • Ectopic – intramuscular<br>• Intervertebral disc transplant<br>• Segmental – femur (endochondral) and mandible (intramembranous) | 1 to 8 months | Direct viral/non-viral particle injection or implant of transduced/ transfected cells with and without scaffolds<br>• Viral transduction – adenovirus<br>• Non-viral transfection – plasmids and vectors<br>• Organic scaffolds – allograft, chitosan, collagen, coral, gelatin and silk fibroin<br>• Inorganic scaffolds – HA, PA and PCL | 2 x $10^{10}$ viral particles<br>0.01 to 5 x $10^{7}$ cells | 2 to 2.5 fold (bone) | Biomechanical testing, biochemistry, CT(micro), cytochemistry, histology, histomorphometry, MRI, radiography, RT-PCR and SEM |
| | Small Models *#11* | Mouse, Rabbit and Rat | | 1 to 16 weeks | | 25 to 250 μg<br>0.2 to 2.5 x $10^{11}$ viral particles<br>0.1 to 4 x$10^{6}$ cells | 1.03 to 5 fold (bone) – one study showed 21 fold | |
| **FGF-2** | Large Models *#1* | Dog | • Furcation – dental root<br>• Irradiation<br>• Drill – calvaria<br>• Segmental – radius (endochondral) | 6 weeks | Direct implantation of transduced cells within scaffold<br>• Inorganic scaffolds – HA, PA66, PLGA and TCP | unknown | Enhanced periodontal bone regeneration | Biochemistry, clinical examination, CT (micro), DXA, histology, histomorphometry, immunohistochemistry, pQCT, radiography, RT-PCR and SEM |
| | Small Models *#5* | Mouse, Rabbit and Rat | | 1 to 20 weeks | | 0.0625 to 5 x $10^{6}$ cells | 2 to 2.7 fold (bone) – one study showed 53.5 fold | |
| **PDGF** | Small Models *#4* | Mouse and Rat | • Ectopic – subcutaneous<br>• Segmental – alveolar ridge and femur | 10 d to 6 weeks | Direct viral/non-viral particle injection or implant of transduced/ transfected cells with and without scaffolds<br>• Viral transduction – adenovirus<br>• Non-viral transfection – plasmids and vectors<br>• Organic scaffolds – collagen, methylcellulose and silk<br>• Inorganic scaffolds – PLGA and mesoporous glass | 5.5 x $10^{8}$ to 5.5 x $10^{9}$ PFU/mL<br>1 x $10^{6}$ cells | 1.7 to 2 fold (bone) | Backscatter SEM, biochemistry, biomechanical testing, histology, histomorphometry, immunohistochemistry, micro CT, northern blot and RT-PCR |
| **TGF-β3** | Small Model *#1* | Mouse | • Ectopic - subcutaneous | 30 d | Direct implantation of transduced cells within scaffold<br>• Viral transduction – recombinant adeno-associated virus<br>• Inorganic scaffold – PLLA/ PEG scaffold | 1 x $10^{6}$ cells | 3D cartilage constructs | Histology, immunohistochemistry and western blot |
| **VEGF** | Small Models *#3* | Mouse and Rabbit | • Ectopic – subcutaneous<br>• Segmental – femur, radius and tibia (endochondral) | 4 to 16 weeks | Direct delivery of non-viral particles or implantation of transfected cells<br>• Non-viral transfection – plasmid vectors<br>• Organic scaffolds – collagen, calcium phosphate and calcium carbonate | 20 μg<br>5 x $10^{6}$ cells | 1.6 to 2 fold (bone)<br>Enhanced vascularisation | CT (micro), histology, histomorphometry, immunohistochemistry, RT-PCR and radiography |
| **Wnt 1, 3A, 4, 5A, 6 and 10B** | Small Models *#8* | Chick, Mouse and Rat | • Drill – calvaria<br>• SCID mouse<br>• Transfected embryo | 4 d to 12 weeks | Direct implantation of transfected/ transduced cells with and without scaffolds<br>• Viral transduction – lentivirus and retrovirus<br>• Non-viral transfection – plasmid vectors<br>• Organic scaffold – HA and TCP<br>• Inorganic scaffold – PLGA | 0.15 to 5 x $10^{6}$ cells<br>N/A (transgenic animal) | 1.25 to 12 fold (bone)<br>1.5 fold (cartilage)<br><br>Wnt-5A reduced bone formation | CT (micro), histology, histomorphometry, immunohistochemistry, *in situ* hybridisation and radiography |
| **3. Combinational Delivery** | | | | | | | | |
| **BMP-2** *plus* (BBP, BMP-7/OP-1, Epo, FGF, integrin, MSCs, Runx2, TGF-β2, tobramycin, VEGF or zoledronic acid) | Large Model *#4* | Dog, Horse and Pig | • Drill – calvaria, orbital bone (lacrimal), and ulna<br>• Ectopic – intramuscular and subcutaneous<br>• Fusion – lumbar<br>• Irradiated – mandible<br>• Segmental – femur and tibia (endochondral) | 1 to 9 weeks | Direct delivery by injection or implant, and indirect delivery by viral/non-viral particle injection or transduced/ transfected cell implant<br>• Organic scaffolds – alginate, allograft, chitosan, collagen, coral and gelatin<br>• Inorganic scaffolds – CMC, CDHA, PEG-diacrylate, PEG-MMP, PLA, PLGA, PPF, TCP and titanium<br>• Viral transduction – adenovirus, baculovirus and lentivirus<br>• Non-viral transfection – plasmids | 5.26 to 120 μg BMP-2<br>2 x $10^{11}$ viral particles | 1.6 fold (bone) | Biochemistry, biomechanical testing, CT (micro), DXA, histology, histomorphometry, immunohistochemistry, *in situ* hybridisation, RT-PCR, PET (micro), radiography, rheology and western blot |
| | Small Models *#38* | Minipig, Mouse, Rabbit and Rat | | 1 to 16 weeks | | 0.0025 to 200 μg BMP-2<br>0.075 to 7.5 x $10^{10}$ viral particles<br>1 to 4.8 x $10^{6}$ cells | 1.1 to 20 fold (bone)<br>4 to 8.3 fold (biomechanics)<br>2 fold (vasculature) | |

