

# Gene Therapy for Bone Regeneration

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**Abstract:** Efficacious bone regeneration could revolutionize the clinical management of many bone and musculoskeletal disorders. Bone has the unique ability to regenerate and continuously remodel itself throughout life. However, clinical situations arise when bone is unable to heal itself, as with segmental bone loss, fracture non-union, and failed spinal fusion. This leads to significant morbidity and mortality. Current attempts at improved bone healing have been met with limited success, fueling the development of improved techniques. Gene therapy in many ways represents an ideal approach for augmenting bone regeneration. Gene therapy allows specific gene products to be delivered to a precise anatomic location. In addition, the level of transgene expression as well as the duration of expression can be regulated with current techniques. For bone regeneration, the gene of interest should be delivered to the fracture site, expressed at appropriate levels, and then deactivated once the fracture has healed. Delivery of biological factors, mostly bone morphogenetic proteins (BMPs), has yielded promising results both in animal and clinical studies. There has also been tremendous work on discovering new growth factors and exploring previously defined ones. Finally, significant advances are being made in the delivery systems of the genes, ranging from viral and non-viral vectors to tissue engineering scaffolds. Despite some public hesitation to gene therapy, its use has great potential to expand our ability to treat a variety of human bone and musculoskeletal disorders. It is conceivable that in the near future gene therapy can be utilized to induce bone formation in virtually any region of the body in a minimally invasive manner. As bone biology and gene therapy research progresses, the goal of successful human gene transfer for augmentation of bone regeneration draws nearer.

**Keywords:** BMP, bone formation, bone morphogenetic protein, bone regeneration, fracture healing, gene therapy, ossification, osteogenesis, osteoblast differentiation.

## INTRODUCTION

The United Nations and the World Health Organization have declared the years 2000-2010 as the "Bone and Joint Decade" to promote public awareness of bone and musculoskeletal disorders. This underscores the increasing impact these disorders have on health as well as the quality and longevity of life. In 2000, more visits to physicians' offices were made for musculoskeletal conditions than for any other reason [Statistics, 2000]. Furthermore, there were 56 million physician visits for musculoskeletal injuries in 2000, accounting for 62 percent of all injury-related visits [Cherry and Woodwell, 2002]. In total, musculoskeletal disorders cost the United States alone \$215 billion yearly [Praemer *et al.*, 1999]. Coupled with this heightened awareness of musculoskeletal issues is the completion of the Human Genome Project (HGP). The HGP and the continuing identification of disease genes present an extraordinary opportunity for gene therapy and the treatment of human disorders. Although the use of gene therapy in bone regeneration is still at an experimental stage,

continuing advances in gene delivery technologies and the identification of novel osteogenic factors will undoubtedly make gene therapy an increasingly important part of treating bone-related disorders.

## CLINICAL SIGNIFICANCE OF BONE REGENERATION

For the most part, fractures in healthy individuals are able to heal themselves. However, failures of this regenerative process have a significant impact on all measures of patient outcome. There are at least three common clinical scenarios where there is an identified need to augment bone regeneration. (1) Segmental bone loss: segmental bone defects, frequently referred to as critical-sized defects, are common problems facing the orthopedic surgeon due to the increase in musculoskeletal trauma, tumor resection with bone loss, and revision joint arthroplasty. Because segmental defects cannot heal spontaneously, some forms of biological augmentation are needed to bridge the defect. (2) Fracture non-union: of the 5.6 million fractures sustained annually [Einhorn, 1995], the vast majority heals uneventfully with standard fracture management. Augmentation of normal fracture repair would allow more rapid mobilization, rehabilitation, and return to pre-morbid function. In addition, it has been estimated that 5-10% of fractures go on to delayed union or non-union [Praemer *et*

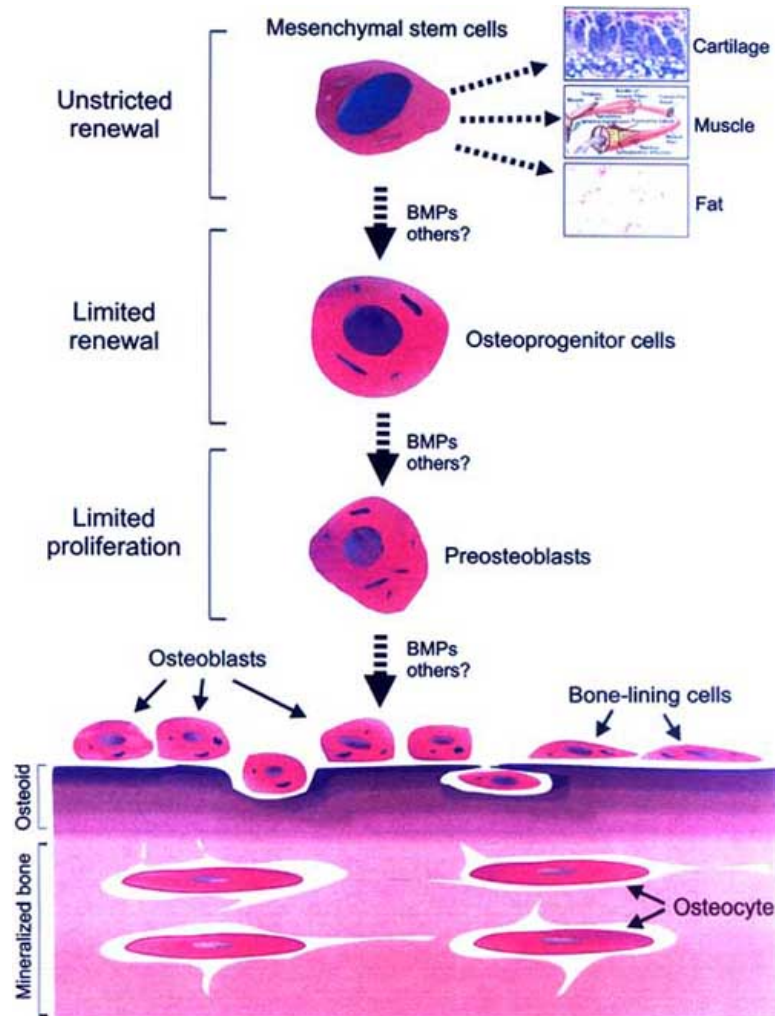
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*al.*, 1999]. Causes of impaired healing include infection, high-energy injuries with significant bone loss and soft tissue compromise, osteoporosis causing a decrease in bone mineral density, diabetes and smoking causing disturbances to microcirculation, and specific fracture patterns [Dickson *et al.*, 1994; Kwiatkowski *et al.*, 1996; Marshall *et al.*, 1996; Sarmiento, 2000; Siris *et al.*, 2001]. There are a multitude of clinical options to treat segmental defects and non-unions, including bone allograft, bone autograft, vascularized bone graft, augmentation with recombinant bone morphogenetic proteins (BMPs), and amputation. Despite many advances, poor patient outcomes suggest that better solutions are still needed. (3) Spinal fusion: each year over 200,000 spinal fusions are performed; failure to achieve a bony union occurs in 10-40% of patients with a single level fusion, and increases as multiple levels are attempted [Boden, 2002]. The gold standard for attaining bony union in spinal fusions, as well as segmental defects and non-unions is autologous bone graft, usually from the iliac crest. However, harvesting this graft is not without complications: multiple surgical sites

