

Growth Factor Interactions in Bone Regeneration

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Bone regeneration is a complex process regulated by a large number of bioactive molecules. Many growth factors and cytokines involved in the natural process of bone healing have been identified and tested as potential therapeutic candidates to enhance the regeneration process. Although many of these studies show an enhancement of the bone regeneration process by a single drug therapy, *in vivo* bone regeneration is the result of a complex interplay between the applied growth factor and various endogenous produced growth factors. To investigate these growth factor interactions, various studies have investigated the effect of growth factor combinations on bone regeneration. This review provides an overview of the growth factor and cytokine combinations tested in translational bone regeneration studies and shows that their interaction may result in an enhancement or inhibition of bone formation.

Introduction

BONE IS, AFTER BLOOD, the most commonly transplanted tissue. Worldwide, an estimated 2.2 million grafting procedures are performed annually to repair bone defects in orthopedics, neurosurgery, and dentistry.¹ Given the demographic challenges of a growing and aging population, this number is expected to increase. The increasing number of grafting procedures and the disadvantages of current autograft treatments (e.g., limited graft quantity and donor-site morbidity) and allograft treatments (e.g., reduced rate of graft incorporation and risk of disease transmission) drive the quest for alternative methods to treat large bone defects.²⁻⁶

The ability of devitalized, demineralized bone to induce ectopic bone formation and the recognition that proteins were responsible for this bone induction launched the promising strategy of bone tissue engineering based on bioactive molecules.^{7,8} Since then, various proteins with an important role in this autoinductive process were isolated and investigated for their therapeutic potential in bone regeneration, including bone morphogenetic proteins (BMPs), transforming growth factor-beta (TGF- β), fibroblast growth factor (FGF), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), epidermal growth factor, parathyroid hormone (related protein) (PTH/PTHrP), and interleukins (IL). Although many studies show an enhancement of the regeneration process by applying one of these proteins, the

exact molecular coordination of the bone regeneration process has not been fully defined. Moreover, as various growth factors and cytokines are involved simultaneously in natural bone healing, the combined addition of several factors in specific temporal fashion is likely to be more effective in exogenous stimulation of healing.

Normal bone regeneration (e.g., during fracture healing) is a complex process that involves a large number of growth factors and cytokines for its regulation. No matter how influential one factor may appear in the process, its action in isolation may have little effect without interaction with endogenously produced growth factors and cytokines. Therefore, determination of growth factor interactions is essential to understand and/or control the process of bone regeneration. This review provides an overview of the combinations of growth factors that have been investigated in translational bone regeneration studies. Since most research focuses on combinations with BMPs due to their potent osteoinductive capacity, these growth factors will play a central role in this review.

BMPs and Their Combinations

Over 20 members of the BMP family have been identified and at least 7 of them have documented osteoinductive capacities.⁹ The biologically active form of BMPs consist of 30–38 kDa proteins composed of two disulfide-linked polypeptide subunits. On the basis of their amino acid sequence homology, the osteoinductive BMPs have been divided into

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separate subgroups, which include the BMP-2/-4 group and the osteogenic protein-1 or OP-1 group (BMP-5 to -8).¹⁰⁻¹² Most research in the field of bone regeneration focuses on the commercially available BMP homodimers. However, BMP members can also form heterodimers consisting of, for example, one BMP-2 and one BMP-7 subunit and displaying much higher affinities for BMP receptors than homodimers.¹³ Given the number of BMP genes, the possible number of combinations is large. So far, native BMP-heterodimers have not been identified *in vivo* and their separation from homodimers in experimental studies remains difficult because of the biochemical similarity between BMPs.^{11,14}

Although BMPs are involved in numerous developmental and pathophysiological processes, their effects on bone formation have been studied most extensively. In bone, they are synthesized by skeletal cells such as osteoblasts and sequestered in the extracellular bone matrix.^{7,15,16} *In vitro*, BMPs can differentiate mesenchymal stem cells into the osteoblastic phenotype.^{17,18} When implanted ectopically, the osteoinductive BMPs can initiate the complete cascade of bone formation, including the migration of mesenchymal stem cells and their differentiation into osteoblasts.¹¹ This bone induction occurs through endochondral as well as intramembranous ossification and results in the formation of normal woven and/or lamellar bone.^{11,19}

In contrast to the numerous studies in which the effect of separate BMP homodimers was investigated, few studies focused on the combined administration of BMP family members (Table 1).²⁰⁻²⁵ So far, none of the studies using BMP homodimers has been able to show an enhanced effect of the combinations.²³⁻²⁵ Neither the *in vitro* capacity to induce osteogenic cell differentiation nor the *in vivo* capacity to induce ectopic bone showed an effect of administration of homodimer mixtures compared to an equal dose of one of the BMP homodimers separately. In contrast to the homodimer mixtures, several recombinant BMP heterodimers (particularly BMP-2/-6, BMP-2/-7, and BMP-4/-7) were more potent *in vitro* and *in vivo* compared to a corresponding

amount of their homodimers.^{24,25} This increase in osteoinductive activity was even 20-fold in case of BMP-2/-7. Also, transduction studies using nonviral or adenoviral osteoinductive BMP vectors showed more efficient induction of osteoblast differentiation and spinal fusion.^{18,21,26-28}

A possible explanation for the different effects between homodimers and heterodimers may be found in the BMP signaling pathway (summarized in Refs.²⁹⁻³¹). The BMP signaling pathway converges at the receptor and intracellular signaling level. BMPs bind to two different types of serine/threonine kinase receptors, known as type I and type II receptors (Fig. 1). The type I receptors are subdivided into three different activin receptor-like kinases (ALK), known as ALK-2, ALK-3, and ALK-6. Of these type I receptors, ALK-3 and ALK-6 have a high affinity for BMP-2/-4 group and a low affinity for the OP-1 group,^{32,33} whereas ALK-2 preferentially binds the OP-1 group.³³⁻³⁵ In contrast to the type I receptors, the three type II receptors (BMPRII, ActRIIA, and ActRIIB) appear to bind most BMPs. Upon BMP binding, the receptors form multimers, most likely a tetramer consisting of two pairs of type I and type II receptors.³⁶ This allows the activation of the type I receptor and subsequent intracellular signaling. The combination of ALK-3 or ALK-6 with ALK-2 type I receptor signaling was shown to be more potent in inducing transcriptional activation than signaling by either receptor alone.¹² Binding of heterodimers may be more likely to induce such a combined receptor activation than homodimers. Another explanation for the enhanced activity of heterodimers may lie in differential Noggin antagonism of BMP action, which was shown to be decreased for heterodimers compared to homodimer signaling.³⁷

