Gene therapy approaches to regenerating bone

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Abstract

Bone formation and regeneration therapies continue to require optimization and improvement because many skeletal disorders remain undertreated. Clinical solutions to nonunion fractures and osteoporotic vertebral compression fractures, for example, remain suboptimal and better therapeutic approaches must be created. The widespread use of recombinant human bone morphogenetic proteins (rhBMPs) for spine fusion was recently questioned by a series of reports in a special issue of *The Spine Journal*, which elucidated the side effects and complications of direct rhBMP treatments. Gene therapy—both direct (in vivo) and cell-mediated (ex vivo)—has long been studied extensively to provide much needed improvements in bone regeneration. In this article, we review recent advances in gene therapy research whose aims are in vivo or ex vivo bone regeneration or formation. We examine appropriate vectors, safety issues, and rates of bone formation. The use of animal models and their relevance for translation of research results to the clinical setting are also discussed in order to provide the reader with a critical view. Finally, we elucidate the main challenges and hurdles faced by gene therapy aimed at bone regeneration as well as expected future trends in this field.

Keywords

Gene therapy; Bone regeneration; Tissue engineering; Viral vectors; Nonviral vectors

1. Introduction

Bone formation and regeneration therapies continue to require optimization and improvement because many skeletal disorders remain undertreated. Nonunion fractures, especially those in anatomical locations suffering from a low blood supply such as the distal radius or scaphoid bone, do not have optimal therapies [1, 2, 3]. Five percent of all scaphoid factures are nonunion injuries that cannot heal and are accompanied by severe pain and morbidity [3]. Ten percent of all fractures are nonunion injuries that never heal [4].

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Osteoporotic vertebral compression fractures are the most common fragility fractures in the United States. Accounting for approximately 700,000 injuries per year, these injuries lead to prolonged hospitalizations and result in high health care costs [5]. Current therapeutic strategies include implantation of autologous bone grafts for nonunion fractures [6, 7, 8], vascularized bone grafting in one- or two-stage operations for fractures in anatomical sites with a poor blood supply [3], and vertebroplasty or balloon tamp reduction for vertebral fractures [9]. Those methods are hampered by donor-site morbidity; a limited supply of autologous bone grafts [8]; complicated two-step surgeries [3]; and in some cases, such as vertebral fracture repair, by lack of clinical results [10, 11].

Recombinant human bone morphogenetic protein–2 and rhBMP-7 have been used clinically throughout the last decade to promote fracture repair and bone formation in cases of spinal fusion [7, 8, 12, 13]. However, recent evidence has indicated serious flaws in the use of rhBMP-2 as well as in published reports regarding its application in spinal fusions. A recent review of 13 original industry-sponsored rhBMP-2 studies found that the authors reported 10 to 50 times fewer complications in cases treated with rhBMP-2 than were found in the manufacturer's original FDA summaries [14]. Another review highlighted side effects in the central and peripheral nervous systems associated with rhBMP-2 use, which could explain the high rates of nerve root irritation seen in clinical practice [15]. Moreover, osteolysis (bone resorption by the body) following spinal fusion procedures utilizing rhBMP-2 was found in 54% of cases [16]. All these critical reports were published in a special issue of *The Spine Journal* dedicated to revision of the growing use of rhBMPs in orthopedic medicine. However, it is important to note that therapies based on the use of rhBMP–2 and rhBMP-7 are the only biological solutions currently available to avoid bone harvesting [7, 8, 12, 13]. A systematic review performed in the UK determined that addition of BMP treatment to conventional intervention is more effective than conventional intervention alone for establishing union of acute open tibial fractures [4]. Moreover, a 44% reduction in tibia failure to heal was noticed when rhBMP-2 was administered on a collagen sponge [17]. rhBMP-7 was also used with beneficial results for long-bone nonunion fractures [8]. However, this treatment requires megadoses of the protein—as high as 1.5 mg protein/ml matrix [8, 18]—and thus is not always cost-effective [4].