and incisions lead to a decrease in weight bearing status and function, and to an increase in blood loss, operative time, risk of fracture, risk of infection, and pain. Furthermore, autograft does not necessarily lead to fusion. These complications underscore the need to understand the biomechanical and cellular events critical to bone formation, and to develop innovative strategies to enhance bone regeneration. These techniques may lead to other applications in reconstructive medicine, including dental surgery and plastic surgery. In addition, aspects of medical care, including endocrinology and rheumatology, may be improved with this knowledge of bone formation.

## PREREQUISITES TO BONE REGENERATION

Bone is one of the few organs that retains the potential for regeneration into adult life, and is the only tissue that can undergo continual remodeling throughout life. The molecular mechanism underlying bone formation is a complex and highly coordinated process (Fig. 1). During skeletogenesis, bone formation can occur through two



**Fig. (1). Schematic representation of osteoblast differentiation.** Osteoblastic lineage differentiation is a multi-stage process that originates from pluripotent mesenchymal stem cells and through osteoprogenitor cells, preosteoblasts, and mature osteoblasts, resulting in formation of bone-lining cells and osteocytes. The proliferation rate decreases as the differentiation process progresses. Several growth/differentiation factors, such as BMPs, play a role in regulating osteoblast differentiation.

different pathways, intramembranous ossification or endochondral ossification [Olsen *et al.*, 2000]. In the case of intramembranous ossification (e.g. the flat bones of the skull, the mandible, part of the clavicle), osteogenesis occurs directly in condensations of mesenchymal cells [Ducy and Karsenty, 2000; Ducy *et al.*, 1999; Reddi, 1998]. With endochondral ossification (e.g., long bones), mesenchymal cells first form a cartilage anlage prefiguring the future skeletal elements, and then bone is formed to replace the cartilage [Ducy and Karsenty, 2000; Ducy *et al.*, 1999; Reddi, 1998]. Bone regeneration following a fracture progresses through sequential phases similar to endochondral ossification, starting with chemotaxis and mitosis of mesenchymal stem cells. By day three, these cells have become adherent and reach maximal proliferation [Reddi, 2001]. By day five, chondroblasts begin to differentiate. Days seven and eight bring maximal chondrogenesis. On day nine, cartilage hypertrophy as well as mineralization has begun. Angiogenesis then sets the stage for osteoblast differentiation on days 10-11. The process of remodeling is then begun on the endochondral bone. Although the molecular events underlying bone regeneration remain undefined, it is generally postulated that osteogenesis is a sequential multistep cascade that recapitulates most, if not all, of the cellular events occurring during embryonic skeletal development [Olsen *et al.*, 2000; Reddi, 1998].

In general, successful bone regeneration rests on the presence of at least four crucial elements, namely osteoinduction, differentiation of osteoblasts leading to production of osteoid matrix, osteoconduction, and mechanical stimulation (Fig. 1). Bone morphogenetic proteins (BMPs) are essential for the first requirement, osteoinduction, which is the activation of various cytokines or growth factors to attract osteoblasts and/or their progenitor cells to the repair site and induce them to produce bone. Marshall Urist first reported these factors, now known as BMPs, in the 1960s when he discovered that demineralized bone induced *de novo* bone formation in various animal models [Urist, 1965]. BMPs belong to the TGF- $\beta$  superfamily and are the active osteoinductive factors in bone regeneration [Alden *et al.*, 2002; Groeneveld and Burger, 2000; Kingsley, 2001; Linkhart *et al.*, 1996; Reddi, 1998; Sun *et al.*, 2003; Yamaguchi, 1995]. The importance of BMPs in bone regeneration will be discussed in depth later.

The production of osteoid matrix, which is the precursor to bone formation, is the second element necessary for bone formation. Since only osteoblasts can produce osteoid, osteoblast lineage specific differentiation of mesenchymal stem cells is an essential step in the process of bone formation [Aubin, 1998; Caplan, 2001; Ducy and Karsenty, 2000]. Recent studies have explored the possible sources of osteoblasts, including mesenchymal stem cells [Qi *et al.*, 2003; Tsuchida *et al.*, 2003], muscle-derived stem cells [Asakura *et al.*, 2001; Bosch *et al.*, 2000; Lee *et al.*, 2001; Young *et al.*, 2002], peripheral blood buffy-coat cells [Viggeswarapu *et al.*, 2001], bone marrow cells [Bruder and Fox, 1999; Yamaguchi *et al.*, 1996], and the connective tissue [Beresford, 1989]. Although the molecular mechanisms underlying bone formation remain to be defined, BMPs seem to play an important role in osteoblast

differentiation [Aoki *et al.*, 2001; Bachner *et al.*, 1998; de Jong *et al.*, 2002; Katagiri *et al.*, 1994; Kitching *et al.*, 2002; Rickard *et al.*, 1994; Sakano *et al.*, 1993; Thies *et al.*, 1992; Yamaguchi *et al.*, 1996; Yamaguchi *et al.*, 1991].

The third requirement for bone regeneration is osteoconduction, referring to the three-dimensional structure, or scaffold, that allows the ingrowth of bone stimulated by the first two requirements. An osteoconductive material is not sufficient to produce bone; rather it provides the architecture for osteogenic cells to congregate, adhere, and interact. Examples of osteoconductive materials include freeze-dried bone allograft, ceramics, and natural and synthetic polymers [Vaccaro, 2002]. Once the above three factors have collaborated to produce viable bone tissue, the bone must remodel to become functional and durable. Mechanical forces exert strain on bone that stimulates both osteoblasts and osteoclasts to remodel the bone structure to better resist the strain, also known as Wolff's law [Huiskes, 2000; Wolff, 1892]. With inadequate strain to induce sufficient remodeling, disuse mode remodeling will remove bone [Frost, 2001]. Thus, adequate mechanical strains must be present to maintain the newly regenerated bone.