Transforming Growth Factor-Beta

TGF- β s are multifunctional growth factors with a broad range of biological activities in various cell types in many different tissues. Three isoforms of TGF- β have been found in humans (TGF- β 1 through - β 3), which all consist of 25 kDa

TABLE 1. COMBINATIONS OF BONE MORPHOGENETIC PROTEINS

Growth factors	Form (dose)	Setting	Outcome parameters	Effect	Ref.
BMP-2/BMP-7	Homodimers (100 μ g/100 μ g)	Tooth extraction defect in primates	Bone area	n.s. ^a	23
BMP-4/BMP-7	Hetero- and homodimers	Critical-sized femoral defect in rats	Bone volume Mechanical strength	Homodimers n.s. ^a Heterodimers synergistic ^b	25
Various BMP combinations	Hetero- and homodimers	Intramuscular implants in mice/rats	Bone volume	Homodimers n.s. ^a Heterodimers synergistic ^b	24
BMP-2/BMP-7	Gene therapy	Critical-sized calvarial defect in mice	Bone volume	Additive effect ^c	26
BMP-2/BMP-7	Gene therapy	Subcutaneous implants in mice	AP activity Calcium content Phosphate content	Synergistic ^c	27
BMP-2/BMP-7	Gene therapy	Posterolateral spinal fusion model in rats	Bone volume AP activity Osteocalcin	Synergistic ^c	21

^aCombination of homodimers compared each homodimer alone.

^bHeterodimers compared to homodimers.

^cAdenovirus vectors encoding BMP-2 (AdBMP-2) and BMP-7 (AdBMP-7) compared to each vector alone
AP, alkaline phosphatase; BMP, bone morphogenetic protein; n.s., not significant.

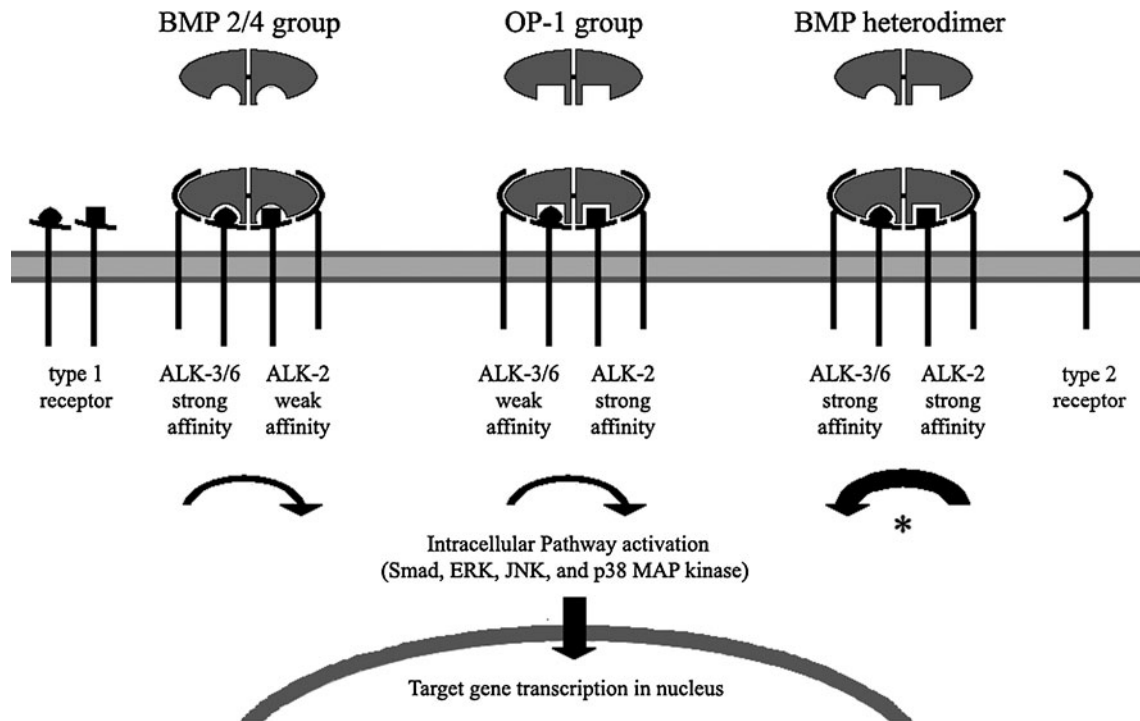


FIG. 1. Signal transduction via BMP receptors. Upon BMP binding, the receptors most likely form tetramers consisting of two pairs of type I and type II receptors that subsequently activate the intracellular signaling pathways. Since the three type I receptors (ALK-2, ALK-3, and ALK-6) have different affinity for the various BMP molecules, the potency of the intracellular signaling activation varies between the different BMP-receptor complexes. The combination of ALK-3 or ALK-6 with ALK-2 type I receptor signaling was shown to be more potent in inducing transcriptional activation than signaling by either receptor alone (*). ALK, activin receptor-like kinase; BMP, bone morphogenetic protein.