Gene therapy approaches to bone regeneration are being studied extensively to provide much needed improvements in bone regeneration. Unlike protein-based therapy, gene delivery induces the production of physiological, rather than pharmaceutical, amounts of growth factor over time. Delivery of the gene is much cheaper than delivery of the protein and can be better controlled; in addition, when compared with rhBMP delivery, ex vivo gene therapy was found to be more efficient [19]. Gene therapy, therefore, may provide a better clinical solution to pathological disorders currently treated with rhBMPs. However, among the more than 1300 clinical trials conducted between 1990 and 2007, only 8.2% involved the delivery of growth factors and most of these targeted the cardiovascular system [20]. The fact that rhBMP use is now being reevaluated might help to promote the massive research that has been performed in various animal models into the clinical arena. "

Viral vectors for gene delivery are the most popular vectors used in clinical trials as well as in research due to their high efficiency. Use of nonviral vectors is increasing [20], however, in response to safety issues associated with the use of retroviral vectors [21, 22] and adenoviral vectors [23]. Since brief expression of osteogenic genes is sufficient for bone formation, the use of nonviral vectors can be valuable for bone regeneration applications [24, 25]. Following direct adenovirus-mediated gene delivery, *BMP-6* and *BMP-9* were found to be the most potent inducers of osteogenic differentiation among 14 different *BMP* genes, followed closely by *BMP-2* [26, 27]. This finding was also apparent using nonviral techniques [28, 29, 30], demonstrating the potential of gene therapy in the orthopedic field.

Gene therapy is usually categorized as either in vivo, in which the gene is delivered directly into recipient cells in the site of interest, or ex vivo, in which the gene of interest is inserted in vitro into a targeted cell population (usually stem cells or fibroblasts) and the cells are delivered to the desired site in vivo [31]. Those two gene delivery strategies are usually termed "in vivo gene delivery" and "cell mediated gene delivery", respectively. When gene is delivered into the desired tissue, either directly or by using a cell mediator, a complex cascade of events follows that results in expression of the inserted DNA and in an effect on the expressing cells or the cellular environment. Multitude of factors can affect this process, as recently reviewed [32, 33]. Only few studies compared in-vivo and cell-mediated gene therapy for bone repair. One of those studies demonstrated that while bone formation capacity was similar using both strategies, the use of transfected cells allowed for better control of bone formation [34]. In addition, ex-vivo gene therapy enables better control over the identity of recipient cells in contrast to in-vivo gene delivery in which it is difficult to target the gene to a specific population of cells. The main advantage of in vivo gene delivery approach is that is does not require the complex process of cell isolation, characterization and expansion. Yet, recent studies have shown possible strategies to overcome these disadvantages. Kimelman-Bleich et al. showed that is possible to target gene delivery to a population of host progenitors using an implantation of a biodegradable scaffold [35]. Another study suggested a "same-day" approach in which stem cells were transduced on the same day of isolation and implanted in vivo without an expansion phase [36]. Most orthopedics-oriented approaches include ex vivo gene therapy because of the added benefits of a cellular component, which allows for fast and predictable bone formation. BMP-expressing mesenchymal stem cells (MSCs) are usually used because of their osteogenic [37] and ability to act in both an autocrine and paracrine fashion by differentiation of implanted cells and recruitment of host cells [38].

Here, we review recent advances in gene therapy research aimed at bone regeneration or formation ex vivo and in vivo. The use of animal models and their role in clinical translation are also discussed in order to provide the readers with a critical view. Finally, we discuss the main challenges and hurdles faced by gene therapy aimed at bone regeneration as well as expected future trends.

2. In Vivo Gene Therapy for Bone Regeneration

2.1. Introduction

Table 1 summarizes the studies reviewed in this section.

The first attempts at direct gene delivery aimed at bone regeneration were reported as early as 1996 [39]. Nonviral vectors used at the time included naked DNA delivery and an array of methods designed to enhance the poor efficiency of gene delivery associated with naked DNA delivery, such as dividing DNA delivery into several constitutive injections [40], implementation of gene-activated matrices (GAM) [32], and use of sonoporation and electroporation [28, 29, 30, 35]. Viral vectors, which were more efficient but, alas, raised some safety issues, were also used. Adenoviral vectors were used first and met with satisfying results [26, 41], which were somewhat hampered by the immune system's response to bone formation [42]. Adeno-associated viral vectors (AAVs) were used successfully, mainly when combined with bone allografts [43]. Finally, retroviral and lentiviral vectors were used as well and had a positive influence on bone formation and regeneration [44]. In this section we will review studies performed using in vivo gene delivery for bone formation and regeneration.

