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Growth factor delivery for bone tissue repair: an update

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Growth factors (GFs) are endogenous proteins capable of acting on cell-surface receptors and directing cellular activities involved in the regeneration of new bone tissue. The specific actions and long-term effects of GFs on bone-forming cells have resulted in exploration of their potential for clinical bone repair. The concerted efforts have led to the recent approval of two GFs, bone morphogenetic protein-2 and osteogenic protein-1, for clinical bone repair, and human parathryroid hormone (1-34) for augmentation of systemic bone mass. This review provides a selective summary of recent (2001 – 2004) attempts for GF delivery in bone tissue regeneration. First, a summary of non-human primate studies involving local regeneration and repair is provided, with special emphasis on the range of biomaterials used for GF delivery. Next, efforts to administer GFs for systemic augmentation of bone tissue are summarised. Finally, an alternative means of GF delivery, namely the delivery of genes coding for osteogenic proteins, rather than the delivery of the proteins, is summarised from rodent models. To conclude, future avenues of research considered promising to enhance the clinical application of GFs are discussed.

Keywords: biomaterials, bone regeneration, gene delivery, growth factors, systemic bone regeneration

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1. Introduction

Growth factors (GFs), also known as cytokines, are endogenous proteins that act on a wide variety of cells and direct their actions via cell-surface receptor binding and activation. The actions of GFs on cells are long lasting, so that a single exposure of cells to GFs results in long-term (weeks to months) cellular effects. The observed cellular effects are multifaceted, typically manifested as:

- an onset of vectorial migration (chemotactic effect)
- a stimulation of cell division (mitogenic effect)
- an induction of cellular differentiation (morphogenic effect)
- an initiation of programmed cell death (apoptotic effect)
- a modulation of metabolic activity
- a combination of the above

GFs are typically multimodal, exhibiting a different mechanism of action depending on the concentration and/or exposure time, as well as the phenotype of the target cell. Because developmental bone formation is an orchestrated cellular process tightly controlled by the actions of GFs, GF therapy is an obvious strategy when the bone integrity is compromised and bone tissue needs to be repaired. Such a strategy aims to enhance the local presence of bone-depositing osteoblasts, either by attracting the cells to the repair site or by inducing the proliferation of local undifferentiated, precursor cells, followed by the transformation of precursor cells into osteoblastic phenotype. Angiogenesis is an integral part of this process, as it provides the necessary nutritional support for the newly formed tissue, as well as providing a cell source for further

remodelling of the tissue formed at the outset. There is no magic bullet when it comes to the choice of the appropriate GF for bone repair. Mitogens such as platelet-derived growth factors (PDGFs), morphogens such as bone morphogenetic proteins (BMPs), metabolic regulators such as insulin-like growth factors (IGFs), as well as angiogenic proteins such as basic fibroblast growth factor (bFGF), have all been used with some degree of success in bone repair in animal models.

Although GFs are a logical choice for enhancing bone repair, there is an important distinction between the endogenous utilisation of GFs for bone induction and modelling, and the exogenous application of GFs for bone repair. The former typically involves local production of minute quantities of the proteins continually, whose activities are regulated by binding to extracellular matrices and presentation to the cells. Exogenous GFs, on the other hand, rely typically on the one-time application of supra-physiological concentrations of the proteins, whose local presence needs to be maintained throughout the repair process. When GFs are used for local bone repair, the critical drug delivery challenge is to identify a biomaterial carrier that maintains a sufficient concentration of the proteins at the application site for the duration of the repair process, while providing an appropriate support for the healing process. The latter requires the biomaterial carrier to provide an appropriate mechanical environment, to degrade at a tailored rate (to match the tissue formation) and to exhibit sufficient cell compatibility and porosity for induction of functional tissues. When GFs are used for systemic augmentation of bone tissue, the critical drug delivery challenge is to deliver a high dose of the proteins to bones throughout the body, with minimal distribution to extraskeletal sites. This requires the GFs to be 'bone-seeking', with an ability to exhibit preferentially an affinity to bones. Unlike local regeneration, systemic therapy is possible with multiple administration of GFs. In both systemic and local therapy, stimulation of bone repair relies on the local presence of the exogenous proteins. That is, no further pharmacological activity is achieved once protein levels are reduced to below a threshold concentration. To circumvent this limitation, delivery of the GF genes, rather than the proteins, has been receiving much attention in recent years. The gene delivery approach allows local production of the proteins to modulate the healing process, and may better mimic the endogenous bone regeneration and healing process. The critical drug delivery challenge in this case is to design gene carriers that can effectively transfer a therapeutic gene into an endogenous system, either directly to the local cells *in vivo*, or indirectly to expanded cells *ex vivo* intended for administration into a repair site.

This manuscript will summarise recent (2001 – 2004) progress in GF delivery for bone repair. The use of GFs has recently been summarised by the authors [1], and this review is intended to update the concepts explored in the previous review. The organisation of this review is similar to the previous review, in which the GFs used in local and systemic bone regeneration are discussed separately. Critical observations from animal studies have been emphasised, with special attention on the drug delivery aspects of GF administration. Other researchers have also reviewed the GF therapy for bone tissue engineering [2-8], and the reader is encouraged to consult these reviews for an independent point of view. Finally, efforts towards an alternative approach to the delivery of GFs, namely the delivery of osteogenic genes, in which specific expression of gene products are intended to accelerate bone repair, are summarised. The review is restricted to rodent models in the latter case, so as to provide the spectrum of genes and delivery modes utilised for bone repair.

2. Local delivery of growth factors

The potential of a GF needs to be evaluated in a non-human primate model before efficacy testing in clinical studies. It has been recognised that the threshold/optimum dose for exogenous application of BMPs varies among species at different phylogenetic levels [9,10], and non-human primate studies are appropriate to guide the dose selection in clinical studies. Among the GFs delivered in non-human primates are members of the transforming growth factor (TGF) family, such as TGF-β_1 and TGF-β_2, members of the BMP family, such as BMP-2, BMP-7 (also known as osteogenic protein-1, OP-1) and growth differentiation factor (GDF)-5 (also known as cartilage-derived morphogenetic protein-1), bFGF, PDGF and IGF-I [1]. As a result of multi-centre studies [11-14], recombinant human BMP (rhBMP)-2 has recently been approved for clinical intervention in acute tibia fractures treated with intramedullary nailing and in interbody fusion of the lumbar spine with specific types of spinal devices (LT-CAGE® lumbar tapered fusion device, and InterFIX™ and InterFIX RP™ threaded fusion devices [Medtronic, Inc.]). BMP-7, as a component of an implantable device, was approved as an alternative to autograft for long bone non-unions, in which the use of autograft is unfeasible and alternative treatments have failed, and in revision spinal fusions. In addition to the recombinant proteins, naturally-derived osteogenic 'cocktails' were shown to be effective in clinical studies [15], but their clinical application is likely to be hampered due to difficulties in quality control/assurance of the drug product (i.e., unlike relatively pure recombinant proteins, naturally isolated protein mixtures are more difficult to obtain with a consistent composition and a desired osteopotency).