homodimers sharing 60%–80% similarity in their amino acid sequence. TGF- β is synthesized by many different cell types and is stored as an inactive complex with latency-associated peptide in the extracellular bone matrix.³⁸ Another major source of this factor are platelets in the blood clot formed after a fracture. Release of the protein from the complex and subsequent induction of its bioactivity is tightly regulated and can occur through different mechanisms.³⁹ In general, TGF- β stimulates migration of osteoprogenitor cells and is a potent regulator of cell proliferation, cell differentiation, and extracellular matrix synthesis.^{40,41} Its use for bone regeneration has been evaluated in various experimental settings that show both stimulatory and inhibitory effects on bone formation. Overall, stimulatory effects on bone healing and bone formation predominate.⁴⁰ Although no osteoinductive capacity is reported in rodent studies, intramuscular bone induction was found in a TGF- β -loaded collagenous matrix in primates.^{42,43}

In several studies attempts were made to enhance bone regeneration by combining TGF- β and BMPs (Table 2).^{42–50} To investigate a possible interaction between TGF- β and BMP during bone induction, the combination was always compared to similar dosages of each of the individual growth factor. Despite the differences in experimental settings, isoforms used and delivery vehicles, most studies showed an additive or synergistic effect of the combination. Ectopic implantation studies in primates showed that TGF- β enhanced BMP-7-induced bone formation in a dose-responsive manner.^{42,43} The largest synergistic effect was

seen after coadministration of low TGF and BMP dosages combined with culture-expanded bone marrow stromal cells in RGD-modified alginate hydrogels.⁴⁹

Although the exact molecular actions of the growth factor combinations are not fully understood, several effects have been proposed by which the different isoforms of TGF- β synergize with BMPs. In the early phase after implantation, both growth factors can directly increase the local pool of osteoprogenitor cells by stimulating their migration.⁴⁰ Since the circulation is one of the sources of osteoprogenitor cells during ectopic BMP-induced bone regeneration,⁵¹ cell recruitment may also be indirectly enhanced by their combined effect on angiogenesis. It has been suggested that TGF- β 1 and BMP-7 synergistically interact to enhance angiogenesis and vascular invasion since their coadministration increased vessel formation in a chick chorioallantoic membrane assay and type IV collagen expression in ectopic ossicles in primates.^{42,52} The mitogenic effect of TGF- β may further increase the osteoprogenitor cell pool by stimulating cell proliferation. *In vitro*, this mitogenic effect did not modify the osteoinductive responsiveness of the cells to BMP.⁵³

Apart from their combined effects on angiogenesis, cell recruitment and proliferation, TGF- β and BMPs also interact during osteoblast differentiation (extensively reviewed by Janssens *et al.*⁴⁰). Despite the conflicting results of numerous *in vitro* experiments, it seems that TGF- β 1 may have stimulatory effects on osteoblast differentiation during the early stage and an inhibitory effect on differentiation and mineralization at later stages. This altered biological effect would

TABLE 2. COMBINATIONS WITH TRANSFORMING GROWTH FACTOR-BETA

Growth factor dose	Setting	Outcome parameters	Effect ^a	Ref.
15 ng TGF- β ^b 2 mg BMP-2	Intramuscular implants in mice	Bone area	Synergistic	48
0.5–15 μ g TGF- β 1 20–100 μ g BMP-7	Intramuscular implants in primates	Bone area AP activity	Synergistic	43
0.5–100 μ g TGF- β 1 20–100 μ g BMP-7	Intramuscular and calvarial defect implants in primates	Bone area AP activity Collagen expression	Synergistic	42
10 ng TGF- β 1 15 mg bBMP ^c	3-mm segmental radial defect in dogs	Bone area	Additive	46
140 ng TGF- β 2 25–400 ng bBMP ^c /mg implant	Subcutaneous implants in rats	Bone (semiquantitative) AP activity	Synergistic	44,45
12 μ g TGF- β 2 25 μ g BMP-2	Titanium implant model in dogs	Implant fixation Bone area	Additive (implant fixation) Nonsignificant (bone area)	50
20 ng TGF- β 3 200 ng BMP-2	Subcutaneous implants in mice	Bone area	Synergistic	49
0.2 μ g TGF- β 3 2 μ g BMP-2	Critical-sized femoral defect in rats	Bone volume Mechanical strength	Nonsignificant	47

^aEffect of combination of growth factors compared to TGF- β and BMP alone.

^bTGF- β extracted from platelets by acid/ethanol procedure and chromatography.

^cBMP extracted and purified from bovine bone.

TGF- β , transforming growth factor-beta.

seem to be the result of crosstalk between the intracellular signaling pathways of BMP-2 and TGF- β 1. Although TGF- β 1 seems to inhibit matrix mineralization at later stages, the *in vivo* consequences of these inhibitory effects might be limited since expression of TGF- β receptors is downregulated when cell differentiation progresses. This results in a decreased responsiveness to TGF- β 1 at later differentiation stages.

Fibroblast Growth Factor

FGFs are considered potent regulators of cell growth and wound healing. The FGF family consists of 25 members, which range in size from 17 to 34 kDa, share a 16%–65% similarity in amino acid sequence, and fall into different subgroups.⁵⁴ In bone, they are produced by various cells, including osteoblasts, macrophages, and endothelial cells, and are stored in their active form in the extracellular bone matrix. When released or secreted, FGFs act in an autocrine and paracrine way as a mitogen on many cell types. In addition to their mitogenic effects, FGFs are involved in a number of other cellular processes, including angiogenesis, wound healing, and cell differentiation. During fracture healing, distinct groups of FGFs are differentially expressed, suggesting an active stage-specific role for FGF signaling during the repair process.⁵⁵ In the early phase, FGF-1, -2, and -5 genes are expressed, which is associated with a rapid increase in local cell population.^{55,56} At later stages, expression of other FGFs is upregulated, suggesting that these growth factors are involved in the regulation of chondrogenesis and osteogenesis.⁵⁵ The best studied family member on bone regeneration is FGF-2 (also known as bFGF). Although FGF-2 alone is not capable of inducing ectopic bone formation, it plays an important role in the regulation of normal bone healing.⁵⁷ Exogenous FGF-2 enhances callus

formation and stimulates bone healing in various orthopedic applications.^{58–64}

In various studies FGF-2 or FGF-4 was combined with BMPs in an attempt to enhance bone regeneration and it was shown that the effects of FGF were time and dose dependent (Table 3). Using a local BMP-2 delivery vehicle in combination with subcutaneous FGF-4 injections for 3 consecutive days, FGF administration at an early stage after implantation (days 2–4) increased the amount of newly formed bone, whereas its administration at later time points (days 6–8 or 9–11) had no effect.⁶⁵ When implanted in a delivery vehicle, low FGF dosages in combination with a single BMP dose synergistically enhanced BMP-induced bone formation; however, bone formation was inhibited by coadministration of high FGF dosages.^{66–69} The shift from an enhanced to an inhibitory effect occurred between ~16 and 2000 ng FGF per delivery vehicle.