2.2. Viral vectors

Ectopic bone formation obtained using various adenoviruses encoding for several *BMP* genes [26, 41] demonstrated the potential use of these vectors for in vivo gene delivery aimed for bone regeneration. Those vectors were not only delivered by injection into the desired site. Adenoviral particles encoding for the *BMP-7* gene (adBMP-7) were mixed with a bovine-derived collagen carrier and implanted in an ectopic site in a mouse model; this experiment showed induced bone formation as well [45]. However, the immune response to the presence of these vectors slowed bone formation in mice treated with adBMP-2 [42] and in rats injected with adBMP-9 [41]. When adBMP-9 was injected into the thigh muscles of athymic rats and Sprague-Dawley rats, the bone volume achieved in the athymic rats was three times higher than that noted in the immunocompetent animals [41]. This work illustrated the effect of the immune response on bone formation when a viral vector is used and the need to circumvent this response.

After the osteogenic effect of adenovector-mediated gene delivery of *BMPs* was established in an ectopic site [42], efforts were made to use this strategy to obtain bone formation at the site of a bone defect. Attempts were made to achieve bone regeneration in several defect models; for example, an adenovirus encoding for the *BMP-6* gene was used for bone repair in a rabbit ulnar osteotomy model [46], and an adenovirus encoding for the *BMP-2* gene was implemented to promote bone regeneration in a femur segmental critical-sized defect in rats [47]. One of the first attempts, made in 2000, involved a rabbit femur segmental critical-sized defect model. Seven weeks following adBMP-2 injection, robust bone formation was noted in defect sites and some of the defects were bridged by new bone [48]. When a similar approach was used to promote bone regeneration in a segmental critical-sized defect in a rat model, however, only 50% of the defects were bridged with mature bone not containing cartilage islands 8 weeks after gene delivery [49].

To improve these results and increase the rate of bone formation, Betz et al. studied the effect of the timing of gene delivery following the creation of the bone defect [50]. Because fracture healing is a well-orchestrated process in which cells and soluble factors appear and act in defined times [51], it made sense that gene delivery should coincide with the appropriate therapeutic window, that is, when targeted cells reside in the defect site. When an injection of adBMP-2 was delayed until 10 days after creation of the defect, most defects later bridged with bone. This compared favorably with findings of no bridged defects when gene delivery occurred at the same time as defect creation and 50% bridged defects when adBMP-2 was delivered 1 day after defect creation [50]. These results demonstrate the importance of the correct timing of gene delivery and are in accordance with findings of studies performed using nonviral gene delivery [35].

The effect of vector dose was also examined in a rat segmental defect model. A high dose of adBMP-2 injected 5 days after defect creation resulted in 100% bridging of bone defects 8 weeks after gene delivery, unlike unfavorable results in animals in which lower doses of vector were used [47]. However, when adenovectors were tested in large-animal models, the results were not consistent. Injection of adenovectors encoding for either *BMP-2* or *BMP-6* into osteochondral defects made in a weight-bearing femoral condyle in a pony model resulted in bone formation but failed to provide long-term healing [52]. However, adBMP-2 injected into bone defects in an osteoporotic sheep model induced fast defect healing, which was characterized by higher callus stiffness during the initial stages of the healing process, compared with results in untransduced controls [53].

Another acellular in vivo gene therapy approach for regenerating bone that recently gained attention is recombinant adeno-associated virus (rAAV)-coated allografts [54, 55]. Following the successful calvarial defect regeneration performed using human MSCs that

were implanted into the defect site and then transduced using a AAV-BMP2 vector, an acellular approach was perused [56]. The initial idea behind this technology was to introduce angiogenic and osteoclastogenic signals to cortical surfaces of massive allografts in order to induce their "revitalization" via vascular invasion and remodeling of necrotic bone [57]. Subsequently, to provide maximal biomechanical healing, rAAV vectors were developed to express osteogenic genes capable of inducing a new bone collar spanning the entire length of the allograft without resorbing it [58, 59]. In a murine femoral allograft model designed to heal a 5-mm mid-diaphyseal defect [43], the best results were obtained using a self-complementary 2.5 serotype vector encoding human *BMP-2* (scAAV2.5-BMP2), which achieved a 25-fold increase in transduction efficiency over the standard rAAV2 vector [59]. In vivo dose-response studies demonstrated that the optimal dose was 4.2×10^8 particles per mm^2 allograft surface, which induced a new cortical shell indistinguishable from that formed by live autografts. These rAAV-coated allografts achieved a 3-fold increase in graft bone volume compared with autografts, an increase due to decreased resorption. This led to biomechanical superiority over both allografts and autografts, and torsional rigidity equivalent to that of unfractured femur.