With the approval of effective GF therapies for local bone healing, the challenge for the next-generation clinical devices will be an improvement in the current standard of care. Improved therapies are desirable for the following reasons:

- to increase the assurance of healing, as existing therapies are < 100% effective in the approved clinical indications (i.e., rhBMP-2 application in spinal fusion reports failure rate of 5.6% after 24 months [14], rhBMP-2 in fracture healing require a > 20% secondary interventions [11], and OP-1 application in spinal fusion reports a success of 55 – 75% depending on the evaluation criteria [16])

- to limit excessive bone formation sometimes seen in clinical studies [12] that may limit bone induction at the site of therapy
- to widen the scope of application for bone sites not already approved
- to accelerate the healing process and restore the lost function as quickly as possible
- to improve delivery of the therapy, and, in particular, to establish minimally invasive approaches for device application, for example, by injectable delivery of the therapy as opposed to existing modes of implantable delivery

These improvements are likely to emerge by employing new GFs or enhancing the design of the current delivery systems and/or biomaterial carriers with which the osteogenic proteins are implanted.

Towards these goals, several non-human primate studies using rhBMP-2 and OP-1 have recently been reported (Table 1). The impetus for the primate studies with the GFs already approved for human use is multifaceted. First, a better understanding of regenerative events at specific anatomical sites, such as the changes in cellular events and carrier integrity, will lead to better understanding of osteoinductive device performance in clinics, especially when the devices are implanted at different sites. Given the well-reported histological results, the time course of cell infiltration, osteogenic differentiation, initial bone deposition and long-term remodelling are beginning to be better understood [27,28,31]. Such well-controlled cellular studies, although possible [10], are difficult to conduct in clinical studies. Primate studies have also explored critical device properties that contribute to better performance, such as the exploration of an appropriate therapeutic dose or improvement of mechanical properties of existing implants. Only primate studies can mimic the clinical situation, especially for the unique mechanical environment present in the spinal fusion indication [23]. Augmentation of existing devices with bone marrow cells has been attempted with promising results, as this procedure incorporates cellular elements that are absent in existing devices [17]. In addition to cellular effects, it is likely that the presence of other cytokines, such as bFGF, may contribute to the beneficial effect of bone marrow. Finally, new biomaterial carriers are continually explored to overcome certain limitations of the existing clinical delivery systems [21]. The biomaterials are generally regarded to act as 'depots' of the delivered growth factors, maintaining a high enough concentration of the proteins during formation of new mineralised tissue. Whether this is by direct binding or simply by acting as a physical barrier to prevent free diffusion of the proteins is open for debate [32]. The latter is likely to be the case, given the wide array of biomaterials used for successful bone induction: collagen, gelatin, PLGA, various hydroxyapatites (HAs) and tricalcium phosphate (TCP) (Table 1). One critical limitation of the exisiting clinical delivery systems, absorbable collagen sponge in the case of rhBMP-2 and demineralised bone matrix from bovine in the case of OP-1, is the lack of mechanical strength, limiting the therapy to non-compressive environments or utilisation of protective cages in compressive environments. Mechanically-resilient HA [26] and TCP [31] capable of effectively allowing bone regeneration are welcome additions to the repertoire of biomaterials in this aspect. It is also possible to impregnate the collagen sponges with particulate biomaterials to enhance its compressive strength [22,23]. A granular HA–TCP composite has already been tested in a pilot clinical study, in which 20/20 patients receiving rhBMP-2 exhibited successful posterolateral spine fusion based on radiographic assessment [33]. The mineral-based biomaterials are known to bind BMPs avidly [32,26], but direct demonstration of this has not been attempted in primates, only in smaller animal models. With mechanically-resilient carriers, biomaterial degradation is a critical challenge, as they can, in theory, impede mineralised bone deposition, unlike the malleable collagenous implants. Combination biomaterials, such as the gelatin sponges coated with polymeric PLGA for increased durability may offer a compromise between faster degradation (as compared with mineral-based carriers) and better space creation (as compared with collagenous carriers).

Apart from rhBMP-2 and OP-1, the only other GFs utilised in non-human primates were bFGF (a potent angiogenic factor and a mitogenic protein especially on preosteoblast cells) and TGF-β_3 (a regulator of cell proliferation and differentiation). Whereas the beneficial effect of bFGF was evident in the utilised model [30], the beneficial effect of TGF-β_3, despite clear evidence of unique microscopic changes at the implant site, was not obvious [31]. No dose-response studies were reported for TGF-β_3 and it is likely that a higher dose might have revealed a more robust response in this animal model (rhBMP-2 concentrations used in spinal fusion is > 1 mg/ml, whereas the TGF-β_3 dose was 0.05 mg/ml in this study). The bFGF dose in the primate ulnar osteotomy model (~ 200 µg/site) appeared to be lower than the BMP-7 dose used in a previous study (~ 1 mg), although direct comparison of the two studies is difficult given the differences in the implanted biomaterial carriers (gelatin versus bovine demineralised bone matrix, respectively) and the created surgical defects (unspecified thinner osteotomy versus 2 cm defect, respectively). Preliminary studies have also indicated this protein to be potent in inducing osteogenic activity. For example, enhanced mineralisation in bone marrow cells as compared with BMP-2 (Figure 1). Nevertheless, proteins more potent than available BMPs may lead to more cost-effective therapies with reduced chance of side effects, as a lesser quantity of the exogenous protein needs to be administered. Now that the BMPs have become the standard therapy, it will be imperative to compare the potency of bFGF (and other GFs) head-to-head with BMPs, so that the true potential of the emerging GFs can truely be assessed.

Table 1. Protein GFs shown to be effective in non-human primate models of local bone regeneration.