## ADVANTAGES OF USING GENE THERAPY FOR BONE REGENERATION

### Direct Delivery of Recombinant BMPs for Bone Regeneration

Historically, the use of demineralized bone matrix and/or purified recombinant human BMPs (rhBMPs) has been a mainstay in bone regeneration studies [Frenkel *et al.*, 1993; Gerhart *et al.*, 1993; Sandhu *et al.*, 2001]. Although promising results have been extensively documented, one of the major caveats is the stability and biological activity of the delivered rhBMPs [Groeneveld and Burger, 2000; Uludag *et al.*, 2001]. Multiple carriers have been tested to maintain retention of recombinant human BMPs (rhBMPs) delivered directly to the site of interest. Most currently used carriers are able to maintain levels of about 50% of initial dose for less than one week, with subsequent degradation to 15% at two weeks, and 5% at three weeks [Seeherman *et al.*, 2002]. Thus, directly delivered protein may not be active for long enough to promote effective bone formation. Another major limitation of the use of rhBMPs is associated with the cost of producing recombinant proteins; this may in many ways be preventing the broader use of rhBMPs. For example, 0.002 mg of rhBMP-2 can be extracted from one kilogram of powdered normal bone; this physiologic concentration is adequate for normal fracture healing [Takaoka *et al.*, 1988]. However, this concentration is not sufficient to heal critical size defects by definition. Furthermore, rodents have required concentrations of 0.01 mg/ml to accelerate fracture healing, while non-human primates require as much as 1.5mg/ml. The requisite concentration is even higher in spinal fusion models [Seeherman *et al.*, 2002]. Thus, the need for these supraphysiologic concentrations of osteogenic factors can be prohibitively expensive in a clinical setting. Although numerous efforts are being made to improve the efficacy of direct use of recombinant osteogenic proteins, many of the

above limitations can be readily overcome by using gene therapy approaches.

### **Therapeutic Gene Delivery for Bone Regeneration**

Gene therapy may represent an ideal approach towards augmenting bone regeneration as it enhances the first three conditions needed for bone regeneration: gene therapy can enhance osteoinduction via expression of growth factors, induce osteoblast differentiation and facilitate the production of osteoid matrix, and utilize an osteoconductive apparatus. Thus, many strengths of gene therapy are especially relevant to bone regeneration. While first conceived as a systemic treatment for hereditary single-gene defects [Kay and Woo, 1994; Thomas *et al.*, 2003], localized gene therapy is well suited for bone formation because of the ability to deliver genes to a discrete site. In the case of bone regeneration, transient expression is also a desirable benefit and readily available with existing gene transfer techniques. Thus, gene therapy in bone regeneration has the unique ability to deliver gene products to precise anatomic locations at elevated levels for an extended duration.

Traditionally, gene delivery can be accomplished by using viral vectors or non-viral means. Non-viral approaches include delivery of naked DNA/plasmids by direct injection, liposome-mediated transfection, particle-mediated delivery (e.g. gene gun), microseeding, electroporation, and polymer-DNA complex implantation [Bonadio *et al.*, 1999; Dubruel *et al.*, 2003; Ferry and Heard, 1998; Hoeller *et al.*, 2002; Lee *et al.*, 2003; Luu *et al.*, 2003; Park *et al.*, 2003]. These techniques are of interest because they are often less costly and able to sustain gene expression as compared to direct delivery of proteins (i.e. recombinant BMPs) to a critical size defect. However, the use of non-viral vectors is restricted by their relatively low efficiency of gene transfer, and hence, a lower level of transgene expression, although some studies are attempting to overcome this limitation.

Gene transfer mediated by viral vectors represents the most common approach in gene therapy studies. Five major types of viral vectors can be divided into two groups based on whether their genomes persist in a non-integrated form (i.e., adenovirus and herpes virus) or become integrated into host chromosomes (i.e., retrovirus, adeno-associated virus, and lentivirus) [Thomas *et al.*, 2003]. This categorization is important in that the latter group tends to be better suited for applications requiring persistent and stable genetic alteration [Thomas *et al.*, 2003]. However, under different circumstances, such as bone regeneration, transient transgene expression is advantageous. In this regard, recombinant adenoviral vectors may be the most appealing. In addition, adenoviral vectors mediate the highest level of transgene production [Gerdes *et al.*, 2000]. As a result, the majority of previous work in bone regeneration has used adenovirus vectors, with only a small number of studies using retroviral vectors. Further discussion of the relative advantages of different vectors are beyond the scope of this paper, and the reader is directed towards several recent reviews for a more in-depth discussion [Alemany *et al.*, 2000; Breyer *et al.*, 2001; Hoeller *et al.*, 2002; Palu *et al.*, 2000; Pandya *et al.*, 2001; Robbins *et al.*, 1998; Thomas *et al.*, 2003].

In addition to the selection of proper gene delivery vehicles, it is equally important to choose an appropriate route of gene delivery. As discussed above, in the case of bone regeneration, local gene delivery is the most desired method. There are two main strategies in local gene therapy: direct delivery (*in vivo*) and transplantation of genetically modified osteoblasts or their progenitor cells (cell-based or *ex vivo*). The *in vivo* approach tends to be straight-forward, faster, and less costly, whereas the *ex vivo* method is theoretically safer and more effective because genetic manipulations take place outside the patient's body, and the transduced osteoblasts or progenitor cells can serve as not only a source of osteogenic factor production, but also as a primer site for bone formation. As discussed in the following section, both methods are currently used for bone regeneration.