The greatest obstacles facing gene therapies for bone regeneration surround safety concerns. Although major concerns about this technology have abated because rAAV is a replication-defective nonintegrating vector that is derived from a nonpathogenic virus [60], the potential for cellular transformation and vector genome mobilization cannot be entirely eliminated. Biodistribution studies have shown that the frequency of such highly improbable events is further diminished by the lack of vector dissemination [61, 62] and by rapid clearance of the vector from cell turnover at 3 to 4 weeks [57, 58, 61]. Thus, it seems that rAAV-coated allografts hold great promise for bone regeneration.

Use of a retrovirus or lentivirus for bone induction can be attractive because it ensures sustained expression of the osteogenic transgene and avoids the immune response elicited by adenovectors. Given that retrovectors only affect dividing cells, we can expect that periosteal cells, which are active following fractures, will serve as the vectors' targets [44]. When such a vector was used to deliver a fused *BMP-2/4* gene into a femoral fracture in a rat model, healing was achieved at a rate similar to that of untreated controls and was followed by production of massive amounts of ectopic bone, which eventually remodeled. No transgene activity was found in distant sites, a finding that demonstrated the safety profile of the chosen vector [44]. In an effort to accelerate fracture healing and avoid the excessive bone formation noted when *BMP-2/4* was delivered, Rundle et al. used the same vector for delivery of the *cyclooxygenase-2 (COX-2)* gene [63]. These authors demonstrated that overexpression of *COX-2* in a fracture site resulted in faster healing (3 weeks vs. 5 weeks in the control group) and avoided formation of ectopic bone. Again, gene expression was limited to the defect site [63]. However, no long-term analysis of gene expression and transgene fusion to the host DNA was performed, thus challenging any safety profile of those strategies.

2.3 Nonviral vectors

Nonviral vectors are attractive alternatives because they elicit a minimal immune response on the part of the host. In addition, when the aim is bone formation, transient gene expression—the type of expression achieved using nonviral vectors—is desirable because it limits the amount of newly formed bone and decreases the chance for bone malformation. To improve the poor efficiency of bone formation obtained using naked DNA injections, Osawa et al. divided their plasmid *BMP-2* dose into 2, 4, or 8 daily injections instead of 1 dose [40]. These researchers found that bone formation occurred more frequently when more injections were used. This increased efficiency of bone formation using naked DNA is important because it allows use of a delivery method bearing minimal risk of tissue damage.

Gene-activated matrices (GAMs) were developed to improve the poor gene delivery observed when naked DNA was used and to amplify the structural integrity of newly formed bone by implanting a scaffold within the bone defect site. Basically, this method includes a biodegradable matrix (or scaffold) containing plasmid DNA of a therapeutic gene. In an animal model this matrix is implanted in the area of interest, an ectopic or a defect site, and therapeutic DNA is slowly delivered to surrounding cells. Fang et al. used GAMs in a rat femoral osteotomy model to deliver plasmid DNA encoding for the *BMP-4* gene with or without another plasmid encoding a portion of the parathyroid hormone gene, *PTH1–34* [32]. The investigators observed bridging bone after a few weeks but only in animals in which *BMP-4* was used. The osteotomy site was bridged by bone faster when both plasmids were used. Importantly, in vivo BMP-4 protein production was noted for only 4 weeks following gene delivery, despite the fact that bone continued to form even after 12 weeks. These results demonstrate that direct gene delivery is suitable for bone regeneration, and that transient expression of an inserted gene is advantageous in this setting.