**Table 5.** *In vivo* bone tissue engineering utilising growth factors including BMP-2/OP-1, BMP-7, FGF, PDGF, PTH, PTHrP, TGF-β3, VEGF and Wnt proteins. (continued - part 3)

| Growth Factor(s) | Animal Model | | Defect location and type | Time | Delivery system | Dose/ Conc. | Defect regeneration | Analysis methods |
|---|---|---|---|---|---|---|---|---|
| **3. Combinational Delivery (continued)** | | | | | | | | |
| **BMP-7/OP-1**<br>*plus* (BBP, BMP-2, BMSCs, blood, bone marrow, FGF-2, IGF-1, pamidronate, PDGFb, PTH (1-34), MSCs, osteoblasts, TGF-β1, TGF-β3, TSP-1 or VEGF) | Large Models<br>*#7* | Baboon, Dog and Horse | • Drill – calvaria and femur<br>• Ectopic – intramuscular and subcutaneous<br>• Fusion – lumbar<br>• Osteotomy – tibia<br>• OVX – osteopenia<br>• Segmental – mandible and metacarpal (endochondral and intramembranous) | 2 to 16 weeks | Direct delivery by injection or implant, and indirect delivery by viral/non-viral particle injection or transduced/ transfected cell implant<br>• Organic scaffolds – allograft, chitosan, collagen, gelatin and silk fibroin<br>• Inorganic scaffolds – calcium carbonate, calcium phosphate, CMC, HA, PCL, PDLLA and TCP<br>• Viral transduction – adenovirus<br>• Non-viral transfection – plasmids | 5 to 5,000 µg – one study used 125mg<br>0.2 to 2 x $10^{11}$ viral particles | 1.4 to 5.3 fold (bone)<br>3 fold (osteoid) | Biochemistry, biomechanical testing, CT (micro), cytochemistry, DXA, histology, histomorphometry, immunohistochemistry, MRI, radiography, RT-PCR and SEM |
| | Small Models<br>*#19* | Mouse, Rabbit and Rat | | 1 d to 48 weeks | | 2 to 200 µg<br>0.00075 to 5.5 x $10^{11}$ viral particles<br>1 to 4 x $10^{6}$ cells | 1.2 to 15 fold (bone)<br>1.2 to 1.4 fold (biomechanics) | |
| **FGF-2**<br>*plus* (17β-estradiol, BMP-7/OP-1, estrogen, IGF-2, PTH (1-34), melatonin or VEGF) | Small Models<br>*#11* | Mouse, Rabbit and Rat | • Drill – calvaria, condyle and femur<br>• Ectopic – intramuscular<br>• OVX – osteopenia | 1 to 48 weeks | Direct infusion or scaffold implant<br>• Organic scaffolds – collagen and gelatin<br>• Inorganic scaffolds – titanium | 0.001 to 100 µg<br>200 to 1,000 µg/mL | 1.1 to 3.3 fold (bone)<br>8 fold (osteoid) | Biochemistry, CT (micro), DXA, histology, histomorphometry, immunohistochemistry, radiography and XTM |
| **PDGF**<br>*plus* (bFGF, BMP-2, BMP-7, BMSCs,IGF-1, osteogenin, TGF-*β*1 and VEGF ) | Large Models<br>*#4* | Dog | • Drill – calvaria<br>• Ectopic – subcutaneous<br>• Osteotomy – mandible<br>• Segmental – femur | 4 to 18 weeks | Direct delivery by implant, and indirect delivery by viral particle injection<br>• Organic scaffolds – chitosan, collagen, fibrinogen and methylcellulose<br>• Inorganic scaffolds – Brushite, calcium phosphate, ePTFE, TCP and titanium<br>• Viral transduction – adenovirus | 5 µg/mL<br>2 x $10^{10}$ viral particles | 1.4 to 2.3 fold (bone) | Flow cytometry, histology, histomorphometry and micro CT |
| | Small Models<br>*#6* | Mouse, Rabbit and Rat | | 4 to 8 weeks | | 0.05 to 200 µg<br>0.001 to 0.05 µg/mL | 2.5 to 10 fold (bone) | |