## **CURRENT USE OF GENE THERAPY IN BONE REGENERATION**

### **Biological Factors Used to Enhance Bone Regeneration**

The time course of bone formation highlights some of the strengths of gene therapy. New bone formation is modulated by a variety of osteogenic factors, including PDGF [Aubin *et al.*, 1995; Nash *et al.*, 1994], FGFs [Aubin *et al.*, 1995; Canalis *et al.*, 1988; de Crombrugge *et al.*, 2001; Nakamura *et al.*, 1998], IGF-1 and IGF-2 [Deasy *et al.*, 2002; Gazzero *et al.*, 1998; Linkhart *et al.*, 1996], TGF- $\beta$  [Cheifetz *et al.*, 1996], VEGF [Tarkka *et al.*, 2003; Uchida *et al.*, 2003] and BMPs [Seeherman *et al.*, 2002]. These factors may affect different points along the bone formation process, including chemotaxis, proliferation, and differentiation. However, thus far some of the BMPs are the only group that can initiate and sustain the entire bone formation cascade [Barnes *et al.*, 1999; Einhorn and Lee, 2001; Sakou, 1998]. BMPs comprise a large and diverse family, many of which may have a distinct role in the cascade of bone morphogenesis (Table 1). At least, 15 BMPs have been identified in humans and rodents [Reddi, 2001]. BMP-1 is a metalloprotease and has very little osteogenic activity [Sarras, 1996], while BMP-3 has been shown to antagonize other bone forming BMPs [Bahamonde and Lyons, 2001; Cheng *et al.*, 2003; Daluiski *et al.*, 2001]. While the osteogenic capacity of the remaining BMPs has been the focus of numerous studies [Cheng *et al.*, 2003; Valcourt *et al.*, 1999; Yamaguchi *et al.*, 1996], BMP-2 and BMP-7 (a.k.a., OP-1) have been most extensively studied both in animal studies and in clinical trials (Table 2).

### **Use of BMP-2 in Bone Regeneration**

BMP-2 is one of the most studied BMPs in bone regeneration, and there are now clinical trials with promising results [Boden *et al.*, 2002; Boden *et al.*, 2000; Govender *et al.*, 2002]. Though initially used as a recombinant protein, BMP-2 mediated gene therapy has been widely pursued. Lieberman *et al.*, showed that new bone formation from rhBMP was noticeably different from adenovirus mediated BMP-2, in which rhBMP-2 created lace-like bone spanning defects, even with large quantities of protein, whereas the BMP-2 transduced cells formed robust, coarse trabecular bone [Lieberman *et al.*, 1999]. These results suggest that gene therapy can improve upon recombinant proteins for

**Table 1. Members of BMP Family and Their Possible Functions in Development and Skeletal Tissues**

BMP subfamily	BMP* designation	Generic name	Year cloned	Phenotype of genetic disruption [References]	Functions in development and musculoskeletal system	
BMP2/4	BMP2	BMP2A	1988	embryonic lethality. delayed primitive streak, small allantois, lack of amnion, heart defects, decreased number of primordial germ cells (PGCs). [Zhang & Bradley, <i>Dev.</i> 122:2977 (1996)]	bone & cartilage morphogenesis/heart	
	BMP4	BMP2B	1988	embryonic lethality. lack of allantois and PGCs, posterior truncation, head defects, lack of optic vesicle. [Winnier <i>et al. Genes Dev</i> 9:2105 (1995)]	bone morphogenesis	
BMP3	BMP3	osteogenin	1988	increased bone density. [Daluski <i>et al. Nat Genet</i> 27:84 (2001)]	negative regulator of bone density	
BMP7	BMP5	BMP5	1990	short ear phenotype, defects in skeleton, lung and kidney. [Kingsley <i>et al. Cell</i> 71:399 (1992); King <i>et al. Dev Biol.</i> 166:112 (1994)]	bone morphogenesis	
	BMP6	Vgr-1	1990	mild delay of sternum ossification. [Solloway <i>et al. Dev Genet.</i> 22:321 (1998)]	bone morphogenesis, hypertrophy of cartilage/skin	
	BMP7	OP-1	1990	skeletal defects, kidney agenesis, eye defects. [Dudley <i>et al. Genes Dev.</i> 9:2795 (1995); Luo <i>et al. Genes Dev.</i> 9:2808 (1995)]	bone morphogenesis, eye and kidney development	
	BMP8	OP-2	1992	Defects in spermatogenesis and epididymis. [Zhao <i>et al. Dev</i> 125:1103(1998)]	bone formation	
	BMP9	GDF-2	1994	not known	cholinergic neuron differentiation, hepatocyte growth, hematopoiesis, bone formation	
	BMP10	BMP10	1995	not known	expression restricted to heart	
	BMP11	GDF-11	1995	Defects in A-P patterning of axial skeleton. [McPherron <i>et al, Nat Genet</i> 22:260(1999)]	A-P patterning of axial skeleton	
	GDF-5,6,7	BMP12	GDF-7 or CDMP-3	1995	commissural interneurons of spinal cord. Hydrocephalic abnormalities growth defects in seminal vesicle. [Lee <i>et al, Genes Dev</i> 12:3394(1998); Settle <i>et al, Dev Bio</i> 234:138(2001)]	ligament and tendon development
		BMP13	GDF6 or CDMP2	1994	defects in joints, cartilage, and ligament formation. [Settle <i>et al. Dev Bio</i> 254(1):116-130(2003)].	ectopic induction of tendon and ligament, cartilage development
		BMP14	GDF-5 or CDMP-1	1994	Brachypodism in mice (limbs shortened with reduced number of bones). Dominant negative mutation in humans causes severe limb shortening and dysmorphogenesis. [Storm <i>et al. Nature</i> 368:639(1994); Storm and Kingsley, <i>Dev</i> 122:3969(1996);Thomas <i>et al. Nat Gen</i> 17:58-64(1997)]	joint formation, chondrogenesis
BMP-15		GDF-9B	1996	increased ovulation rate & infertility. Sterile due to defects in oogenesis. [Dong <i>et al. Nature</i> 383:531 (1996)]	ovulation and female fertility	

\* Note: BMP-1 is excluded because it functions as a protease rather than a bone-forming factor.

bone regeneration. Park *et al.*, compared the effects of adenoviral vectors and liposomes on bone regeneration of a critical size defect of rat femur model with cell-mediated BMP-2 gene transfer [Park *et al.*, 2003]. While both treatment groups healed the critical size defect, the liposome group took 6 weeks to heal, whereas the adenoviral group completed healing within 4 weeks. These studies suggest that the time course and the quality of repair can be modulated with different gene transfer techniques.