So far, little is known about the mechanism underlying the dose- and time-dependent FGF effects. The time-dependent effect may suggest that FGF plays an important role in cell recruitment and expansion during the early phase of bone regeneration. This is also suggested *in vitro*, where successive exposure of mesenchymal stem cells to FGF followed by BMP was equally successful in inducing calcium deposition compared to combined growth factor exposure during the same period.^{70,71} *In vivo*, the combined angiogenic and migration effects of FGF could indirectly or directly enhance the recruitment of mesenchymal stem cells to the implantation site.⁵² Further, the mitogenic effect of FGF likely stimulates the proliferation of the local osteoprogenitor cells. BMPs can subsequently commit the enlarged osteoprogenitor cell pool toward the osteoblastic lineage, which ultimately results in the enhanced local bone formation.

Interestingly, *in vitro*, FGF-2/-9 were shown to stimulate mesenchymal stem cell proliferation and reversibly inhibit their differentiation in a dose-dependent manner.^{71–73} An inhibitory effect on the production of differentiation markers

TABLE 3. COMBINATIONS WITH FIBROBLAST GROWTH FACTOR

Growth factor dose	Setting	Outcome parameters	Effect ^a	Ref.
10 ng FGF-2 1 µg BMP-2	Onlay of implants on rabbit cranium	Bone area	Additive at 3 weeks Synergistic at 6 and 9 weeks	166
0.8 µg FGF-2 0.1 µg BMP-2	Subcutaneous implants in rats	AP activity Calcium content Collagen content	Synergistic on AP activity and calcium content, inhibitory on collagen content at 2 weeks, nonsignificant at later times	167
Cells exposed to 2.5/50 ng/mL FGF-2/BMP-2 for 6 days	Implants with seeded cells subcutaneous in rats	Semiquantitative histologic score	Synergistic	70
0.1 µg FGF-2 0.6 µg BMP-2	Bone chamber model in rabbits	Bone area Bone ingrowth	Inhibitory	168
0.016–50 µg FGF-2 2 µg BMP-2	Intramuscular implants in rats	Bone area AP activity	Synergistic at a dose ≤400 ng inhibitory at a dose ≥2 µg	66
0.001–5 µg FGF-2 5 µg BMP-2	Intramuscular implants in mice	BMD Bone area	Synergistic at a dose ≤10 ng inhibitory at a dose ≥100 ng	68
0.016–5 µg FGF-2 2 µg BMP-2	Intramuscular implants in rats	BMD, BMC Bone area AP activity	Synergistic at a dose ≤16 ng inhibitory at a dose ≥80 ng	67
0.2 mg FGF-2 1 mg BMP-2	Femoral osteotomy in rabbits	Bone area	Additive compared to BMP-2 alone ^b	84
12.5 µg FGF-2 5 µg BMP-2	Implants seeded with cells and cultured for 1 week placed in rabbit spinal fusion model	Fusion determined by radiographs and palpation	Additive	169
0.1 mg FGF-2/kg/day for 3 days [BMP] unknown	Subcutaneous BMP implants in rats and three FGF injections	Bone density	Synergistic with FGF administration at days 2–4	65

^aEffect of combination of growth factors compared to FGF and BMP alone.

^bControl group FGF alone was missing.

FGF, fibroblast growth factor; BMD, bone mineral density; BMC, bone mineral contents.

like alkaline phosphatase activity and induction of mineralization were also seen in cultures containing FGF-2/-9 in combination with BMP-2 in a 1:5 ratio or with osteogenic medium.^{71,73,74} However, in cultures containing FGF-2 and BMP-2 at a ratio of 1:10 or higher, the combination had a stimulatory effect on the bone differentiation markers compared to BMP alone.⁷⁰ These studies suggest a ratio-dependent FGF/BMP effect in which the mitogenic FGF stimulus overrules the BMP-induced osteogenic differentiation at high FGF concentrations. *In vivo*, the excessive amount of FGF at the time of BMP release might have inhibited bone induction.

In an attempt to further elucidate the mechanism behind the dose-related FGF effect, expression of genes associated with BMP signaling was studied in cells surrounding ectopically implanted FGF-2/BMP-2 delivery vehicles.⁶⁸ Whereas BMP-2 alone induced an upregulation of ALK-3 and BMPRII receptors, exposure to BMP-2 with FGF-2 resulted in an additional upregulation of ALK-6 receptor expression.⁶⁸ *In vitro*, this FGF-enhanced ALK-6 expression augments BMP-induced Smad signaling and expression of alkaline phosphatase activity.⁷⁵ Further, FGF-2 stimulated expression of Smad 6 (an inhibitory Smad) in a dose-dependent manner, which may explain the inhibition of BMP-2-mediated differentiation at higher FGF concentrations. Although it is not known whether these changes in gene expression profiles are responsible for the *in vivo* physiological response, they clearly show a dose-dependent crosstalk between the FGF and BMP signaling pathways.