Protein	Delivery system	Model	Critical observation(s)	Ref.
rhBMP-2	PLGA matrix containing rhBMP-2	Mandiblar osteotomy defect	Co-delivering bone marrow cells along with rhBMP-2/PLGA matrix greatly enhanced the extent of bone formation. Bone marrow cell alone was also as effective, but not rhBMP-2/PLGA alone. Small sample size in treatment groups hampers conclusive results‡	[17]
rhBMP-2	Collagen sponge	Mandibular and cleft osteotomy in *Macaca fascicularis*	rhBMP-2-induced new bone was subsequently able to 'accept' functional titanium implants. Aged animals (~ 20 years) responded to rhBMP-2 similarly to that of younger animals (6 – 9 years). rhBMP-2 induced bone formation more effectively than autogenous bone graft in a cleft osteotomy	[18]*
rhBMP-2	Gelatin/PLGA sponge	Alveolar mandible defect in Rhesus monkeys	2.8 mg rhBMP-2 yielded radiographic evidence of bone formation initially at 4 weeks with full effect at 12 weeks, unlike no bone formation in control (no implant) group. The deposited bone underwent maturation in both lamellar and trabecular spaces between 6 and 12 weeks. Significant variation in induced bone was noted among the animals	[19]
rhBMP-2	Gelatin/PLGA sponge	Mandibular osteotomy defect (30 mm) in Rhesus monkeys	9 mg of rhBMP-2 gave sufficient bone for implant placement in 20 weeks, where radiographically new bone was indistinguishable from the original bone structure. Histological analysis indicated rapid, unorganised (woven) bone deposition initially, followed by remodelling after 15 weeks. The high dose of implanted was noted, but no attempts were made to explore the necessary threshold dose for osteoinduction	[20]
rhBMP-2	Calcium/phosphate cement	Fibula osteotomy defect in baboon	An injectable calcium/phosphate cement (α-BSM™) facilitated bony bridging at > 0.35 mg/ml rhBMP-2 concentration (8 weeks), unlike the surgical control (no intervention) and the injection of the cement alone (0 mg/ml rhBMP-2)	[21]
rhBMP-2	ACS embedded with HA–TCP particles	Bilateral posterolateral intertransverse process arthrodesis in Rhesus monkeys	HA–TCP (5:95 and 15:85 ratios) embedded in an ACS gave easier radiographic monitoring of bone formation, faster resorption of carrier, and better histological bone formation in the centre of fusion mass, as compared with 60:40 HA–TCP embedded ACS. The authors state that "failed osteoinduction results in rabbits have a high predictive value for failure in primates, (and) successful rabbit results do not ensure success in primates". Growth factor concentration, rather than the dose, was proposed as the critical parameter for the implant success	[22]
rhBMP-2	ACS embedded with HA–TCP or allograft bone particles	Bilateral posterolateral intertransverse arthrodesis in Rhesus monkeys	Successful fusion (L4-L5) with only one of three autogenous bone grafts, whereas six of six rhBMP-2/ACS implants augmented with ceramic particles/ bone chips gave successful fusion. There was no ectopic bone with both particulate-embedded sponges, which gave an equivalent response at rhBMP-2 doses (3 mg) that was lower than the historical results in this animal model	[23]
rhBMP-2	ACS	Alveolar osteotomy defects in cynomolgus monkeys	A relatively low dose of rhBMP-2 implantation with ACS (0.1 mg/defect) did not result in significant benefit in new bone induction and osseointegration as compared with control (no rhBMP-2 treatment), indicating significant regenerative capability of the alveolar site	[24]
rhBMP-2	ACS	Osteotomy defect in cranium of Rhesis monkeys	0.1 mg rhBMP-2/site induced 71% bone in treated defects, whereas controls (ACS alone) gave 28% bone in defects. The rhBMP-2 receiving animals exhibited good osseointegration and no apparent adverse effects on animals	[25]
OP-1 and rhBMP-2	Bovine demineralised bone matrix	Mandibular osteotomy defect in baboons	OP-1 in the utilised model provided greater extent of cementogenesis, but reduced alveolar bone formation as compared with rhBMP-2 application. Simultaneous application of the GFs did not synergistically enhance periodontal regeneration	[26]

*Summary of previously published studies. ‡Authors' conclusions. §Note that the BMP-2 used in this study was derived from an *Escherichia coli* expression system, unlike the Chinese hamster ovary-derived BMP-2 used in other studies.

ACS: Absorbable collagen sponge; bFGF: Basic fibroblast growth factor; DEXA: Duel X-ray absorbtiometry; HA–TCP: Hydroxyapatite–tricalcium phosphate (15:85%); OP: Osteogenic protein; PLGA: Poly(lactic-co-glycolic acid); rhBMP: Recombinant human bone morphogenic protein; TGF: Transforming growth factor; α-BSM™: α-Bone Substitute Material (ETEX Corporation).

Table 1. Protein GFs shown to be effective in non-human primate models of local bone regeneration (continued).

Protein	Delivery system	Model	Critical observation(s)	Ref.
OP-1	Porous HA matrix	Calvarial osteotomy defects in baboons	Whereas 100 µg OP-1/site was effective for bone deposition, 500 µg OP-1/site gave a more robust, but occlusive, bone formation pattern at the implant periphery after 30 days. Longer times resulted in complete remodelling of the induced bone, filling the pores of implants. HA from different sources exhibited some, but not overwhelming, differences in the extent of new bone induction. A faster resorbing carrier was needed in this model	[27]
OP-1	Bovine bone-derived collagen	Ulnar (2 cm) osteotomy defect in African green monkeys	1 mg OP-1 induces new radiographic bone at 6 – 8 weeks post-surgery. DEXA analysis indicated robust mineralisation at 12 weeks. Increased cellular proliferation was evident as early as 1 week and throughout the 20-week healing period and, consistent with DEXA, significant bone deposition was evident histologically at 12 weeks onwards	[28]
bFGF	Gelatin	Mandibular osteotomy defect in baboon	bFGF increased new bone formation as a function of implant dose. Induction of new cementum was also increased with bFGF application, and no undesired activities (e.g., ankylosis or root resorption) were noted	[29]
bFGF	Injectable gelatin sponge (open tibia injection)	Ulnar osteotomy defect in cynomolgus monkeys	Complete healing of osteotomy defect in 10 out of 10 animals at 10-weeks with 200 µg/site bFGF, but only 6 out of 10 in control (no bFGF) group. Beneficial effect of bFGF on bone mineral density and mechanical properties	[30]
TGF-β_3	β-TCP matrix	Partial vertebral defect model in baboon (L2-L4)	Unlike sham operation (no implants), β-TCP implants with and without TGF-β_3 (50 mg/ml) were osseo-integrated. TGF-β_3 appeared to accelerate resorption of implants at their periphery and caused deposition of well-organised cancellous bone at the bone–implant interface	[31]

*Summary of previously published studies. ‡Authors' conclusions. §Note that the BMP-2 used in this study was derived from an *Escherichia coli* expression system, unlike the Chinese hamster ovary-derived BMP-2 used in other studies.

ACS: Absorbable collagen sponge; bFGF: Basic fibroblast growth factor; DEXA: Duel X-ray absorbtiometry; HA–TCP: Hydroxyapatite–tricalcium phosphate (15:85%); OP: Osteogenic protein; PLGA: Poly(lactic-co-glycolic acid); rhBMP: Recombinant human bone morphogenic protein; TGF: Transforming growth factor; α-BSM™: α-Bone Substitute Material (ETEX Corporation).

It is also noteworthy that no primate studies have been reported with GDF-5, PDGF and IGF-I, since the compilation of the previous review [1]. A combination therapy, in which multiple GFs are delivered simultaneously to a defect site, was reported in baboons using rhBMP-2 and OP-1 co-administration, but the two GFs did not act synergistically in the chosen animal model. Each protein instead led to a unique morphological transformation at the utilised mandibular osteotomy site [27] (see Table 1). Several studies demonstrated synergistic effects of GF combinations in lower animals, including: FGF-4 and BMP-2 in the rat ectopic model [34]; and IGF-I and TGF-β in a rat fracture [35] and a sheep cervical spine fusion model [36], in which a GF dose (150 µg IGF-I and 30 µg TGF-β_1) gave the optimal response [37]. An early time point in the sheep cervical spine fusion model showed that the bone formation obtained with dosing 150 µg IGF-I and 30 µg TGF-β_1 was equivalent to that obtained by using comparable concentrations of BMP-2 (150 µg), suggesting that other GFs might substitute for the BMPs already approved for clinical application. Studies in primate models would be required to validate these results before these approaches progress to the clinics [36].

Finally, progress towards minimally-invasive delivery of GFs is being made by developing injectable biomaterial carriers that retain the proteins at an application site without significant protein loss over the course of the healing period. The bFGF study of Kawaguchi *et al.* was conducted with an injectable formulation of GF and gelatin, except it was injected in an open defect site, rather than percutanously [30]. A calcium/phosphate-based biomaterial percutanously injected into an osteotomy defect was shown to be effective in delivering rhBMP-2 in a baboon fibula model [21]. The effective concentration of rhBMP-2 was > 0.35 mg/ml, but no information about the injection volume was reported, so that its comparison with effective bFGF concentration could not be performed. Both gelatin and calcium/phosphate are degradable, so that they are not likely to impede new tissue induction *in situ* on injection. It is not clear whether the formulations used have been optimised, as it is possible to engineer the physicochemical properties of biomaterials and modulate the *in situ* retention of GFs, as was demonstrated with synthetic polymers co-injected with rhBMP-2 [38]. Efforts to engineer naturally-occurring biomaterials (such as collagen and its denatured derivative gelatin) to 'solidify' on contact with physiological milieu and sequester the injected proteins will greatly facilitate the regenerative therapies not possible with existing approaches, for example, in closed fractures and in local degeneration such as osteonecrosis.