*Ex vivo* approaches with BMP-2 have worked well with different lines of stem cells, including bone marrow [Lieberman *et al.*, 1999; Tsuchida *et al.*, 2003] and muscle [Lee *et al.*, 2001; Young *et al.*, 2002]. These stem cell transductions all resulted in healing of critical sized bone defects in small animal models. Current work is examining bone induction in stem cells transduced from human fat [Dragoo *et al.*, 2003; Zuk *et al.*, 2001]. In addition, Riew *et al.*, implanted autologous bone-marrow cells transduced with BMP-2 into the paraspinous region in rabbits. While new bone

**Table 2. Current Gene Therapy Studies for Bone Regeneration**

Therapeutic gene	Delivery approach	Experimental model	Experimental Outcomes	References
BMP-2	Recombinant protein (RP)	Rat/Mouse	Healed critical defects	[Yasko, 1992; Lee, 1994; Lieberman, 1999; Chen, 1997; Hong, 1998; Murata, 1999; Musgrave, 1999; Saito, 2003; Uludag, 1999; Uludag, 2000; Whang, 1998]
		Rabbit	Hydrogel carrier as scaffold	[Hong, 1998]
		Sheep/Dog	Healed segmental defects, augmented prosthesis	[Gerhart, 1993; Lovell, 1989; Murakami, 2003; Toriumi, 1991]
		Non-human primate	Laparoscopic anterior lumbar interbody arthrodesis	[Boden, 1998]
		Human	Better fusion rate than autograft, no complications	[Boden, 2000; Govender, 2002; Boden, 2002; Johnson, 1988; Johnson, 1988]
	<i>In vivo</i>	Rat/Mouse	Mixed results in immunocompetent animals	[Musgrave, 1999; Alden, 1999; van Griensven, 2002; Alden, 2000; Engstrand, 2000; Gonda, 2000; Okubo, 2000]
		Rabbit	Healed femoral critical defect	[Baltzer, 2000; Baltzer, 2000]
	<i>Ex vivo</i>	Rat/Mouse	Better fusion rate than autograft, no complications	[Wang, 2003; Lee, 2001; Lieberman, 1998; Lieberman, 1999; Oyama, 1999; Partridge, 2002; Tsuchida, 2003; Lou, 1999]
		Rabbit	Spinal fusion achieved	[Riew, 1998; Cheng, 2001]
	Nonviral	Rat/Mouse	Bone healed faster with adenovirus than liposomes	[Park, 2003]
BMP-7	RP	Rat	Different effect based on stage of osteoblast differentiation	[Li, 1996]
		Canine	Solid spinal fusion, long bone healing	[Cook, 2001; Lovell, 1989; Salkeld, 2001; Cook, 1994; Paramore, 1999]
		Rabbit	Lumbar spinal fusion	[Grauer, 2001]
		Sheep	Lumbar spinal fusion	[Cunningham, 1999; Magin, 2001]
		Human	Bony union achieved	[Friedlaender, 2001; Cook, 2001; Geesink, 1999; Johnsson, 2002; Jeppsson, 1999]
	<i>In vivo</i>	Rat/mouse	Repaired segmental defects	[Franceschi, 2000; Rutherford, 2002; Rutherford, 2003]
	<i>Ex vivo</i>	Rat	Ectopic bone formation	[Cheifetz, 1996; Haaijman, 2000; Krebsbach, 2000]
		Rabbit	Infected periosteal cells with retrovirus	[Breitbart, 1999; Mason, 1998]
BMP-4	RP	Rat/Mouse	Pharmacokinetics study	[Uludag, 2000]
	<i>In vivo</i>	Rat/Mouse	Fracture healing	[Rundle, 2003; Jane, 2002]
	<i>Ex vivo</i>	Rat/Mouse	Healed critical defects	[Gysin, 2002; Wright, 2002; Yamaguchi, 1996]
	Nonviral	Rat/Mouse	Electroporation, GAM (with PTH1-34 cDNA as well)	[Kishimoto, 2002; Fang, 1996; Bonadio, 1999]
BMP-6	RP	Rat/Mouse	Pharmacokinetics study	[Uludag, 2000]
	<i>In vivo</i>	Rat/Mouse	Possibly more potent than BMP-2	[Jane, 2002]
	<i>Ex vivo</i>	Rat/Mouse	Ectopic bone formation	[Gitelman, 1994; Yamaguchi, 1996; Kang, 2004]
BMP-9	<i>In vivo</i>	Rat/Mouse	Spinal fusion and ectopic bone formed	[Helm, 2000; Varady, 2001]
	<i>Ex vivo</i>	Rat/Mouse	Fusions attained, no toxicities	[Dayoub, 2003; Dumont, 2002; Kang, 2004]
LMP-1	<i>In vivo</i>	Rat/Mouse	<i>De novo</i> bone	[Boden, 1998]
		Rat/Mouse	Used Buffy coat cells	[Minamide, 2003; Boden, 1998; Viggswarapu, 2001]
		Rabbit	Spinal fusion achieved	[Boden, 2000]

formation was observed, solid fusion was not achieved [Riew *et al.*, 1998]. Wang *et al.*, recently compared single level spine fusion rates between various combinations of BMP-2 and carriers and found significantly higher fusion rates with *ex vivo* BMP-2 transduction as compared to the gold standard, autogenous iliac crest bone graft [Wang *et al.*, 2003].

Direct *in vivo* delivery of an adenoviral vector expressing BMP-2 has been shown to induce ectopic bone formation when injected into thigh muscles of rats [Alden *et al.*, 1999b; Lieberman *et al.*, 1998; Musgrave *et al.*, 1999] and in rat spinal fusion models [Alden *et al.*, 1999a]. These studies were carried out in immunocompromised animals. However, only inflammation was seen at the injection site in immunocompetent rats [Alden *et al.*, 1999b]. Okubo *et al.*, induced transient immunosuppression in rats with cyclophosphamide and were able to stimulate ectopic bone formation [Okubo *et al.*, 2000]. Interestingly, when a BMP-2 adenoviral vector was directly injected into the fracture site in a rat fracture model, transgene expression was detected in various cell types, including chondrocytes, osteoblasts, and osteoclasts, many of which were incorporated into the newly mineralized bone [van Griensven *et al.*, 2002]. Nevertheless, the development and refinement of *in vivo* techniques are important because they allow for direct injection of viral vectors into the fracture zone, while avoiding the costly and time-consuming steps of harvesting, transduction, and implanting stem cells associated with *ex vivo* approaches.