| **PTH (1-34 and 1-84)**<br>*plus (alendronate, BMP-2, BMP-7, BMSCs, human PDL cells, ibandronate, IL-6, MSCs, pamidronate, rapamycin, tiludronate and zoledronic acid)* | Large Models<br>*#1* | Sheep | • Ectopic – subcutaneous<br>• Fracture – tibia<br>• OVX – osteopenia<br>• Segmental – femur and tibia | 3 months | • Subcutaneous injection | 500IU/day | 2 to 4 fold (bone)<br>PTH alone | Biochemistry, mechanical testing, DXA, FACS, faxitron, histology, histomorphometry, immunocytochemistry, immunohistochemsitry, micro CT, nanoindentation testing, northern blot, QCT, radiography, raman spectroscopy, RT-PCR, SEM and western blot |
| | Small Models<br>*#14* | Mouse, Rabbit and Rat | | 1 to 15 weeks | | 10 to 90 µg/kg/d | 1.2 to 4.1 fold (bone)<br>1.2 to 3.1 fold (mechanics) | |
| **PTHrP peptides (1-36 and 1-86)**<br>*plus* (C-terminal PTHrP (107-139) peptide or PTH) | Small Model<br>*#3* | Mouse | • Diabetes<br>• Knockout – PTHrP<br>• Osteopenia | 1 to 2 months | • Subcutaneous injection | 80 to 100 µg/ kg/d | 1.5 to 3 fold (bone) | Biochemistry, CT (micro), DXA, faxitron analysis, histology, immunohistochemistry, RT-PCR, radiography and western blot |
| **TGF-β3**<br>*plus* (BMP-2, chondrocytes, MSCs, OP-1, Sox9 or TGF-β1) | Large Models<br>*#2* | Baboon and Sheep | • Drill – condyle, humerus and patella<br>• Ectopic – subcutaneous and intra-muscular | 63 to 90 d | • Organic scaffold – chitosan and fibrin<br>• Inorganic scaffold – HA and calcium carbonate | 0.05 to 125 µg TGF-β3 | 5.3 fold (bone) | Biomechanical testing, CT (micro), histology, histomorphometry, immunohistochemistry and RT-PCR |
| | Small Models<br>*#12* | Mouse, Rabbit and Rat | | 1 to 22 weeks | Direct infusion or scaffold implant<br>• Organic scaffolds – alginate and fibrin<br>• Inorganic scaffolds – PEG-PCL, PLGA, PLL and pNIPAm-co-AAc | 0.02 µg TGF-β3 (0.01 to 0.1 µg/ mL) | 12.8 to 13 fold (bone)<br>1.6 to 22 fold (collagen) | |
| **VEGF**<br>*plus* (BMP-2, BMP-4, BMP-7/OP-1 or FGF-2) | Large Model<br>*#2* | Dog and Pig | • Drill – calvaria<br>• Ectopic – subcutaneous and intramuscular<br>• Segmental – femur, ulna (endochondral) and infra-orbital bone (intramembranous)<br>• Sinus floor elevation | 9 weeks | Direct delivery by injection or implant, and indirect delivery by viral/non-viral particle injection or transduced/ transfected cell implant<br>• Organic scaffolds – alginate, biocoral, collagen, gelatin and silk hydrogel<br>• Inorganic scaffolds – calcium phosphate, PLA, PLGA, PPF, octacalcium phosphate and TCP<br>• Viral transduction – adenovirus and retrovirus<br>• Non-viral transfection – plasmids and vectors | 0.4 to 4 µg | 1.6 fold (bone)<br>Enhanced vascularisation | Biochemistry, CT (micro), histology, histomorphometry, immunohistochemistry, *in situ* hybridisation, microangiography, radiography and SEM |
| | Small Models<br>*#17* | Mouse, Rabbit and Rat | | 1 to 16 weeks | | 0.2 to 20 µg<br>5.5 x $10^{11}$ viral particles<br>0.2 to 3 x $10^{6}$ cells | 1.4 to 20 fold (bone)<br>4 to 208 fold (biomechanics)<br>2 fold (vasculature) | |
| **4. Human Trials** | | | | | | | | |
| **BMP-2** | Human<br>*#30* | | • Bone augmentation<br>• Facial reconstruction (cleft, mandible and maxilla)<br>• Long bone fracture and non-union (tibia)<br>• Lumbar fusion | 6 weeks to 6 years | • Organic scaffolds – allograft, autograft, collagen and gelatin<br>• Inorganic scaffolds – fusion cage, HA-TCP, PEEK, PGA, PLGA, PLA and titanium mesh | 0.9 to 100 mg (0.75 to 1.5 mg/ mL) | Enhanced bone healing was observed in the majority of patients | Biochemistry, CT, histology and radiography |