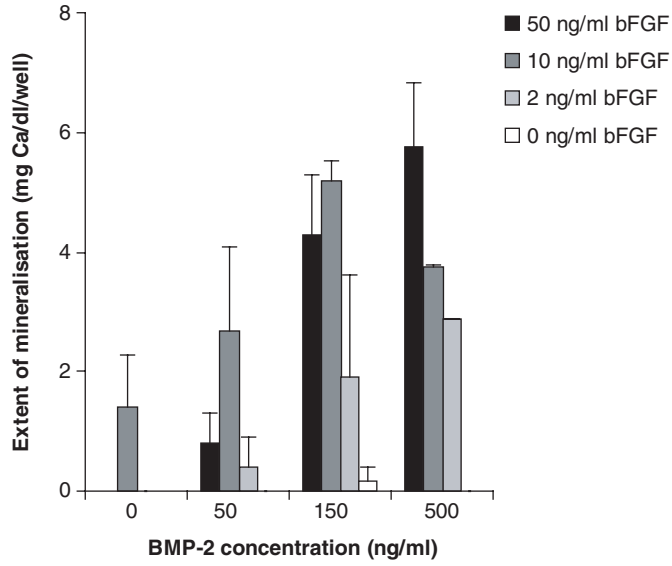

Figure 1. Effects of bFGF and BMP-2 treatment on mineralisation of BMSCs. BMSCs were harvested from ~ 32-week-old female Sprague–Dawley rats and cultured as described in HAQUE T, ULUDAG H, ZERNICKE RF, WINN SR, SEBALD W: Tissue engineering (2005) (In Press). The cells were treated for 1 week with a basal medium (DMEM + 10% fetal bovine serum) containing the indicated GF combinations (0, 50, 150 and 500 ng/ml BMP-2, each BMP-2 concentration supplemented with 0, 2, 10 and 50 ng/ml bFGF). The BMSC were then incubated in an osteogenic medium (DMEM + 10% fetal bovine serum medium supplemented with 3 mM β-glycerolphosphate + 3 × 10^{-8} nM dexamethosone) without any other GFs, and the extent of mineralisation was assessed with a colorimetric calcium assay. Note that: (1) there was increased mineralisation as the concentration of BMP-2 was increased; and (2) bFGF at very low doses (2 – 10 ng/ml) acted synergistically with BMP-2 for an enhanced mineralisation. Even in the absence of BMP-2 (i.e., 0 ng/ml concentration), bFGF was capable of stimulating mineralisation at 10 ng/ml concentration and the highest concentration of BMP-2 (500 ng/ml) required bFGF supplementation for mineralisation after 1 week.
bFGF: Basic fibroblast growth factor; BMP: Bone morphogenic protein; BMSC: Bone marrow stromal cell; GF: Growth factor.

3. Systemic delivery of growth factors

Based on the beneficial effect of GFs in local bone regeneration, systemic administration of GFs has been pursued for the augmentation of bone tissue throughout the skeletal system. Since the previous review, human parathyroid hormone (hPTH) 1-34, has been approved for osteoporosis therapy (see [39] for a review). Other GFs are continued to be explored for this indication and a summary of recent preclinical studies have been provided in Table 2. Unlike the local delivery, these studies have been conducted without a delivery system that targets the proteins specifically to bone. In this case, the proteins distribute throughout the body to establish a certain concentration in each organ, and it is expected that: a beneficial pharmacological activity arises at bone sites due to the attainment of a therapeutic concentration; and no undesired activities are induced at extraskeletal sites due to an inability to reach to a pharmacological concentration at these sites. Most of the studies explored the beneficial effect of bFGF in the female ovariectomised (OVX) rat model, which is an established animal model for type I osteoporosis.

The well-established anabolic effect of IGF-I is still being explored, even though clinical studies involving long-term IGF-I treatment in postmenopausal women whose circulating IGF-I levels were elevated to levels observed in young women confirmed its ineffectiveness in increasing bone mineral density (BMD) or strength [51]. Preclinical studies, however, are yielding additional information about the effect of delivery mode on the obtained anabolic effect. In an IGF-I-deficient MIDI mouse model, IGF-I was shown to be more effective in producing significant increases in total BMD and in femoral BMD when administered three times a day, as opposed to once-daily administration [52]. Due to the short half-life of IGF-I and its rapid clearance by the kidney, a single administration may not sustain free IGF-I levels sufficient to produce the desired pharmacological effects. Studies in mice have revealed the importance of IGF-I in regulating the number of osteoblast progenitors in bone marrow cells. A dose- and age-dependent effect of IGF-I on trabecular bone formation rate has been observed in OVX rats [53]. Investigations into time-dependent effects of daily dosing of IGF-I on the linear growth of femur in newborn mice revealed that trabecular bone volume as well as femoral growth increased with the duration of the therapy [50]. Previous experience indicated a beneficial effect of IGF-I-binding proteins (IGFBPs) when co-delivered with IGF-I, but a recent study indicated that IGFBP-5 administration alone was capable of

Table 2. Systemic administration of growth factors in a preclinical model of postmenopausal osteoporotic (ovariectomised) rodents.

Protein	Route	Dose and schedule of administration	Observations	Ref.
bFGF	i.v.	Daily injections of 200 µg/kg for 14 days	An increase in osteoblast and osteoid surfaces at haematopoietic sites and an attenuation of growth factor effect at fatty marrow sites was observed	[40]
bFGF + PTH	i.v.	Daily injections of 200 µg/kg bFGF for 14 days, followed by 5 days/week injections of PTH at 80 µg/kg	Tibial cancellous bone volume was found to be increased by 15% by the sequential treatment of bFGF and PTH. bFGF alone increased osteoid volume, osteoblast surface and osteoid surface, but not the cancellous bone volume	[41]
bFGF	s.c.	Daily injections of 1 mg/kg for 21 days	A fourfold increase in osteoblasts surface and an eightfold increase in osteoid surface was observed	[42]
bFGF	i.v.	Daily injections of 200 µg/kg for 14 days	An increase in cancellous bone volume was observed. In addition, bFGF was observed to upregulate gene expression for bone matrix proteins and IGF-I	[43]
bFGF ± oestradiol and bFGF ± hPTH	i.v.	Daily injections of 200 µg/kg bFGF for 15 days. A week after bFGF treatment, three times weekly injections of 10 and 80 µg/kg oestradiol for 4 weeks	bFGF treatment showed evidence of new trabeculae that formed osteoid bridges with pre-existing trabeculae. The trabecular connections so established could be maintained with either oestrogen or hPTH	[44]
bFGF ± hPTH	s.c.	Injections of 1 mg/kg bFGF (5 days/week) for 60 days	bFGF was observed to increase trabecular bone mass by increasing trabecular number and connectivity, whereas hPTH increased the trabecular bone mass by thickening existing trabeculae	[45]
bFGF ± oestrogen	s.c.	Daily injections of 1 mg/kg/d of bFGF and 10 µg/kg oestrogen for for 3 weeks (4 days/week)	Increase in osteoid volume, osteoblasts surface and osteoid surface was observed with bFGF treatment. Bone anabolic effect of bFGF was not enhanced by oestrogen combination therapy	[46]
bFGF	i.v.	Daily injections of 200 µg/kg for 7 or 10 days	Bone anabolic effects of the growth factor were observed to start as early as 24 h post-injection of bFGF and to increase with time	[47]
BMP-2	i.p.	1 – 5 µg/mouse/day for 20 day	BMP-2 administration increases number of proliferative activity of marrow stem cells, as well as ostaeoblastic activity *in situ*. The trabecular bone volume in both Type I and Type II osteopenic mice was also increased with systemic BMP-2. The therapeutic outcomes obtained were dependent on the species of the mice utilised, with no adverse systemic effects observed in mice	[48]
IGFBP-5	s.c.	Daily injections of 30 µg/mouse for 8 weeks	Enhancement in bone formation and bone accretion was observed, which was attributed to stimulation of osteoblast activity	[49]
IGF-I	i.p.	Daily injections of 1.21 mg/g for 6, 11 or 16 days	Femoral length and volume and the number of osteoclasts was found to be significantly greater after IGF-I injection	[50]