### Use of BMP-7 in Bone Regeneration

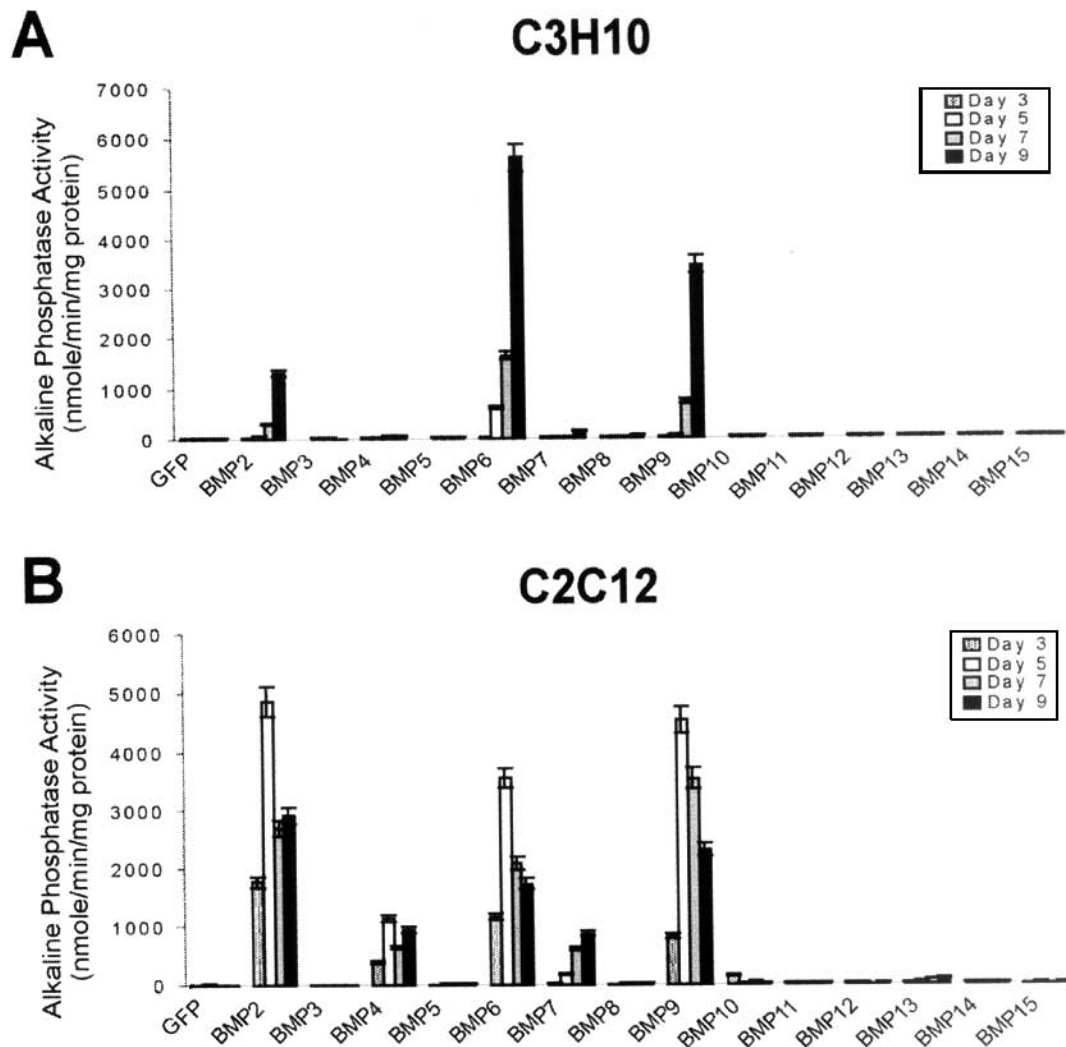
BMP-7 represents another osteogenic factor with a significant body of research. Currently, it is an FDA approved BMP (available as a recombinant protein form and marketed as OP-1) for recalcitrant nonunion of long bones and anterior spinal fusions. BMP-7 has been shown to induce new bone *in vivo*, promote the growth of osteoblasts, and maintain the osteoblast phenotype *in vitro* [Breitbart *et al.*, 1999; Cook and Rueger, 1996; Maliakal *et al.*, 1994]. In animal studies, BMP-7 has been shown to heal critical sized defects in rabbits [Cook *et al.*, 1994b], dogs [Cook *et al.*, 1994a; Salkeld *et al.*, 2001], and non-human primates [Cook *et al.*, 1995]. It has been used in clinical trials for spinal fusion [Jeppsson *et al.*, 1999; Johnsson *et al.*, 2002], treatment of critical defects [Geesink *et al.*, 1999], and tibial nonunions [Friedlaender, 2001; Friedlaender *et al.*, 2001]. Friedlaender *et al.*, showed that OP-1 had similar rates of union when compared to autograft [Friedlaender *et al.*, 2001]. Current research has been directed towards BMP-7 gene therapy to augment bone regeneration. Krebsbach *et al.*, transduced fibroblasts with BMP-7 and observed an osteoblast phenotype, which led to the formation of clinically evident bone [Krebsbach *et al.*, 2000]. These findings suggest that BMP-7 gene therapy can be used to replace the costly recombinant protein approach in bone regeneration. However, recent studies have suggested that BMP-7 may be less biologically active than other BMPs, namely BMP-2, BMP-6, and BMP-9 as was demonstrated *in vitro* [Cheng *et al.*, 2003] as well as in animal models [Li *et al.*, 2003; Kang *et al.*, 2004].

### BMP-6 and BMP-9 as More Osteogenic Factors in Bone Regeneration

While much attention has been directed towards BMP-2 and BMP-7-mediated bone regeneration, it is not clear whether or not they represent the most osteogenic BMPs. We recently conducted a comprehensive analysis of the *in vitro* and *in vivo* osteogenic activity of the 14 types of human BMPs (e.g. BMP-2 to BMP-15) [Cheng *et al.*, 2003; Kang *et al.*, 2004]. Such an extensive analysis had been previously impossible, largely because recombinant BMPs are not readily available for the 14 type of BMPs, and/or are not highly biologically active. To circumvent these problems, we constructed recombinant adenoviruses to express each of the 14 BMPs and infected pluripotent mesenchymal progenitor C3H10T1/2 cells and pre-osteoblastic C2C12 cells. The osteogenic activities of the individual BMPs were then determined by measuring the induction of early and late osteogenic markers, such as alkaline phosphatase, osteocalcin, and matrix mineralization upon BMP stimulation [Cheng *et al.*, 2003]. Our results demonstrated that BMP-2, BMP-6, and BMP-9 are the most potent BMPs in inducing osteoblast differentiation of mesenchymal stem cells, while BMP-2 and BMP-7 also induce osteogenic markers in pre-osteoblast C2C12 cells (Fig. 2). Interestingly, our results also demonstrate that several combinations of BMPs (such as BMP-5 and BMP-10 or BMP-12 or BMP-13, and BMP-7 and BMP-10 or BMP-12 or BMP-13) exhibit significant synergy in their ability to induce alkaline phosphatase activity [Sun *et al.*, 2003]. Taken together, our findings are consistent with the notion that some BMPs are more efficacious for bone regeneration applications, and that the efficacy of osteogenesis may depend not only on types of BMP or combination of BMPs, but also on cell types present.