Insulin-Like Growth Factor

IGF-I and IGF-II are small single-chain polypeptides of ~7.5 kDa that play an important role in bone metabolism and are essential to skeletal growth and maintenance of bone mass. They are synthesized by multiple tissues and elicit their effects in an endocrine, paracrine, and autocrine way. The majority of IGFs exist in complexes, bound to one of the IGF-binding proteins that modulate their biological actions in a cell-specific manner.⁷⁶ IGFs are the most abundant growth factors produced by bone cells and are stored at the highest concentration of all growth factors in the bone matrix. Although there is still debate about its exact role in bone cell proliferation and differentiation, IGF has an anti-apoptotic effect on (pre)osteoblasts and enhances bone matrix synthesis.⁷⁷ *In vivo*, systemic IGF infusion showed an increase in bone formation, bone volume, and/or bone turnover in animal models and clinical trials for osteoporosis.⁷⁸ However, major drawbacks of systemic-free IGF administration are its side effects such as hypoglycemia, intracranial hypertension, headache, fatigue, and dyspnea.⁷⁹ Consequently, localized IGF delivery was explored and showed to enhance bone formation without increasing circulating IGF-I levels.^{80,81} Although insulin can bind to the IGF receptor as well, higher concentrations will be required due to its lower affinity. Since this may cause hypoglycemia, insulin will probably never be considered as a therapeutic agent for bone regeneration.

TABLE 4. COMBINATIONS WITH INSULIN-LIKE GROWTH FACTOR

Growth factors	Setting	Outcome parameters	Effect ^a	Ref.
0.25 mg IGF-1 1 mg BMP-2	Femoral osteotomy in rabbits	Bone area	Additive compared to BMP-2 alone ^b	84
10 ng IGF-I 25 ng TGF- β	Solution with growth factors in a marginal femoral defect in rats	Biomechanical test	Nonsignificant	85
50 μ g IGF-I 10 μ g TGF- β 1	Coated titanium implants in a midshaft tibia fracture in rats	Biomechanical test Callus formation	Additive/synergistic	88
25 ng IGF-1 0.1 μ g TGF- β 1	Gelatin hydrogel in a segmental tibia defect in rats	Bone formation (X-ray)	Nonsignificant	86
25 ng IGF-1 0.1 μ g TGF- β 1	Gelatin hydrogel in a marginal mandibular defect in rats	Bone formation (X-ray)	Nonsignificant	87

^aEffect of combination of growth factors compared to IGF and BMP alone.

^bControl group IGF alone was missing.
IGF, insulin-like growth factor.

A limited number of studies have investigated the effect of combined IGF-I and BMP addition on *in vitro* and *in vivo* osteogenesis. *In vitro*, sequential exposure to BMP-2 followed by IGF-I led to synergistically enhanced mesenchymal cell proliferation and alkaline phosphatase activity.⁸² Similar effects were seen after exogenous BMP-7 and IGF-I administration or cotransfection of both genes in fetal calvarial cell cultures.⁸³ Both studies suggest that IGF-I enhances the mitogenic action as well as the differentiation activity of BMPs. In an *in vivo* bone-implant integration model, combined delivery of IGF-I and BMP-2 from coated titanium screws significantly improved bone formation compared to BMP-2 alone (Table 4).⁸⁴ Thus, the IGF/BMP combination seems to enhance both *in vitro* and *in vivo* osteogenesis.

The combination of IGF and TGF- β has been studied more extensively. Unfortunately, few studies compared the growth factor combination versus single IGF and TGF- β controls and their experimental setup varied greatly, in particular with respect to dose and delivery vehicle (Table 4).⁸⁵⁻⁸⁸ Compared to TGF- β alone, nanogram dosages of the IGF/TGF- β combination in solution or released from gelatin hydrogels did not result in significantly enhanced mechanical properties or bone formation in rat marginal

cortical defects, tibial defects, and mandibular defects, but the release of micrograms of the growth factor combination from a coated titanium implant enhanced the mechanical properties and callus formation in a rat tibial fracture model (Table 4).

Although only the latter *in vivo* study showed enhanced effects of the IGF/TGF- β combination over TGF- β alone, *in vitro* studies clearly suggest an interaction of both growth factors in regulation of bone formation. Synthesis of IGFs and IGF-binding proteins by osteoblasts is regulated by various growth factors, including TGF- β .^{89,90} Further, exogenously administered combinations of IGF-I and TGF- β synergistically enhanced cell proliferation and matrix synthesis in osteoblast cultures.⁹¹⁻⁹⁴ A similar synergistic effect of IGF-I and FGF was seen on cell proliferation and matrix synthesis in osteoblast cultures.^{91,92} Unfortunately, no studies have been performed to investigate the effect of an IGF/FGF combination on *in vivo* bone regeneration.

Vascular Endothelial Growth Factor

VEGF is considered one of the key regulators of angiogenesis during bone formation.⁹⁵ The biologically active

TABLE 5. COMBINATIONS WITH VASCULAR ENDOTHELIAL GROWTH FACTOR

Growth factor dose	Setting	Outcome parameters	Effect ^a	Ref.
Cells expressing VEGF or BMP-2	IM and CD in mice	Bone area	Synergistic (IM) Additive (CD)	101
Cells expressing VEGF or BMP-2	IM in mice	Bone area	Synergistic at 4 weeks Nonsignificant at 8 weeks	108
2 μ g VEGF 6.6 μ g BMP-2	SC and critical-sized FD in rats	Bone volume	Additive (SC) Nonsignificant (FD)	110
12 μ g VEGF 2 μ g BMP-2	Cranial defect in rats	Bone volume	Synergistic at 4 weeks Nonsignificant at 12 weeks	111
Cells expressing VEGF or BMP-4	IM and CD in mice	Bone area	Synergistic (IM) Additive (CD)	107
3 μ g VEGF BMP-4 plasmid	SC implants in mice	Bone area Bone mineral density Bone mineral content	Additive	109

^aEffect of combination of growth factors compared to VEGF and BMP alone.