bFGF: Basic fibroblast growth factor; BMP: Bone morphogenic protein; hPTH: Human parathyroid hormone; IGFBP: IGF-I-binding protein.

stimulating bone deposition, presumably due to its stimulatory effect on osteoblastic activity [49].

Most recent studies have focused on the anabolic effect of bFGF. As before [1], intravenous injection of bFGF was beneficial in stimulating bone deposition, but this was recently extended to the subcutaneous route as well. This is a more acceptable delivery route for clinical application, but a much higher dose of the GF was needed for this route: 1 mg/kg [42] versus 0.2 mg/kg required for the intravenous route [40,41]. The bFGF therapy was most effective at haematopoietic marrow sites, such as the lumbar spine [40], possibly due to differences in the cellularity between this type of marrow and the fatty marrow. The beneficial effect of bFGF was believed to be initiated after the withdrawal of the treatment, but Power *et al.* [47] reported that the increase in histomorphic indices of bone formation began as early as 24 h after initial treatment and was observed to increase with time. The rapid anabolic effects of bFGF have been suggested to be due to conversion of bone-lining cells to osteoblasts, which would thereby augment the osteoprogenitor cell population [47]. Apart from its rapid skeletal effects, bFGF was capable of forming new bone within the bone marrow, as well as establishing osteoid connections between disconnected trabeculae [46]. The trabeculae developed by bFGF therapy formed osteoid bridges with the lattice, suggesting its ability to stimulate new trabeculae [44]. bFGF was observed to increase trabecular bone mass by

increasing the trabecular number and connectivity, as opposed to hPTH (1-34), which acts by thickening the trabecular walls [45]. This finding opens up the possibility of using sequential treatment paradigms involving bFGF and hPTH (1-34) for the treatment of severe osteoporosis.

Successful application of BMPs for systemic therapy has recently been reported. Turgeman *et al.* reported that rhBMP-2 at 0.5 – 5 µg/mouse i.p. daily was capable of stimulating osteoprogenitor cell proliferation in bone marrow, based on colony formation assays *in vitro*, as well as increase in trabecular bone volume in type I and II osteoporosis models [48]. The bone induction was not consistently dose dependent, but a select group of animals exhibited sufficient bone induction to restore the lost bone density in some of the utilised models, but not all. Although hPTH was more effective in enhancing the BMD under similar conditions, the feasibility of utilising BMPs systemically is an exciting prospect for systemic augmentation of skeletal integrity.

4. Adverse effects of systemic growth factor administration

Studies regarding acute toxicity from a single intravenous dose, or sub-chronic toxicity testing with a daily intravenous dose, of rhBMP-2 for 28 days, yielded negative results in rat and dog models [48,54]. The lack of systemic toxicity could presumably be attributed to rapid clearance of rhBMP-2, as observed in pharmacokinetic studies in rat and non-human primate models: $t_{½}$ = 16 and 6.7 min in rats and non-human primates, respectively [54]. Similar to rhBMP-2, no significant physiological disturbances have been reported in rats and non-human primates following intravenous delivery of OP-1 [54]. No harmful renal effects of OP-1 have been shown. Rather, it has been observed that its intravenous administration prevents the loss of renal function associated with ischaemic rat injury [55]. In mice, intraperitoneally-injected OP-1 did not give any ectopic bone induction at the injection site, and 30, 100 and 300 µg/kg OP-1 doses injected for 8 – 12 weeks did not lead to significant histopathological changes in major organs [56]. An antifibrotic effect of OP-1 in renal tissues was suggested as a possible mechanism for renal protection, but such effects of OP-1, if any, on bone tissue, have not been reported in that study. bFGF administration, however, has been recognised to cause significant undesired activities at extraskeletal sites. bFGF acts as a pleiotropic mitogen that influences the growth, differentiation and survival of a variety of cell types [57]. Adverse effects associated with intravenous treatment of rats with bFGF include anaemia, kidney and lung hypertrophy with extramedullary haematopoiesis, defective renal function [46], impaired bone mineralisation [42] and retarded weight gain [46]. Subcutaneous bFGF treatment in rats was found to induce substantial reductions in haematocrit levels and a similar impairment in bone mineralisation as observed during intravenous treatment [46]. This impairment in mineralisation has been found to resume during the bFGF withdrawal period, resulting in the calcification of the abundant osteoid formed post-bFGF treatment, and its conversion to bone [58,47]. Although the mechanism of this defective mineralisation at doses efficacious in stimulating systemic skeletal anabolic effects is not clear, the hypophosphatemia observed in OVX rats is thought to play a role [58]. As in the case of bone mineralisation, the haematocrit levels also are found to return to normal after withdrawal of the GF treatment [47,58].

5. Gene therapy for promotion of bone regeneration

In addition to parenteral administration of GFs, gene therapy approaches to induce functional gene expression are being explored for bone regeneration. This promising therapeutic modality can be accomplished by either inserting a vector containing the genetic sequence of a desired protein directly *in vivo*, referred to here as *in vivo* gene therapy, or by implanting autogenic or allogenic cells that had been transduced with a desired gene, refereed to here as *ex vivo* gene therapy (for review, see [59-61]). Although various animal models have been used to assess the viability of gene therapy for bone repair, this review will focus exclusively on rat-based models to emphasise the range of delivery approaches used and the critical delivery challenges, rather than the therapeutic outcome in different animal models. The nature of the gene carrier plays a significant role in the therapeutic outcome, not only due to its transfection efficiency, but also the response it garners from the host. The most preponderant carriers are adenovirus based, as they can be easily manipulated (with the capacity to carry 5 – 8 kb of exogenous DNA), grown to high titres, and infect both replicating and non-replicating cells [59]. In contrast, retroviral-based carriers can successfully integrate their genetic material into their host genome to yield long-term gene transduction and do not express any background retroviral genes (reducing the immunogenicity of the transduced cell), but are inefficient in infecting non-replicating cells [59]. Much like retroviruses, adeno-associated virus carriers offer long-term gene expression and do not increase the immunogenicity of transduced cells, but are capable of infecting replicating and non-replicating cells [62,63]. Non-viral carriers such as lipophilic molecules and cationic polymers, on the other hand, cause relatively fewer long-term complications (such as immunogenicity and tumorogenicity), but are generally regarded as ineffective carriers capable of providing only relatively short expression periods. The latter, however, might be an advantage when it comes to bone repair, as a transient gene expression is ideal until the normal integrity of the skeletal tissue is restored.