To date, BMP-6 mediated bone formation has been analyzed in a limited number of studies. Earlier experiments demonstrated that BMP-6 was able to promote osteoblast differentiation and bone formation [Gitelman *et al.*, 1995; Gitelman *et al.*, 1994]. Using adenoviral vectors to deliver BMP-2, BMP-4 and BMP-6, Jane *et al.*, recently compared their ability to induce ossification in athymic nude rats. They found that BMP-4 produced ectopic bone through mechanisms similar to endochondral ossification, and BMP-6 seemed to induce bone by way of mechanisms similar to both intramembranous and endochondral ossification, while interestingly BMP-2 induced no bone growth [Jane *et al.*, 2002]. Ultimately the density of ectopic bone formed by BMP-4 and BMP-6 was comparable, but BMP-6 produced the most rapid tissue calcification. These results confirm the varying levels of osteogenic activity among the different BMPs, and BMP-6 may represent a more osteogenic factor than those currently used.

Ironically, BMP-9 is one of least studied BMPs. Nevertheless, our *in vitro* and *in vivo* studies about the potent osteogenic activity of BMP-9 have been supported by several *in vivo* studies reported by Helm's group [Dayoub *et al.*, 2003; Varady *et al.*, 2001]. It was shown that a solid spinal fusion was achieved at 16 weeks after injection of BMP-9 into the paraspinous musculature [Helm *et al.*, 2000]. Similar results have been obtained with *ex vivo* therapy as



**Fig. (2). Distinct osteogenic activity of the 14 types of BMPs.** Subconfluent pluripotent C3H10T1/2 cells (A), and osteoblastic precursor C2C12 cells (B) were infected with AdBMPs and the control AdGFP. Cells were lysed at indicated times for colorimetric assays of alkaline phosphatase activity using *p*-nitrophenyl phosphate as a substrate. Adapted from *Cheng, et al. 2003* with permission.

human mesenchymal cells transduced with AdBMP-9 induced spinal fusion in a rat model [Dumont *et al.*, 2002]. Taken together, these *in vitro* and *in vivo* studies strongly suggest that BMP-6 and BMP-9 may represent more potent osteogenic factors than the prototypic BMPs currently used for bone regeneration.

#### Use of BMP-4 in Bone Regeneration

BMP-4 shares significant sequence homology with BMP-2. In fact, homologous BMP-2 primers were used in the initial sequencing of the gene [Shore *et al.*, 1998]. However, its osteogenic ability has not been studied as extensively as that of BMP-2. BMP-4, as well as BMP-2, has been shown to upregulate osteogenic markers [Valcourt *et al.*, 1999]. Reference was made earlier to Jane *et al.*, which suggested significant osteogenic potential for BMP-4 [Jane *et al.*, 2002]. Rundle *et al.*, used a BMP-4 retroviral vector *in vivo* and found induction of bone growth in a fracture model [Rundle *et al.*, 2003]. Further work on BMP-4 is needed to establish it as a valid osteogenic factor.

#### Use of LMP-1 in Spinal Fusion

While BMPs are considered to be a major group of biological factors that promote bone formation, other non-BMP factors have also been shown to enhance bone formation. For instance, new approaches to spinal fusion involve using LIM mineralization protein-1 (LMP-1), a novel osteoinductive intracellular protein. LMP-1 is a member of the heterogeneous LIM-domain family of proteins whose functions include a variety of fundamental processes, such as transcriptional regulation [Kong *et al.*, 1997], organization of the cytoskeleton [Sadler *et al.*, 1992], and cell type development [Way and Chalfie, 1988]. Unlike BMPs, which are secreted proteins and act by binding to cell surface receptors, LMP-1 is thought to be an intracellular signaling molecule involved in osteoblast differentiation [Boden *et al.*, 1998a]. Recently, increased levels of BMP-2, BMP-4, BMP-6, BMP-7 and TGF- $\beta$ 1 have been detected in LMP-1 transduced cells [Boden *et al.*, 1998a; Minamide *et al.*, 2003]. While much has to be learned about LMP-1 functions, it has been shown that solid spinal fusion can be

achieved in rat and rabbit models using either LMP-1 transduced bone marrow cells or buffy-coat blood cells [Boden *et al.*, 1998b]. These results with LMP-1 are promising, but have yet to be applied to other bone regeneration models.

### Use of PTH1-34 Peptide and Gene Activated Matrix (GAM) in Bone Regeneration

A recent development is the use of a GAM consisting of a collagen sponge that provides the scaffolding to promote cell ingrowth and deliver plasmid DNA directly to the cells involved in fracture repair [Bonadio *et al.*, 1999; Fang *et al.*, 1996]. Local granulation tissue fibrocytes and capillaries migrate into the degradable matrix, uptake, then transiently express the plasmid DNA that stimulates the bone regeneration cascade. Fang *et al.*, reported that a 5mm critical femoral defect in Sprague-Dawley rats healed when treated with GAMs impregnated with BMP-4 cDNA or a fragment of parathyroid hormone PTH1-34 cDNA [Fang *et al.*, 1996]. Both of these gene-activated matrices caused healing of the defects. Interestingly, healing was more rapid, occurring at four weeks instead of nine weeks, when both cDNAs were included in the GAM, suggesting a possible synergistic effect between BMP-4 and PTH1-34. This may be due to induction of PTH-dependent cascades [Yamaguchi *et al.*, 1996]. Similar results were shown in a canine model of a tibial critical defect [Bonadio *et al.*, 1999]. In this model, GAMs containing hPTH1-34 plasmid DNA demonstrated retention and expression of the plasmid for 6 weeks, and bone induction was observed in a dose- and time-dependent manner. However, the critical sized defects did not heal. This procedure offers some theoretical advantages because it uses plasmid DNA and essentially has no gene size limitation or any safety concern regarding the immunogenicity of viral vectors (especially adenoviral vectors), and yet it is inexpensive to produce in large quantities.