CD, calvarial defect; FD, femoral defect; IM, intramuscular implants; SC, subcutaneous; VEGF, vascular endothelial growth factor.

form of VEGF, also referred to as VEGF-A, is a dimeric protein that may consist of different splice variants (121–206 amino-acids) of a single gene. Most cell types produce several VEGF forms simultaneously and their expression can be enhanced by hypoxia or other cytokines. *In vivo*, VEGF induces angiogenesis as well as permeabilization of capillaries.^{96,97} During bone repair, these newly formed vessels and vascular changes are crucial for nutrient supply, transport of macromolecules, and invading cells. The important role of VEGF during bone regeneration has been shown in various experimental models, demonstrating stimulation or disruption of the normal bone regeneration process in response to VEGF administration or inhibition, respectively.^{98–101}

Several *in vitro* studies suggest a BMP/VEGF-regulated coupling between osteogenesis and angiogenesis through reciprocal signaling. Cocultures demonstrated that osteoblast-like cells stimulated the proliferation of endothelial cells by production of VEGF, whereas endothelial cells stimulated the differentiation of osteoprogenitor cells by production of BMP-2.^{102,103} Further, BMP-induced differentiation of pre-osteoblast-like cells enhanced endogenous production of VEGF.^{104–106} These studies show the importance of angiogenesis during osteogenesis and emphasize the possible benefits of combining VEGF and BMPs for bone regeneration.

So far, VEGF has only been combined with BMPs in an attempt to enhance bone regeneration. The combined implantation of VEGF-expressing cells with BMP-2 or BMP-4-expressing cells synergistically enhanced bone formation at an ectopic implantation site (Table 5).^{101,107,108} This effect on bone regeneration was dependent on the amount of cells applied and the ratio of VEGF-/BMP-expressing cells. Interestingly, coimplantation of cells expressing the VEGF-antagonist soluble Flt1 had an inhibitory effect on bone induction by BMP-2- or BMP-4-expressing cells.^{101,107} The enhancement of BMP-induced osteogenesis by VEGF was further investigated using local delivery vehicles releasing recombinant VEGF in combination with a BMP-4 plasmid or recombinant BMP-2.^{109–111} Although these studies showed enhancement of BMP-induced bone formation by VEGF, the effects with recombinant growth factor(s) were less pronounced compared to the studies using *ex vivo* transduced cells.

Several mechanisms could be responsible for the enhanced BMP-2-induced bone formation by VEGF. In addition to BMP, VEGF may stimulate the recruitment of mesenchymal

stem cells.^{112,113} Further, VEGF-induced vascular changes (e.g., increased vascular support network and vascular permeability) could enhance this cell recruitment. In combination with BMP-transduced cells, the VEGF-induced angiogenesis could also have resulted in an increased survival of BMP-expressing cells. Such a larger pool of BMP-expressing cells could have resulted in higher local dose of the osteoinductive factor and a subsequent larger amount of bone as a result of the dose–response relation between BMP and bone formation.

Platelet-Derived Growth Factor

PDGF is considered one of the key regulators of general tissue repair.¹¹⁴ Its family consists of dimeric proteins of ~30 kDa composed of disulfide-linked polypeptides encoded by four different genes (PDGF-A, -B, -C, and -D) which form homodimers (PDGF-AA, -BB, -CC, and -DD) and heterodimers (PDGF-AB). During the early phase of wound healing, platelets are the major source of PDGF. After injury and hemorrhage, platelets aggregate and release cytokine-loaded granules containing various amounts of PDGFs. Upon release, PDGFs stimulate the recruitment of neutrophils, macrophages, and mesenchymal cells, which then serve as an ongoing source of PDGFs during the healing process. PDGF also enhances proliferation of various bone cell types and enhances angiogenesis by its induction of sprouting from adjacent blood vessels and expression of VEGF. Although both PDGF and VEGF stimulate angiogenesis, PDGF stimulates the migration of a population of mesenchymal cells that appears to be different from those stimulated by VEGF.^{115,116} Apart from its positive effect on soft tissue healing, the therapeutic potential of PDGF was also demonstrated in various animal models for skeletal reconstruction.¹¹⁴

The effect of various dosages of PDGF-BB was studied in combination with BMPs in a collagen matrix in a craniotomy defect (Table 6).¹¹⁷ Whereas BMPs caused a dose-dependent increase in radiopacity and bone area in the defect, BMP-induced bone formation was inhibited by PDGF-BB dosages between 20 and 200 µg. Also in combination with demineralized bone matrix, which contains various growth factors, including BMPs, PDGF showed a similar inhibitory effect.¹¹⁸ This inhibitory effect of PDGF on BMP-induced or

TABLE 6. COMBINATIONS WITH PLATELET-DERIVED GROWTH FACTOR

Growth factors	Setting	Outcome parameters	Effect ^a	Ref.
20–100 µg PDGF-BB 30–150 µg BMP	Cranial defect in rats	Radiopacity and bone area	Inhibitory	117
50 µL PRP ^b 200 ng BMP-2	Cranial defect in rats	Bone mineral density Bone mineral content	Synergistic	134
100 µL PRP ^b 15 µg BMP-2	Cranial defect in rabbits	Bone area	Nonsignificant	135
10 µg PDGF-BB 10 µg IGF-I	Periodontal defect in primates	Bone area New tooth attachments	Nonsignificant	129
6 µg PDGF-BB 6 µg IGF-I	Partial-thickness tibial defect in pigs	Callus formation	Synergistic ^c	123

^aEffect of combination compared to PDGF or PRP and BMP alone.

^bPlatelet concentrate containing various growth factors, including PDGF, TGF-β, IGF-I, and VEGF.