With the *in vivo* approach (Table 3), the major emphasis has been on the delivery of BMPs, in which administration of several BMP genes has resulted in either ectopic or orthotopic bone formation. The gene delivery approach has actually enabled assessment of therapeutic potential of some BMPs whose proteins are not readily available in large quantities, for example, BMP-9. Unlike protein delivery, delivery of BMP-2 [72,79],

Table 3. A summary of studies intended for *in vivo* gene delivery for bone induction/repair at ectopic and orthotopic sites.

Gene	Site	Carrier	Observations	Ref.
BMP2	Ectopic	AV	Whereas administration of BMP-2-expressing AV led to ectopic bone formation in athymic nude rat thigh musculature, no bone formed in immunocompetent rat	[65]
			Administration of BMP-2-expressing AV led to bone formation in immunocompromised rat calf muscle	[66]
			Co-administration of BMP-2-expressing AV with atelopeptide type I collagen not only led to induction of ectopic bone, but decreased vector immunogenicity in Wistar rats	[67]
			Transient cyclophosphamide-induced immunosuppression was required to facilitate intramuscular formation of bone by BMP-2 AV. No bone formation was observed without immunosuppresion	[68-70]
			Administration of BMP-2-expressing AV led to greater bone formation in immunocompromised (i.e., athymic) rats than normal rats at thigh muscle	[71]
			Under graft-induced ischaemic degeneration, BMP-2 overexpression led to the induction of ectopic ossification in calf muscle of immunocompetent rats. Administration of BMP-2-expressing AV (i.e., without muscle grafting), however, failed to yield bone formation	[72]
		AAV	BMP-2 AAV delivery induced endochondral bone formation in the hindlimbs of immunocompetent rats	[63,64]
		Plasmid	Transcutaneous electroporation of BMP-2-containing plasmid led to ectopic bone formation in rat	[73]
	Orthotopic	AV	BMP-2 vector increased bone deposition in models of distraction osteogenesis, and critical-sized mandibular and nasal defects	[74-76]
			BMP-2 delivery facilitated spinal fusion following injection into the paraspinal musculature of athymic rats	[77]
BMP3B	Ectopic	AV	As assessed through various radiological, histological and biochemical indices, BMP-3B expressing AV led to the intramuscular formation of bone in nude rats	[66]
BMP4	Ectopic	AV	Administration of BMP-4 expressing AV in the hindlimbs of athymic nude rats led to ectopic ossification	[62,78]
			Whereas administration of BMP-4-expressing AV led to ectopic bone formation in athymic nude rat thigh musculature, no bone formed in immunocompetent rats	[65,79,80]
		AAV	The formation of ectopic bone, via endochondral bone formation, was only observed in the hindlimb musculature of immunocompetent rats treated with BMP-4-expressing AAV	[81]
	Orthotopic	Retrovirus	Administration of BMP-4 hybrid vector to fracture periosteum enhanced repair by markedly increasing callus size in immunocompetent rats	[82]
BMP6	Ectopic	AV	Ectopic bone formation seen in the musculature of normal and athymic rats treated with BMP-6 vector	[65,78]
BMP7	Ectopic	AV	Whereas administration of BMP-7-expressing vector led to ectopic ossification in athymic nude rat thigh musculature, no bone formed in immunocompetent rat	[65]
BMP9	Ectopic	AV	Endochondral bone formation observed with the administration of BMP-9 vector in both immunocompromised (athymic) and immunocompetent rats	[79,80,83]
	Orthotopic	AV	Administration of BMP-9-expressing AV induced significant amount of bone in models of spinal arthrodesis and distraction osteogenesis in athymic rats	[75,84]
TGFB1 (*TGF-β_1*)	Orthotopic	AV	Percutaneous administration of TGF-β_1-expressing AV increased the epiphyseal calcification zone thickness in rat pups. Overexpression of TGF-β_1 led to an increase in VEGF expression	[85]
VEGF	Orthotopic	AV	Treatment of musculature surrounding femoral defect led to an increase in cartilage formation, rate of cartilage replacement with bone, and femoral bone mineral content in immunocompetent rats	[86]
PDGF	Orthotopic	AV	Collagen matrices containing PDGF-encoding AV gave enhanced cellular proliferation, and increased cementum and bone regeneration in alveolar bone defects of immunocompetent rats	[87]

AV: Adenovirus; AAV: Adeno-associated virus; BMP: Bone morphogenic protein; PDGF: Platelet-derived growth factor; TGF: Transforming growth factor; VEGF: Vascular endothelial growth factor.

BMP-4 [79], and BMP-7-expressing vectors [79] did not always result in successful bone induction. This undesired outcome may originate from poor recruitment of proliferating satellite cell and migratory phagocytes [72], or an antibody response towards the vectors, which may attenuate or even negate the desired osteogenic effect [79,80]. Experimental models suppressing the host's immune system, for example, by thymectomy or pharmacological agents (e.g., FK506 and cyclophosphamide), have been shown to improve treatment efficacy [68,79]. Using such an athymic rat model, Li and co-workers compared the volume of ectopic bone formed using a dose of 10^7 plaque-forming-units to reveal the following order of osteogenic potency: BMP-6 > -4 > -9 > -2 > -7 [79]. Although the suppression of an immune response helps to elucidate its influence on vector-mediated protein expression *in vivo*, this approach is not clinically acceptable. Thus, further preclinical animal studies using vectors that are inherently less immunogenic than adenoviral vectors (e.g., retrovirus and non-viral carriers) will be more clinically relevant. Adenoviral co-administration with a 'masking material', such as atelopeptide type I collagen [66], may also decrease the vector's inherent immunogenicity. Despite the abrogating role of the immune system, overexpression of BMP-2 [63,72,92], BMP-4 [81,82], BMP-9 [79,80,83], TGF-β [85], PDGF [87] and vascular endothelial growth factor (VEGF) [86] were shown to elicit formation of new bone tissue in various immunocompetent rat models.

Most cell types used for *ex vivo* gene therapy approach are of mesenchymal lineage, although muscle- and adipose-derived pluripotent cells have also been used [93,94]. Much like the studies on *in vivo* gene therapy, the administration of *ex vivo* modified cells, which overexpressed a variety of GFs, including BMPs and the GF-inducing intracellular proteins LIM mineralisation protein (LMP)-1, typically resulted in the formation of bone at the site of administration. In direct comparison of various carriers, Blum and co-workers noted the superiority of adenoviral-based carriers in transducing bone marrow stromal cells with the BMP-2 gene, which correlated with better regeneration in a rat osteotomy model [89]. The observed effect, however, was not exuberant, and the authors noted the importance of pre-transplant culture conditions on the obtained effect. A lack of dramatic effect was also evident at the subcutaneous site, which routinely yields *de novo* bone following the administration of ~ µg quantities of BMP-2. Highlighting the role of the immune system with this approach, Tsuchida *et al.* [92] demonstrated that BMP-2-expressing autogenic and allogenic cells both exhibited a comparable capacity to repair femoral defects in immunocompetent rats, so long as the immune system of the allograft group was transiently suppressed using FK506. That transient immunosuppression was necessary to elicit the regeneration of bone (as non-treated animals did not show signs of orthotopic bone formation) reflected the capacity of the host's immune system to negate treatment effects [92]. Although the majority of studies in Table 4 demonstrate the *ex vivo*-mediated induction of bone in immunocompetent animals, Tsuchida *et al.* reflect the need to optimise osteogenicity without compromising the host's immune system. In addition to comparing the osteogenic potency of cells transduced to express various BMPs (as in [79]), another area not yet explored is the role of scaffold on the efficiency of *ex vivo* protocols. Although most studies in Table 4 have used either collagen- or gelatin-based scaffold, it is presumed that these biomaterials are adequate, but not necessarily optimal. The importance of scaffolds has been well appreciated in the context of GF delivery for local bone repair, and it is likely that designer scaffolds may also enhance the performance of transduced cells in bone induction.