### Gene Therapy with an Osteoconductive Scaffold

Unlike gene therapy in other disorders, a unique feature of gene therapy in bone regeneration is the use of osteoconductive scaffold in conjunction with gene delivery. An ideal tissue engineering scaffold exhibits at least four critical properties: biocompatible, three-dimensional architecture, osteoconductive, and biodegradable. In addition, scaffolds used with gene therapy must also have the ability to deliver the gene product at an appropriate rate and dose. While the scaffolds are not themselves vectors for gene delivery, they can serve multiple important functions. The currently used scaffolds are usually made of natural polymer (collagen, or hyaluronic acid), synthetic polymers (polylactic acid, or polyglycolic acid), and composites (polylactic coglycolic acid and polypropylene fumarate) [Cao *et al.*, 1998; Crane *et al.*, 1995; Freed *et al.*, 1993; Ishaug *et al.*, 1994; Rivard *et al.*, 1995; Vacanti *et al.*, 1991]. The above-mentioned GAM is a good example of the use of gene therapy with osteoconductive scaffolds. GAM offers a degradable 3-D matrix upon which the plasmid DNA has been incorporated. The architecture therefore offers both delivery of the plasmid as well as an osteoconductive matrix for chemotaxis guiding cell recruitment and attachment,

ultimately leading to bone ingrowth. While there is unlikely to be an ideal carrier system for all biologic applications, current work is attempting to optimize individual systems for specific tissue modalities. For example, hyaluronan shows promise as a carrier for mesenchymal stem cells in the reconstruction of cartilage and connective tissues [Solchaga *et al.*, 1999], while demineralized bone matrix may be better suited for bone formation.

## POTENTIAL PITFALLS ASSOCIATED WITH GENE THERAPY

### Possible Mutations Caused by Integrating Viral Vectors

Integrating viral vectors, which refer mainly to retrovirus, have been used for more than 10 years in clinical trials to achieve stable gene expression in proliferating cells. A known theoretical risk is the insertion of the viral genome into host chromatin causing disruption of a cellular sequence associated with malignancy. Recently, these risks have been manifested in two separate incidents. The first involved retrovirally transduced bone marrow cells inducing leukemia in mice [Li *et al.*, 2002]. In the second case, 2 of 11 patients treated successfully for severe combined immunodeficiency (SCID) developed a leukemia-like disorder apparently caused by retroviral genome integration causing a disruption in the oncogene *LMO2* [Cavazzana-Calvo *et al.*, 2000; Hacein-Bey-Abina *et al.*, 2003]. Gene researchers are now debating whether similar risks apply to other applications of retrovirus and integrating viruses. Nevertheless, current efforts are focused on improving the safety profile of existing vectors as well as developing new vector systems with pre-determined insertion sites.

### Host Immune Response to Viral Vectors

The biggest challenge facing all viral vectors continues to be the host immune response. Adenovirus vectors are the most immunogenic of the different virus vectors and induce multiple components of the immune response. Cytotoxic T cells (CTL) respond to viral gene products as well as 'foreign' transgene products and lead to elimination of the transduced cells. Meanwhile, the humoral antibody response precludes repeated administration of the vector by cytokine-mediated inflammation. This is especially troublesome in adenovirus since the non-integrating genome is gradually diluted by DNA degradation and cell division, and therefore requires periodic repeat infection.

Progress has been made in reducing the CTL response by stripping the vector of all viral genes. This advance also reduces the antibody-mediated cytokine response to specific viral proteins. Retrovirus, lentivirus, and AAV do not seem to suffer from CTL responses; since they integrate into the host genome, antibody responses are also of less concern.

Complications of the immune response surfaced with the death of an 18 year old subject in 1999 directly attributed to the administration of an adenoviral vector. Of note, another patient received the same dose of adenovirus and did not experience the same complications. While there are no clear answers, this incidence suggests that toxic side effects may be a function of vector dose. However, the ability to predict vector-related side effects continues to be hampered by the variability of the immune response between individuals.

### Biosafety of Transgene Expression

The delivery of the transgene tends to be non-specific and not easily controlled. For example, local delivery of a vector can lead to leakage and dissemination to nearby tissues, while systemic delivery leads to unwanted vector uptake by cells in multiple organs. This non-specific uptake is undoubtedly related to systemic immune responses. There have been many efforts to target specific cell types for transduction, which will allow for lower virus titers to be administered.

One other theoretic concern worth noting pertains to the function of the introduced transgene. Take for example the BMPs. While there has been proven bone regeneration with the administration of various BMPs, it is unclear what other effects they may have. If a knockout of BMP-5 displays defects not only in the skeleton, but also the kidneys and lungs, what will be the effect of BMP-5 gene therapy on fully developed kidneys and lungs. The local effect of the BMP at the delivery site is predictable, but systemic effects are not. This suggests that *ex vivo* strategies or direct local protein release may be inherently safer.

### CONCLUDING REMARKS AND FUTURE DIRECTIONS

Significant progress has been made on the effects of growth factors on bone formation. Certain factors, such as BMP-2 and BMP-7, have already been used in clinical trials with promising results. Recent studies suggest that BMP-6 and BMP-9 can be used as potent osteogenic factors. Other growth factors and gene products (such as IGF, TGF, and LMP-1) have also been shown to enhance the bone formation process. However, gene therapy approaches to efficacious bone regeneration have to overcome several major obstacles. First, safer and more efficient gene transfer techniques are needed. Although the currently used viral vectors have a proven biosafety profile, they are not readily accepted by the public, which could affect their ultimate clinical use. An ideal gene delivery system for bone regeneration would be non-viral, locally effective, transient or regulatable. Second, the improvement and refinement of cell-based *ex vivo* gene therapy requires a better understanding of stem cell biology. Several sources of mesenchymal stem cells have been used as recipients of BMP gene transfer thus far with variable outcomes. Stem cells should ideally be harvested in a minimally invasive fashion, transduced, and then re-implanted at the same procedure. Work into different sources of readily available mesenchymal stem cells, such as fat, bone marrow, and skeletal muscle, may hold the key to this strategy. It is conceivable that as stem cell technology progresses, we will have a better means of harvesting and transducing mesenchymal stem cells for bone regeneration. Third, a major area for advancement lies in the development of a biodegradable and cell friendly scaffold. Initial work has begun, with research into different variables affecting gene delivery and osteoconduction. Current efforts are also being devoted to determine optimal pore size for bone and vascular ingrowth, as well as materials to allow optimal elution of proteins [Kuboki *et al.*, 2001]. Further research is needed to explore the geometry of the three-dimensional scaffold to

optimize bone ingrowth. Finally, the molecular mechanisms underlying *de novo* bone formation need to be thoroughly elucidated. This would help us to identify biological factors that transduce a convergent or divergent osteogenic signal during osteogenesis, thereby maximizing osteoinduction by using combinations of growth factors that act synergistically. It is conceivable that in the near future gene therapy can be utilized to induce bone formation in virtually any region of the body in a minimally invasive manner. As bone biology and gene therapy research progresses, the goal of successful human gene transfer for augmentation of bone regeneration draws nearer.

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