**Table 5.** *In vivo* bone tissue engineering utilising growth factors including BMP-2/OP-1, BMP-7, FGF, PDGF, PTH, PTHrP, TGF-β3, VEGF and Wnt proteins. (continued - part 4)

| Growth Factor(s) | Animal Model | Defect location and type | Time | Delivery system | Dose/ Conc. | Defect regeneration | Analysis methods |
|---|---|---|---|---|---|---|---|
| **4. Human Trials (continued)** | | | | | | | |
| **BMP-7/ OP-1** | Human<br>*#32* | • Mandible reconstrutcion<br>• Non-union fracture<br>• Long bone osteotomy<br>• Lumbar fusion<br>• Pseudarthrosis | 2 weeks to 68 months | • Organic scaffolds – allograft, autograft, collagen and xenograft<br>• Inorganic scaffolds – CMC, TCP and titanium | 2.5 to 17.5 mg – one study used 2,000 mg (3.5mg average) | Accelerated bone healing and increased bone tissue and mechanical strength was observed | Clinical assessment, CT, histology, histomorphometry, physical examination, radiography and scintigraphy |
| **PDGF** | Human<br>*#1* | • Periodontitis | 9 months | • Organic scaffolds – allograft | 0.5 to 5 mg/mL | 1.12 to 1.17 fold (bone) | Clinical assessment, histology and radiography |
| **PTH** | Human<br>*#14* | • Healthy adults<br>• Low bone mineral density<br>• Mandibular repair<br>• Postmenopausal women<br>• Vertebral fracture | 2 months to 2 years (one study - 7 d) | • Subcutaneous injection | 20 to 100 μg/d<br>2 to 4 pmol/kg/h | Accelerated bone healing through upregulation of bone markers and resultant bone tissue was observed | Biochemistry, clinical assessment, CT, DXA, histology, histomorphometry, QCT, quality of life assessment, radiography, SEM and TEM |
| **PTHrP peptides (1-34 and 1-36)** | Human<br>*#4* | • Healthy adults<br>• Postmenopausal estrogen deficient females | 2 d to 2 weeks | • Subcutaneous injection<br>• Intravenous infusion | 2 to 80 pmol/ kg/h | Bone formation was activated in postmenopausal females, but inhibited in healthy adults | Biochemistry |