These results demonstrate the efficiency of using GAMs to augment bone formation in defects and highlight the safety of this method. Delivery of the *PTH1–34* gene using GAM was also tested in a canine tibia osteotomy model and found to induce extensive bone formation. Gene activity was noted up to 6 weeks following GAM implantation, and bone formation was monitored up to 6 months. Moreover, this study revealed a connection between bone formation and both the dose of plasmid DNA delivered and the defect size [64]. It seems that the low transfection efficiency of naked DNA and its slow release from the collagen matrix allowed for prolonged gene expression and tissue regeneration. In another study researchers used GAM to deliver a reporter gene, *β-galactosidase*, to analyze the gene expression profile associated with this strategy [65]. To enhance gene delivery, DNA was condensed using poly(ethylenimine) (PEI). A high level of gene activity was noted up to 15 weeks after implantation of GAMs loaded with PEI-condensed DNA, and this was coupled with a high percentage of cells within scaffolds incorporating condensed DNA. A quantitative analysis of *β-galactosidase* gene expression revealed that expression levels in scaffolds incorporating condensed DNA were up to two orders of magnitude higher than those of control conditions [65]. Alginate hydrogel was also used as a substrate for GAM, with modest success in in vivo gene delivery [66]. Further technical improvements will probably result in better gene delivery and tissue responses to GAM implantation.

Aiming at further increasing the efficiency of gene delivery, sonoporation (a transient ultrasound-induced increase in cell membrane permeability) was used to obtain ectopic bone formation in a mouse model. This novel technique was compared to electroporation (electrical field–mediated gene transfer) and found to be much less effective. This first report of ectopic bone formation achieved using ultrasound-assisted delivery of an osteogenic gene (encoding for *BMP-9*) demonstrated the safety of using this method in which gene activity was limited for 2 weeks and no tissue damage was found [30]. In vivo electroporation was also used to obtain regenerated bone in a murine nonunion bone defect model [35]. Following several studies in which ectopic endochondral bone formation had been obtained using in vivo electroporation of plasmid DNA encoding for the *BMP-2, -4*, or *-9* genes [28, 29, 67, 68], the *BMP-9* gene was delivered into a 1.5-mm-long segmental defect created in a mouse radius. To provide a concentration of target cells in the defect site, a collagen sponge was implanted, and 10 days following defect formation and scaffold implantation the *BMP-9* gene was delivered. Using this procedure complete healing of the bone defect occurred 5 weeks following gene delivery. Moreover, no excessive bone formation was noted, and the implanted sponge was populated by host progenitor cells that expressed the delivered gene [35]. This study was the first in which bone regeneration in a nonunion bone defect was achieved using nonviral in vivo gene therapy, and it was among the few successful attempts performed. The calculated use of host progenitor cells coupled

with timed gene delivery and implantation of a scaffold allowed for quick formation of high-quality tissue. Nevertheless, because of safety concerns surrounding the delivery of electrical current, in similar settings in the future sonoporation will probably be preferred from the clinical point of view.

Up-scaling former studies, Park et al. investigated the effect on bone regeneration of using a liposomal vector to deliver the *BMP-2* gene to peri-implant bone defects in a porcine calvaria model [69]. They found that the *BMP-2* gene was efficiently introduced into cells surrounding the bone defect, and that those cells produced BMP-2 protein up to 4 weeks. Subsequently, new bone formation was enhanced compared with control groups. This report demonstrated the potential use of in vivo nonviral gene delivery for bone augmentation in preclinical large-animal models.

3. Ex Vivo Gene Therapy for Bone Regeneration

3.1 Introduction

Table 2 summarizes the studies reviewed in this section.