^cCompared to intraanimal controls, however, absolute IGF-I values were higher or equal compared to combination group. PDGF, platelet-derived growth factor; PRP, platelet-rich plasma.

demineralized bone matrix-induced bone formation could be the result of its inhibitory effect on osteoprogenitor differentiation as indicated by its *in vivo* inhibitory effect on the bone matrix apposition rate and *in vitro* inhibition of the osteoblast phenotypic markers osteocalcin and alkaline phosphatase.^{119–122} As PDGF stimulates active bone resorption, the inhibitory effect could also be attributed to a reduction of available surface for bone formation as the surface is eroded by the increased number of osteoclasts.¹²⁰

The effect of the PDGF/IGF combination has been studied in a partial-thickness tibial defects in pigs.¹²³ Comparison of growth factor-treated defects and empty intraanimal control defects showed that the combination of PDGF/IGF-I increased callus area and thickness, whereas neither of the growth factors alone enhanced regeneration. However, interpretation of these data remains difficult since the absolute callus area and thickness of implants containing IGF-I alone were equal or higher compared to the PDGF/IGF-I combination. In contrast to this ambiguous *in vivo* effect, *in vitro* studies did show enhanced effects of the PDGF/IGF combination. *In vitro*, increased proliferation of several bone cell types, deposition of collagen, and formation of bone matrix was found, compared to the same amount of either of the growth factors individually.^{91,94} Further studies are required to characterize the interaction of these growth factors in bone regeneration.

The combination of PDGF and IGF has been studied more extensively in periodontal regeneration. Several *in vivo* studies showed the formation of substantial amounts of new bone, cementum, and periodontal ligament by a combination of PDGF-BB and IGF-I.^{124–127} In a clinical trial, the local application of a high dose (150 µg/mL) of rhPDGF/rhIGF-I significantly increased alveolar bone formation compared to controls without growth factor.¹²⁸ However, in only one study the PDGF/IGF-I combination was compared to individual growth factor controls.¹²⁹ In this primate model, the tested dose of IGF-I alone had no significant effect on bone formation and the formation of new attachments between the tooth and periodontal bone. Although the PDGF/IGF-I combination significantly enhanced bone and new attachment formation compared to the delivery vehicle alone, no significant differences were seen compared to PDGF alone.

A substance that typically contains PDGF in addition to several other growth factors is platelet-rich plasma (PRP) or platelet gel. The platelets in PRP contain several growth factors, including PDGF, TGF-β, IGF-I, and VEGF, that are

released upon their activation. Since this event also occurs after normal bone injury, the concept to enhance bone regeneration with autologous PRP is obvious. So far, the effects of PRP in various clinical and animal models of bone regeneration are ambiguous, with both enhancement of bone regeneration and lack of additional effects reported.^{130–133} In combination with BMP-2, human PRP improved angiogenesis and resulted in enhanced bone healing compared to BMP-2 alone in a rat cranial defect.¹³⁴ However, no statistically significant differences of platelet growth factor release was seen after combined implantation of rabbit PRP/BMP-2 gels compared to fibrin/BMP-2 gels in rabbit cranial defects.¹³⁵ Moreover, in a clinical trial, the combination of PRP and BMP has shown to be even less effective than BMPs alone.¹³⁶ In addition to the variation in experimental setups between these studies, growth factor concentrations in PRP are known to be variable between species and individuals,¹³⁷ all adding to the confusion around effectivity of PRP and combinations with BMPs.

Cytokines

The term cytokine encompasses a large and diverse family of proteins that historically refers to the immunomodulating agents (ILs, interferons, tumor necrosis factors, etc.). However, as more was learned about them, it appeared that these molecules play an essential role in the complex crosstalk between the immune and skeletal system. Although the mechanisms are still poorly understood, it has become apparent that many cytokines are involved in bone metabolism and are able to stimulate or inhibit the formation and function of osteoblasts, osteoclasts, or their precursor cells.^{138–141}

Despite the large number of available cytokines, only IL-11 has been studied in combination with BMP-2 in bone regeneration models (Table 7). IL-11 is produced by various cells and has several biological activities, including roles in hematopoiesis, immune responses, and bone metabolism.^{142,143} *In vitro*, IL-11 enhances osteoclast formation and bone resorption, but also stimulates expression of osteoblastic markers in mesenchymal progenitor cells.^{144–146} During osteogenesis, IL-11 synergizes with BMP-2 in a dose-dependent fashion to enhance the *in vitro* osteoblastic differentiation of progenitor cells.¹⁴⁶ Although the mechanical properties of the formed bone were not significantly different compared to BMP-2 alone, a similar synergistic increase in bone formation rate was seen after combined implantation of IL-11 and BMP-2 in a rat ectopic and rabbit segmental bone defect

TABLE 7. COMBINATIONS WITH CYTOKINES

Growth factors	Setting	Outcome parameters	Effect ^a	Ref.
20–200 µg IL-11 6 µg BMP-2	SC implants in rats	Calcium content	Synergistic	147
200 µg IL-11 1 mg BMP-2	Segmental bone defect in rabbits	Bone volume Mechanical strength	Synergistic (bone volume) Nonsignificant (mechanical strength)	148

^aEffect of combination compared to IL-11 and BMP alone. IL, interleukin.

TABLE 8. COMBINATIONS WITH SYSTEMICALLY ADMINISTERED HORMONES

<i>Growth factor dose</i>	<i>Setting</i>	<i>Outcome parameters</i>	<i>Effect^a</i>	<i>Ref.</i>
20 and 40 µg/kg/day PTH 5 µg BMP-2	SC BMP-2 implants in rats	AP activity Calcium content	Synergistic	156
10 µg/kg/day PTH 5 µg BMP-2	SC BMP-2 implants in mice	Bone mineral density Calcium content	Synergistic	157
10 µg/kg/day PTH 6.5 µg BMP-2	BMP-2 implants in SC and FD in rats	Bone mineral density Bone volume	Synergistic (SC) Additive (FD)	158
10 µg/kg/day PTH 200 µg BMP-7	BMP-7 implants in a partial-thickness tibial defect in rabbits	Bone mineral content Bone volume Mechanical strength	Nonsignificant	159
0.2 and 0.4 µg/kg/day PGE ₂ 5 µg BMP-2	SC BMP-2 implants in rats	AP activity Calcium content	Synergistic	156
50 and 100 ng/kg/day VitD ₃ 5 µg BMP-2	SC BMP-2 implants in rats	AP activity Calcium content	Synergistic	156

^aEffect of combination compared to the hormone and BMP alone.
PTH, parathyroid hormone; VitD₃, 1- α ,25-dihydroxyvitamin D₃.

model.^{147,148} Overall, these results show that IL-11 actions predominantly enhance BMP-2-induced bone formation.