**Abbreviations: 1,24,25[OH]3 D3** *(1,24,25-trihydroxyvitamin D3),* **2MD** *(2-methylene-19-nor-(20S)-calcitriol),* **BBP** *(BMP binding protein),* **BMP-2/7** *(bone morphogenetic protein 2/7),* **CDHA** *(calcium deficient hydroxyapatite),* **CHBB** *(cortico-cancellous human bone block),* **CHPA** *(cholesterol-bearing pullulan nanogel with acrylol residue),* **CMC** *(carboxymethylcellulose),* **CPC** *(calcium phosphate cement),* **CT** *(computerised tomography),* **DBBB** *(deproteinised bovine bone block/porcine collagen),* **DBM** *(demineralised bone matrix),* **DXA** *(dual energy x-ray absorptiometry),* **Epo** *(erythropoietin),* **FGF** *(fibroblast growth factor),* **FTIR** *(fourier transform infrared spectroscopy),* **HA** *(hydroxyapatite),* **HAA-PVAH** *(hyaluronic acid and poly-vinyl alcohol functionalised with hydrazide groups),* **ID2** *(1α-hydroxyvitamin D2),* **ID3** *(1α-hydroxyvitamin D3),* **MMP** *(matrix metalloproteinase),* **MRI** *(magnetic resonance imaging),* **MSC** *(mesenchymal stem cell),* **OP-1** *(osteogenic protein-1),* **ORX** *(orchiectomised),* **OVX** *(ovariectomised),* **PCL** *(poly caprolactone),* **PDLLA** *(poly-D, L-lactic acid),* **PEEK** *(polyetheretherketone),* **PEG** *(poly ethylene glycol),* **PET** *(positron emission tomography),* **PGA** *(poly glycolic acid),* **PLA** *(poly lactic acid),* **PLG (***poly D,L-lactide-co-glycolide),* **PLGA** *(poly lactic co-glycolic acid),* **PLL** *(poly-L-lysine),* **PLLA** *(poly-L-lactide),* **pNIPAm-co-AAc** *(poly-(N-isopropylacrylamide-co-acrylic acid)),* **PPF** *(poly-propylene fumarate),* **pQCT** *(peripheral quantitative CT),* **PTFBA** *(perfluorotributylamine),* **PTFE** *(polytetrafluoroethylene),* **PTH** *(parathyroid hormone),* **PTHrP** *(parathyroid hormone receptor-related protein),* **PUR** *(polyurethane),* **PVA** *(poly-vinyl alcohol),* **PX** *(p arathyroidectomized),* **rhBMP-2** *(recombinant human bone morphogenetic protein-2),* **RT-PCR** *(real time polymerase chain reaction),* **SEM** *(scanning electron microscopy),* **Sox9** *(SRY (sex determining region Y)-box 9),* **TCP** *(tricalcium phosphate),* **TEM** (*transmission electron microscopy),* **TGFβ** *(transforming growth factor β),* **VEGF** *(vascular endothelial growth factor),* **VitD3** *(vitamin D3),* **Wnt** *(wingless-type MMTV integration site family), and* **XTM** *(X-ray tomographic microscopy).* ***# - number of publications.***