6. Expert opinion: future considerations in growth factor delivery

6.1 Local delivery

Now that the first-generation GFs have become available for clinical bone repair, the research community is expected to devote significant resources to new and improved approaches for bone repair. Based on the published literature, it is not clear whether an obvious GF candidate exists that can replace the already approved GFs for local bone repair, namely the rhBMP-2 and OP-1. Several other GFs, such as the bFGF and TGF-βs, seem to be capable of initiating an effective repair process equivalent to the exisiting GF/biomaterial combinations, although head-to-head comparisons, especially in primate models, are sparse. Most primate studies are 'proof-of-principle' studies only, as the limited number of animal subjects utilised (typically less than four/treatment group) does not allow a thorough statistical evaluation of the outcome measures.

Improvements in GF formulations, however, are more likely to yield significant advances over the exisiting therapeutics. Although minimally invasive approaches will enable convenient application of exisiting therapeutics, they are also likely to expand the scope of clinical bone repair. Injectable delivery of GFs in simple buffers is preferable in a clinical setting from a regulatory perspective, as complications, if any, related to biomaterial development are eliminated in this way. Although simple buffer injections could be effective for some GFs in small animal models, in which the healing period is relatively short (for example, rhBMP-2 in a rat fracture model [111]), co-injection with biomaterials is likely to be the reality in a clinical setting, in which the healing period will be significantly longer. Biopolymers such as gelatin, which has been successfully used in primate models, could be engineered to deliver some GFs (e.g., bFGF and TGF-β), but not others (e.g., BMP-2 and VEGF) in an injectable format in rodents [112]. A good correlation between the biopolymer residence time and the protein residence times suggested the feasibility of controlling the GF delivery rate with the co-injected carrier, again supporting the need to engineer the biomaterial carriers to control *in situ*

Table 4. A summary of studies intended for *ex vivo* gene delivery for bone induction/repair at ectopic and orthotopic sites.

Gene	Site	Vector	Cells	Scaffold	Observations	Ref.
BMP2	Ectopic	AV	BMSC	HA	BMP-2-producing BMSCs increased ectopic bone formation in the peritoneum of syngeneic rats	[88]
				Ti mesh	No significant difference in ectopic bone formation relative to unmodified BMSC controls in immunocompetent rats	[89]
		Retrovirus	BMSC	Ti mesh	30 days following subcutaneous implantation in immunocompetent rats, no significant difference in amount of bone formed relative to unmodified BMSCs	[89]
		Cationic lipids	BMSC	Ti mesh	Significantly lower frequency of ectopic bone formation with cationic lipid-modified BMSCs relative to unmodified control	[89]
	Orthotopic	AV	BMSC	Collagen sponge	BMP-2-expressing BMSCs increased bone regeneration in models of spinal fusion, and critical-sized mandibular defects	[90,91]
				Ti mesh	Significantly higher percentage of new bone formed in BMP-2-modified BMSCs relative to unmodified control. Better orthotopic regeneration than either retrovirus- or cationic lipid-modified BMSCs	[89]
				Collagen	Femoral defects repaired with both autogenic and allogenic BMP-2-expressing BMSCs in immunocompetent rats, with immunosuppressant administered to allograft group	[92]
				DBM	Application of BMP-2-expressing BMSCs enhanced bone formation and torsional strength of femoral defects in immunocompetent and nude rats	[95,96]
		Retrovirus	BMSC	Ti mesh	Retrovirus-modified BMSCs did not increase the percentage of bone in calvarial defects in immunocompetent rats relative to unmodified BMSCs	[88]
		Cationic lipids	BMSC	Ti mesh	Lipid-modified BMSCs were unsuccessful in bridging critical-sized calvarial defects in immunocompetent rats	[88]
		Liposome	BMSC	Collagen sponge	Liposome-transduced BMSC was effective in repairing critical-size mandibular defects immunocompetent rats, but not as effective as AV-mediated gene transfer	[90]
BMP4	Ectopic	Retrovirus	BMSC	Gelfoam	Implantation of BMP-4-expressing BMSCs led to ossification in immunocompetent rats	[97]
	Orthotopic	Retrovirus	Muscle-derived cells	Gelfoam	Modified cells repaired of cranial bone defects in immunocompetent rats	[98]
				Collagen sponge	BMP-4-expressing cells improved healing of femoral defects with extensive soft tissue damage in immunocompetent rats	[99,100]
			BMSC	Gelatin	BMP-4-expressing cells repaired calvarial defects in immunocompetent rats	[101]
				Collagen sponge	Although BMP-4-expressing BMSCs enhanced healing of femoral defects in Fischer 344 rats, results were inferior relative to BMP-4-modified muscle derived cells	[100]
BMP7	Orthotopic	AV	BMSC	Bone, collagen	Treatment with BMP-7-modified BMSCs enhanced posterolateral spine fusion in Lewis rats, whereas unmodified BMSCs did not	[102]
				Gelatin	BMP-7-transduced cells repaired calvarial defects in immunocompetent rats	[103]
			Fibroblasts	Collagen, gelfoam	Fibroblast-expressing BMP-7 led to the repair of femoral defects via endochondral bone formation in immunocompetent rats	[104]

AV: Adenovirus; BMP: Bone morphogenic protein; BMSC: Bone marrow stromal cell; HA: Hydroxyapatite; LMP: LIM mineralisation protein; Ti: Titanium.

Table 4. A summary of studies intended for *ex vivo* gene delivery for bone induction/repair at ectopic and orthotopic sites (continued).