Many cell types have been used for ex vivo gene therapy applications aimed at bone regeneration. In this section we will focus on the use of MSCs as gene delivery vehicles and therapeutic agents. One of the first reports of using MSCs for this reason was published in 1999. Rat bone marrow–derived MSCs were infected with adBMP-2 and used to heal a critical-size femur segmental defect in syngeneic rats. Almost all defects treated with the BMP-2–producing cells were healed after 2 months—results similar to those obtained when rhBMP-2 was used alone. However, the bone properties of femurs that received implants of BMP-2–expressing MSCs were superior to those of femurs treated with the protein alone [37]. Similar results were noted couple of years later when human MSCs infected with adBMP-2 were used in order to regenerate a radial defect in a mouse model. Interestingly, stem cells isolated from on osteoporotic bone yielded the same results, a finding that suggests that stem cells retain osteogenic potential [70]. In another study conducted at that time, investigators compared the osteogenic activity of a genetically engineered stem cell line with that of Chinese hamster ovary (CHO) cells transduced to express the *BMP-2* gene using nonviral transfection and subsequent clone generation. This study concluded that MSCs expressing the *BMP-2* exerted both paracrine and autocrine effects: the implanted cells recruited host cells to the defect site while differentiating themselves. In addition, compared with the CHO cells, which also expressed the *BMP-2* gene, the engineered MSCs induced more bone formation [38]. Those early studies highlighted the potential use of adult stem cells as gene delivery vehicles. Unlike direct gene delivery, the method does not depend on the local cell population in the defect site. Moreover, as naïve MSCs are highly capable of bone formation [71], the additional expression of an osteogenic gene enhances the cells' activity and results in efficient bone formation and tissue regeneration. In this section we will review studies performed using ex vivo gene delivery aimed at bone formation and regeneration.

3.2. Cell-mediated gene therapy using viral vectors

The selection of a viral vector for ex vivo gene therapy is based on the vector's transduction efficiency, the choice of animal model (lentiviral vectors, for example, do not transduce murine cells at the same level of efficiency as other mammalian cells [72]), and safety considerations. Adenovectors are widely selected because they have a high level of transduction efficiency and because even transient expression of genes from the *BMP* family usually results in bone formation [21, 22]. For example, adBMP-2–transduced rabbit MSCs that were implanted subcutaneously in nude mice induced robust ectopic bone formation 4 weeks after implantation [73]. Since MSCs are mainly isolated from bone marrow or

adipose tissue, the effect of different sources of MSCs on the outcome of ex vivo gene therapy using adBMP-2 was analyzed. No difference in bone formation was noted between human bone marrow–derived MSCs and human adipose tissue-derived MSCs when transduced with adBMP-2 and implanted in athymic rats to obtain spinal fusion. Importantly, bone volume in rats that received implants of engineered MSCs was significantly greater than bone volume in rats treated with implants of naïve MSCs [74]. Human bone marrow–derived MSCs transduced with adBMP-2 were also used to regenerate tissue in mandibular bone defects in NOD/SCID mice; the results were complete bone regeneration [75]. Taken together, it seems that MSCs are highly useful for ex vivo bone gene therapy applications.

These engineered cells also proved useful in large-animal models. When MSCs transduced with adBMP-2 were implanted in a large-scale skull defect in a porcine model, encouraging results were reported. All defects were completely repaired after 6 months both radiographically and histologically. The bone formed by transduced MSCs was significantly thicker and stiffer than bone formed in defects in which naïve MSCs had been implanted [76]. Similar results—and even faster bone formation (3 vs. 6 months)—were reported for another study of adBMP-2–transduced MSCs that had been implanted in a porcine cranial defect [77]. Importantly, in that report the absence of adenovector was assured by immunochemical staining. These reports strongly buttress the feasibility of a clinical ex vivo gene delivery approach mediated by adenovector.

In an effort to achieve a more evidence-based grounding for vector choice, a comparison was made of ex vivo genetic modification of rat MSCs by adenoviral, retroviral, and cationic lipid vectors encoding for the *human BMP-2* gene, with bone formation (in an ectopic or orthotopic critical-size defect created in the rat cranium) as the end point. Bone formation in the defect site was observed in all conditions, but adenovector-transduced MSCs displayed a statistically significant increase in bone formation compared with the other vectors [78]. Although it seems as though adenovector was the most efficient of these vectors, it is important to note that such a comparison is not straightforward. Factors such as vector concentration, mechanism of gene delivery, exposure time [78], and even the carrier used can affect cell efficiency in bone formation.

Retroviral and lentiviral vectors were also used for ex vivo bone gene therapy. This approach involves the highly efficient gene delivery and prolonged gene expression that can be achieved using lentivectors, as they are capable of integrating the transgene into the host cells' genome. For example, complete defect healing occurred when *BMP-4* was delivered by lentivector into rat MSCs that had been implanted in a segmental defect in the rat calvaria. Naïve MSCs did not induce healing, and yielded less bone mineral density than transduced cells [79]. In another study, retroviral vector carrying the *osterix* gene (a zinc finger–containing transcription factor expressed in osteoblasts) was applied to mouse bone marrow–derived MSCs. *Osterix*-overexpressing MSCs induced almost complete healing of calvarial bone defects in a mouse model [80].