to BMP-8a) have a known osteochondral function (Even *et al.*, 2012). BMP-2 specifically, is a disulphide-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 21 fold in large animal models (He *et al.*, 2009; Wikesjo *et al.*, 2008), and 1.1 and 50 fold in small animal models (Ishihara *et al.*, 2008; Tolli *et al.*, 2011) (Table 5). To date, despite the wealth of reported studies there remains a lack of consensus concerning the optimum rhBMP-2 dose for effective bone defect repair. Applications of rhBMP-2 have utilised dosages between 5 μg and 100 mg in large animal studies (Gu *et al.*, 2011; Nilsson *et al.*, 1986), and 0.1 μg and 5 mg in small animal models (Hayashi *et al.*, 2009; Whang *et al.*, 1998). A few small animal studies have also used higher dosages up to 150 mg (Dohzono *et al.*, 2009; Hou *et al.*, 2012). One explanation for the observed variable success and diverse dosages is the number of different size models and defects investigated. Large animal models included dog (Hussein *et al.*, 2012), goat (Li *et al.*, 2010b), horse (Tsuzuki *et al.*, 2012), monkey (Bai *et al.*, 2009), pig (Abbah *et al.*, 2011) and sheep (Gu *et al.*, 2011). Small animal models included mouse (Yu *et al.*, 2010b), rabbit (Liu *et al.*, 2013) and rat (Iyomasa *et al.*, 2012). Bone defects investigated included both endochondral and intramembranous bone segmental defects (Boerckel *et al.*, 2012; Kirker-Head *et al.*, 1998; Wikesjo *et al.*, 2008), lumbar fusions (Akamaru *et al.*, 2003; Fu *et al.*, 2009) and drill defects (He *et al.*, 2009; Levi *et al.*, 2010), in addition to ectopic implants intramuscularly (Luca *et al.*, 2010b; Saito *et al.*, 2003) and subcutaneously (Fu *et al.*, 2010; Kimura *et al.*, 2010). Consequently, it may be more informative for authors to reference concentration and volume within given defect dimensions rather than a simple dosage as a standard between models. This would aid comparison between studies and animals enabling direct assessment of dosage and defect regeneration correlation. However, employed rhBMP-2 concentrations vary considerably, from 1.6 μg/mL to 1.5 mg/mL for large 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.

*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-originated 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, resulting from 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 3.3-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), α4-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 (Supplementary Table 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 differentiate 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.*, 2002), monkey (Cook *et al.*, 2002) and sheep (Cipitria *et al.*, 2013)) 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 (Blokhuis *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; Haidar *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. Lammens *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 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 ($2 \times 10^{10}$ in large animals (Zhang *et al.*, 2007), and 1.8 to $2.5 \times 10^{11}$ in small animals (Dunn *et al.*, 2005; Zhang *et al.*, 2012b)) and transduced/transfected cells ($1 \times 10^5$ to $5 \times 10^7$ in large animals (Chaofeng *et al.*, 2013; Zhu *et al.*, 2010), and $1 \times 10^5$ to $2 \times 10^6$ 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 from 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 animal models, 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 animal models, 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 animal models, 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 ovariectomised 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 transfected cells ($6.25 \times 10^4$ and $5 \times 10^6$ 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, growth factor selection should be made 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 varying dosages. Following combination administration

of FGF-2 with BMP-2 (van der Stok *et al.*, 2013), BMP-7 (Ma *et al.*, 2007), oestrogen (Iwaniec *et al.*, 2003), IGF-1 (Madry *et al.*, 2010), melatonin (Takechi *et al.*, 2008), PTH (1-34) (Lane *et al.*, 2003a), VEGF (Behr *et al.*, 2012) or 17β-estradiol (Lane *et al.*, 2003a) in small animal models, including mouse (Behr *et al.*, 2012), rabbit (Madry *et al.*, 2010) and rat (Nakamura *et al.*, 2005), 1.1 to 3.3 fold (Fujimura *et al.*, 2002; Lane *et al.*, 2003a) increased bone formation was observed. Dual combinations evidently have important ramifications for bone tissue formation and defect regeneration albeit at magnitudes smaller than FGF-2 delivery alone. It is difficult to interpret whether interactions between dual factors resulted in synergistic or antagonistic signalling. Indeed, it is well known that FGF-2 inhibits BMP-2-driven bone formation by interfering with the signal transduction pathway, demonstrating a need for careful co-administration, possibly in a spatiotemporal manner. Analysis of parameters other than bone may help elucidate the mechanisms in play and aid comparison between studies. Although all studies reported increased bone formation, many did not compare to either growth factor individually or investigate non-bone tissues, such as cartilage production. Comparison to single factors would distinguish between synergistic and antagonistic signalling (greater or lower fold increase following dual combination, respectively).