Gene	Site	Vector	Cells	Scaffold	Observations	Ref.
				Gelatin	Regardless of irradiation treatment, critical-size calvarial defects were repaired with BMP-7-expressing cells in immunocompetent rats, unlike autologous bone graft	[105]
					Application of BMP-7-expressing cells led to repair of alveolar defects, but noggin overexpression inhibited osteogenesis in immunocompetent rats	[106]
BMP9	Ectopic	AV	BMSC	None	BMP-9-expressing BMSCs formed ectopic bone in thigh musculature of athymic rats	[107]
	Orthotopic	AV	BMSC	None	Spinal fusion was achieved with BMSCs expressing BMP-9 in athymic rats	[108]
LMP1	Orthotopic	Plasmid, AV	BMSC	Devitalised bone	Spinal fusion was achieved in all athymic rats treated with modified BMSCs, whereas no bone formation observed with non-transduced BMSCs. Similar results were obtained with LMP-1-transduced BMSCs in a posteroelateral fusion model in rabbits	[109,110]

AV: Adenovirus; BMP: Bone morphogenic protein; BMSC: Bone marrow stromal cell; HA: Hydroxyapatite; LMP: LIM mineralisation protein; Ti: Titanium.

levels of the proteins. This goal was also recently pursued with a synthetic terpolymer, poly-D,L-lactic acid-*p*-dioxanone-poly ethylene glycol, whose degradation rate was controlled to induce orthotopic repair in rats [113]. In addition to protein retention that determines local concentration of the proteins [32], recent studies of Maeda *et al*. also indicated the need for gradual release of the proteins *in situ* [114]. Using several collagen 'minipellet' formulations, these investigators noted the importance of both the initial burst and the subsequent sustained release in the extent of *de novo* bone formed subcutaneously [114]. Given an equivalent implant dose, fast-releasing formulations were found to result in better bone formation. Equally important, simple additives such as amino acids, glycerol and sucrose, were effective in modulating the BMP-2 interactions with collagen, and controlling the *in vivo* release rate. Such biomolecules are more readily acceptable for clinical formulations as compared with new biomaterials whose safety profile requires a more thorough assessment. Formulating the GF delivery systems in a minipellet format might also be advantageous over the one-piece scaffolds, such as collagen sponges, which might restrict cell migration especially to the central region of an implant.

In contrast to diffusible delivery systems, GFs immobilised in proteolytically-sensitive scaffolds could provide an 'on-demand-release-system' whose GF content is released *in situ* as a result of the proteolytic activity of extracellular proteins and cells [115]. Using a rat calvarial osteotomy model, matrix metalloprotease-sensitive synthetic hydrogels were shown to be supportive of BMP-2-induced bone formation [115]. The animal model utilised in this study was a rodent model, which gave a robust bone induction even with a collagen/rhBMP-2 implant of equivalent rhBMP-2 dose. It is likely that the beneficial effect of such a controlled release system is more readily manifested in larger animal models, in which implantation dose and burst release become more significant as the bone healing process is prolonged.

6.2 Systemic delivery

It is likely that no new GF will be available for systemic therapy in the near future, after the approval of hPTH (a possible exception might be novel analogues of hPTH with improved therapeutic index). A critical need in systemic therapy is the drug delivery systems that specifically target the systemically administered proteins to bones. Towards this goal, model proteins (albumin and lysozyme) were derivatised with bone-seeking bisphosphonates, and the bisphosphonate–protein conjugates were found to give up to sevenfold increased bone deposition of the proteins depending on the bone site, post-injection time and the route of administration [116]. The protein-targeting efficiency, defined as the ratio of 'BP–protein delivery to a bone site' to 'delivery of native proteins to the same site', was increased as a function of post-injection time after a single administration of the proteins in rats [116]. This was indicative of not only better initial targeting, but also better retention of bisphosphonate (BP) conjugates at bone sites after the initial protein clearance. The imparted bone affinity was dependent on the conjugation chemistry [117], and the tether length between a protein and bisphosphonate [118]. Approaches to link multiple BP to a single protein attachment site also yield bone-seeking proteins with a lesser degree of modification [119]. Collectively, a foundation for targeting proteins to bone is being established, and efforts to apply this bone-targeting approach to GF delivery to bone are underway. The critical issue in this respect is the need to modify the GFs chemically, without losing their pharmacological activity. Modifying proteins without a loss of activity is feasible, such as biotination of bFGF and BMP-2 [1], but whether the same observation holds true for bisphosphonate modification remains to be seen.

In the absence of a bone-specific drug delivery approach, the clinical utility of GFs for systemic therapy will rely on minimising their undesired activities at extraskeletal sites. The extraskeletal rhBMPs (rhBMP-2 and -7 in particular) appear to be tolerated to some extent in rodent models, but more thorough studies will be required in larger animals to evaluate their probable side effects. The undesirable effects of GFs were generally reversible after the cessation of GF therapy, so that a good control of the administration dose might allow one to strike the right balance between an effective stimulation of bone formation at skeletal sites and undesirable activities at extraskeletal sites. It is likely that the local concentration of a GF is the primary determinant of the obtained pharmacological activities. If so, pharmacokinetics and biodistribution studies can shed light on GF concentrations in various organs as a function of the dose and frequency of the administered therapeutics. Instead of the efficacy studies, it will be more productive to determine the skeletal/non-skeletal ratio of the GFs first, and then utilise the optimal administration regimen (i.e., where skeletal/non-skeletal delivery of GFs is maximised) for the efficacy studies. The potential of GF candidates for systemic therapy is more realistically assessed in this way.

6.3 Gene therapy

Perhaps the most significant activity in recent years has been the concerted efforts to deliver osteogenic genes for bone repair, rather than proteins. As evident in Table 3 and Table 4, several types of delivery systems were utilised for this purpose, but the desired outcomes were not always secured in preclinical models. Viral carriers have been the major choice in these preclinical studies, but immunological complications of viral carriers and possible long-term complications (which are difficult to assess in rodent studies) may hamper final application of the designed systems in a clinical setting. Non-viral systems are advantageous in this respect, but significant efforts are needed to further engineer these systems to make them as effective as viral carriers. Specific information on: the duration of transgene expression *in vivo*, hence the induced pharmacological activity; the development of ectopic bone formation; and the capacity to target specific cells populations, are critical issues that need to be addressed [57,61]. Immunological issues associated with viral carriers have generally been considered to be the underlying basis for inconsistencies in achieving bone repair, but a critical issue not thoroughly explored is the correlation between *in situ* protein levels and the success of bone repair. Ample information on this correlation is available for numerous preclinical models in the case of protein delivery, and it is clear that a threshold dose needs to be administered for the initiation of the repair process. This needs to be explored with the gene therapy approaches, as well as by specifically determining *in situ* levels of the overexpressed proteins (i.e., not solely evaluating qualitative gene expression at the repair site). The expression of exogenous genes typically peak within the first days of therapy, and their expression lasts for a few weeks in animal models [87,120]. Changes in the *in situ* protein levels are likely to depend on individual proteins, which is difficult to guess *a priori*. Nevertheless, protein levels are also likely to peak at the early days of gene delivery and gradually reduce to baseline levels over time. Such a protein profile is not unlike the well-studied protein therapy, in which one-time application of a protein therapeutic results in local build-up of the therapeutic agent that is gradually lost from the repair site. In this respect, gene and protein therapies should exert similar pharmacodynamic effects in bone repair.

An important issue in the gene delivery approach will be assessing the relative merit of delivering the GF genes themselves, which typically act on cell-surface proteins (such as BMPs), compared with intracellular proteins, which mediate GF actions without the need to act on extracellular proteins (such as LMP-1). As the latter does not rely on extracellular secretion to exert its effects, it might provide a faster mechanism for bone repair. However, its efficacy might be limited by the transfection efficiency of the cells, as non-transfected cells will be immune to exogenous gene expressed in the neighbouring cells.

Finally, it must be stated that a major advantage of gene therapy is the elimination of large-scale protein production. Developing viral carriers, however, as is the case for biomaterial carriers, will require considerable development time. Considering that the protein delivery approach has gained momentum since the late 1980s with the cloning of BMPs; and that clinical devices are now beginning to be available, gene delivery is likely to remain as a potentially useful approach for orthopaedic indications for the next 5 – 10 years. As with protein delivery, the potential of gene therapy will be fully realised once studies on non-human primates are conducted.

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