An interesting approach aimed at circumventing the laborious, time-consuming, and highly expensive culture phase of ex vivo gene therapy was recently published. This novel "same-day" ex vivo regional gene therapy consists of buffy-coat cells extracted from rat bone marrow, which were lentivirally transduced with *BMP-2* and then implanted in a rat femoral defect at the same sitting. As a control, traditional ex vivo gene delivery (in which cells are isolated, cultured, transduced, and implanted in the defect site) was also used. Similar healing rates were radiographically determined for both methods. Interestingly, the same-day ex vivo approach yielded radiographic evidence of bone healing and higher bone volume. The results of this study suggest an attractive strategy that will be cost effective in

clinical use [36] and will also minimize the risk of MSC transformation during prolonged culture periods. However, substitution of a nonviral method of gene delivery could further enhance the clinical applicability of this approach.

3.3 Cell-mediated gene therapy using nonviral vectors

An interesting approach that combines in vivo and ex vivo gene delivery strategies using naked DNA for gene delivery was reported by Huang et al. [81]. Poly(lactic-co-glycolic acid) scaffolds loaded with plasmid DNA encoding for the *BMP-4* and *VEGF* genes were implanted subcutaneously in NOD/SCID mice along with human bone marrow–derived MSCs. The combined delivery of all three factors—plasmid DNA for *BMP-4*, plasmid DNA for *VEGF*, and the MSCs—resulted in a significant increase in the quantity of newly formed bone compared with any factor alone or any two factors combined [81]. A similar strategy was adopted in another study, in which MSCs were seeded onto a *BMP-2*–containing scaffold, cultured in a perfusion bioreactor, and then implanted subcutaneously in a rat model [82]. This approach can be advantageous because the cellular component enhances the poor transfection efficiency of naked DNA use.

Naked DNA can also be used to generate a cell clone with which bone defects can be treated. MSCs transfected with an inducible *BMP-2*–encoding plasmid (under a Tet-off system, in which *BMP-2* is not expressed when doxycycline is administered) were subjected to a limiting dilution assay and a stable clone was identified [83]. The same type of cells was used for bone formation in an ectopic site, radial defect regeneration, and spinal fusion in a mouse model; and was even combined with a structural allograft [83, 84, 85, 86]. Although those cells constitute an excellent experimental model with which one can study the influence of factors such as external mechanical loading or oxygen supplementation on bone formation [87, 88], such clones cannot be used in a clinical setting.

Liposome-mediated gene delivery to cultured cells has been widely used to increase the efficiency of nonviral gene delivery. A study was conducted to compare liposome-mediated and adenoviral *BMP-2* gene transfer to rat MSCs and the further use of these cells in the healing of critical-size defects in the rat mandible. Use of liposomes was not as effective as adBMP-2 in the rapid healing of defects [89]. Despite the lower efficacy of this method, the higher safety of liposome use enhances the clinical applicability of this method.

Delivery of *basic fibroblast growth factor* in a similar fashion to rabbit MSCs that were implanted in a 15-mm critical-size segmental bone defect in the rabbit radius resulted in enhanced osteogenic properties of the cells followed by increased bone formation and capillary regeneration [90]. Rat MSCs transfected with the *BMP-7* gene and implanted in a distraction osteogenesis site in a rat model exhibited greater bone formation and earlier mineralization in the distracted callus when compared with naïve MSCs [91]. Similar results were noted in a rabbit model of mandibular lengthening. Using liposomes, rabbit MSCs were transfected with plasmid DNA encoding for the *osterix* gene, and the cells were then implanted in the distracted zone. Bone formation was noted when naïve cells and transfected MSCs were implanted. However, the engineered cells produced greater bone mineral density, thicker new trabeculae, and larger volumes of newly formed bone in the distraction zones [92]. Taken together, those studies show that liposome-mediated gene delivery into MSCs can promote bone regeneration, albeit with a somewhat low efficiency.