**PDGF**

PDGF is a potent mitogen for the induction of angiogenesis from progenitor cells of the mesenchymal lineage. There are 5 isoforms including PDGF-A, B, C, D and a heterodimer AB. Homodimer BB constitutes a dimeric glycoprotein of PDGF that has been administered *in vivo* within small animal models, including the minipig (Herford and Cicciu, 2012), mouse (Ranly *et al.*, 2005), rabbit (Lee *et al.*, 2001b) and rat (Kaipel *et al.*, 2012) for bone tissue engineering strategies (Supplementary Table 4).

*Direct administration of PDGF*

Direct administration of PDGF *in vivo* has been shown to increase bone tissue formation and defect regeneration between 1.5 (Herford *et al.*, 2012) and 2.4 fold (Moore *et al.*, 2009), following dosages ranging from 0.01 (Ranly *et al.*, 2005) to 80 μg (Nash *et al.*, 1994) (two studies have also employed dosages up to 750 μg (Herford and Cicciu, 2012; Herford *et al.*, 2012)). One study by Park *et al.* (2000) demonstrated up to 10 fold increased bone formation; however, this was compared to healing within blank control defects. The same study demonstrated only a 1.5 fold increase in comparison to control defects filled with scaffold alone. Evidently, dependent on the controls used and comparisons made within individual studies, care should be taken when drawing conclusions regarding growth factor efficacy for bone regeneration *in vivo*. Careful consideration should also be afforded to which parameters are used to quantify growth factor efficacy, as Moore *et al.* (2009) reported 9 fold increased union but only 1.9 to 2.4 fold increased bone volume. Standardisation of comparable readouts across related 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 $5.5 \times 10^8$ to $5.5 \times 10^9$ 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 $2 \times 10^{10}$/mL 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.*, 2010) 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 teriparatide (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 (Delmas *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 increased bone formation, 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 during defect preparation and following surgery. 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 osteocyte-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 (osteopaenia 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 ovariectomised 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 level 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 OP-1 (Ripamonti *et al.*, 2010) or MSCs (Mrugala *et al.*, 2008) within large animal models has 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 $rhVEGF_{165}$ 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 $rhVEGF_{165}$ 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 $VEGF_{165}$ 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 over 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 and demonstrated accelerate mineralisation and osteoid deposition' (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 1.5 fold increased cartilage formation and 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 therefore 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 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 Triplett *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 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 vertebrae, 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 particles (Dawson *et al.*, 2009), polyetheretherketone (Klimo and Peelle, 2009), and poly(lactic co-glycolic acid) (Johnson *et al.*, 1988b; Katayama *et al.*, 2009).

However, in the last few years a number of studies have questioned 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 and renal complications, wound complications, increased inflammation and increased cancer risk (Carreon *et al*., 2008; Mesfin *et al*., 2013; Moshel *et al*., 2008). Fu *et al*. (2013) stated, "early journal publications misrepresented the effectiveness and harms through selective reporting, duplicate publication, and underreporting", in their assessment of rhBMP-2 use in spine fusion surgery (Fu *et al*., 2013). Thus, standardisation of analysis methodology and readout parameters would benefit comparison between studies regarding BMP-2 safety and efficacy.

**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 measuring 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 (Johnsson *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; Fabeck *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 fulfil 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 combinations 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**

**Reviewer I:** Can biomaterials/scaffold influence the capability of stem cells to promote bone repair by themselves, without the use of growth factors? If yes, 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 I:** 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.