Systemic Regulators

In contrast to the local application of the previously mentioned growth factors and cytokines, some systemically administered hormones also enhance bone formation. Of particular interest is PTH, a major systemic regulator of bone metabolism. It is secreted from the parathyroid glands and travels through the bloodstream to act upon bone. Whereas continuous PTH infusion causes the bone disease osteitis fibrosa, its intermittent subcutaneous administration enhances bone formation.^{149,150} The anabolic effect of intermittent PTH on bone has made it an effective treatment for osteoporosis in humans, where it was shown to increase bone mass and reduce fracture rate.¹⁵¹ So far, the mechanism behind this dual effect is still not completely understood. It has been suggested that PTH-induced stimulation of bone formation is due to an increase in osteoblast number. This increase in osteoblast number is not dependent on osteoblastogenesis, rather is the result of activation of existing bone-lining cells that undergo hypertrophy and resume matrix synthesis.^{149,152–155} Another proposed method for its effect is the inhibition of osteoblast apoptosis.^{149,153}

Since PTH acts upon cells committed to the osteoblastic lineage, it has been combined with osteoinductive BMPs (Table 8). Ectopically, systemic PTH treatment increased the local BMP-2-induced bone formation and reversed the age-related decrease in osteoinductive potential of BMP-2.^{156–158} Although systemic PTH treatment enhanced healing of a partial-thickness tibial defect, no significant effect of BMP-7 and no synergistic effect of the PTH/BMP-7 combination were seen.¹⁵⁹ In a critical-sized femoral defect, the effects of BMP-2 and PTH alone were opposite and their combination synergistically enhanced bone formation.¹⁵⁸ The discrepancy between these studies may be due to the different orthotopic models. In the partial-thickness defect, endogenous production of BMPs was sufficient to heal the defect. Consequently, PTH enhanced this endogenously induced bone formation and addition of BMP-7 had no significant effect.¹⁵⁹ The interference of this local autoinduction was less in the critical-

sized defects where no spontaneous healing was seen in empty defects and bone formation was significantly enhanced by BMP-2.¹⁵⁸

Apart from the PTH/BMP-2 combination, BMP-2 has also been combined with systemic administration of prostaglandin E₂ and [1,25-dihydroxy]vitamin D₃.¹⁵⁶ In bone, the anabolic actions of systemically administered prostaglandin E₂ increases bone formation and bone mass.^{160,161} In combination with BMP-2, a low dose of prostaglandin E₂ significantly increased alkaline phosphatase activity and calcium content of ectopic implants in rats.

In addition to PTH, vitamin D is an important regulator of bone mineral homeostasis. It plays an important role in osteoblast/osteoclast communication by stimulating the production of receptor activator of nuclear factor- κ B ligand (RANKL) by osteoblasts, which enhances the recruitment and activation of osteoclasts.¹⁶² In addition to the stimulation of osteoblastic activity, it also inhibits osteoblast apoptosis. Despite its complex actions on both osteoblast and osteoclast activity, vitamin D treatment of osteoporotic patients resulted in a modest enhancement of bone mineral density.^{163–165} Systemic administration of vitamin D₃ significantly increased alkaline phosphatase activity and calcium content of ectopic BMP-2-loaded implants.¹⁵⁶ So far, the mechanism behind this effect is not known and further studies are required to characterize this vitamin D₃-BMP-2 interaction.

Summary and Future Directions

Ever since the discovery of the autoinductive capacity of bone by bioactive peptides, researchers have attempted to enhance bone regeneration by modulating the cellular behavior using growth factors, cytokines, and hormones. Although their exact role during the regeneration process has not been fully defined, many of these molecules are able to enhance bone formation with varying potency and efficacy. As shown by the studies summarized in this review, many of these growth factors interact during bone formation. Depending on the combination of growth factors, their routes of delivery, the dosage used, and the animal model, this interaction could result in an enhancement or inhibition of bone formation compared to the individual growth factors.

Synergistic effects on bone formation are seen for most combinations, which in the case of fabricated BMP heterodimers even resulted in a 20-fold increase in bone formation compared to homodimers. Since these translational studies are performed to develop new therapies for bone regeneration, growth factor combinations with *in vitro* inhibitory effects are unlikely studied in translational bone regeneration studies. This may be the reason that only few studies with nonsignificant or inhibitory effects of combination therapies could be found.

To date, the fragmentary results on combinations of usually two growth factors with a limited number of growth factor concentrations and timing suggest that this is a rather understudied area of investigation in bone regeneration, despite its tremendous potential and virtually endless possible combinations. It is recommended that future studies address these challenges by systematically analyzing the many possibilities and that new, high-throughput screening techniques will be required to achieve this. Overall, this review shows we are only beginning to understand the possibilities of applying multiple growth factors and cytokines *in vivo*, the signaling pathways involved, and their convergence points.

Bone regeneration based on the delivery of bioactive molecules is a promising strategy that could revolutionize the way bone grafting procedures are performed in orthopedics, neurosurgery, and dentistry. Despite the success of current single-drug treatments, our knowledge of the bone regeneration process and growth factor interactions is still limited. Therefore, further molecular, cellular, and translational studies are required to obtain a better understanding of the actions and interactions of the different regulators of the regeneration process. In addition, the challenge in translational research will be to improve local delivery vehicles and their pharmacokinetic profiles. So far, many of the growth factor combinations have been released from relatively simple delivery vehicles with unknown pharmacokinetic profiles. New delivery vehicles with adjustable release profiles would help identify the optimal amounts, ratio, timing, and release sequence of these regulators to optimize the bone regeneration process and ensure consistent success in clinical applications.

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