Nucleofection is the use of electroporation to transfect cells in vitro by nonviral means [93]. When human MSCs were nucleofected with either *BMP-2* or *BMP-9* genes and later implanted in ectopic sites in NOD/SCID mice, bone formation was induced 4 weeks after cell implantation [93]. The same technique was used to achieve posterior spinal fusion in a mouse model in which porcine adipose tissue–derived MSCs were implanted. After

nucleofection with *BMP-6*–encoding plasmid, the cells were injected into the lumbar paravertebral muscle in immunodeficient mice. Large bone mass formed adjacent to the lumbar area, which produced posterior spinal fusion of two to four vertebrae in the treated animals [94]. Following this study, porcine adipose tissue–derived MSCs nucleofected with a *BMP-6*-encoding plasmid were used to repair vertebral bone defects in a rat model with great success [95]. Interestingly, the delivery of more than one osteogenic factor increased the extent of bone formation. The nucleofection-mediated delivery of a *BMP2/Runx2* bicistronic vector into adipose tissue–derived MSCs, which were then seeded onto PLGA biodegradable scaffolds that was later implanted in the dorsal subcutaneous spaces of BALB/c-nu mice, resulted in superior bone formation than that obtained using nucleofection-mediated delivery of *BMP-2* alone [96]. It seems that nonviral methods are viable tools for ex vivo gene therapy for bone regeneration.

4. Animal Models and Clinical Translation

To assess the clinical potential of a gene therapy strategy it must be evaluated in an appropriate animal model. Since gene therapy for bone repair will be used initially to treat difficult clinical scenarios, the animal models selected need to simulate these clinical situations. Therefore, critical-size bone defect models have become popular because the gene therapy strategy must be successful in a defined biological environment [97, 98].

Rodent models are attractive because the cost of the animals is low and they are readily available. A significant advantage of using a murine model is the availability of transgenic animals, which allow one to evaluate molecular responses to in vivo and ex vivo gene therapy strategies [38, 62].

The rat segmental defect model is frequently used to assess different types of gene therapy strategies. There are a number of features of this model that make it quite useful. First, a critical-size defect (range 5–8 mm) simulates a significant bone defect in humans. In addition, by stripping the periosteum in and around the bone defect the biological environment is further compromised [37, 46]. Second, healing in the defect can be assessed using plain radiographic, microCT, and biomechanical testing. This allows one to assess the quality of the bone repair, which is a critical element in determining the feasibility of using any gene therapy strategy in a clinical setting [36, 37, 46]. Third, it is very easy to harvest cells from rat bone marrow, adipose tissue, or muscle [37, 99, 100]. These cells can easily be grown in culture and transduced. This facilitates the assessment of ex vivo gene therapy strategies that can be adapted for the clinic. Fourth, the rat bone is large enough to be treated with a plate or an external fixator, both of which simulate fixation devices used in humans. Finally, the availability of nude rats allows one to test human cells to demonstrate proof of concept so that human cells can be adapted for ex vivo gene therapy strategies [37, 46, 99, 100].

Rodent models can be used to demonstrate proof of concept that a particular gene therapy strategy has clinical potential; however, for these strategies to be adopted in the clinical setting they first need to be tested in larger animals. These animal models can include canine, ovine, equine, or nonhuman primate bone repair models [97, 98, 101, 102, 103]. Potential advantages are associated with these models, including the following: 1) the bone gaps created are similar in size as those seen in humans; 2) bone stabilization techniques simulate treatments used in humans; and 3) if one plans to use an ex vivo strategy, the number of cells that are needed and the cell carriers that can be used may be similar or even the same as those used in humans.

In summary, an effective animal model needs to simulate the clinical problems seen in humans. Essential to a rodent model is a step-wise approach in which healing, local protein

production, and the biology of repair are demonstrated. This information can then be used to fine-tune the strategy in larger animals before considering an evaluation in humans.

5. Challenges, Hurdles, and Future Trends

Nearly no method of direct gene delivery aimed at bone formation has reached the clinical trial phase. One reason for this is the widespread use of autologous bone grafts and rhBMPs for nonunion fractures and for spinal fusion applications. Implantation of autologous bone grafts is the gold-standard treatment for nonunion fractures or massive bone loss, and as much as 1.5 million bone-grafting operations are performed each year in the US [6, 7, 8]. rhBMP–2 and rhBMP-7 are widely used in various orthopedic applications [7, 8, 12, 13] such as establishment of bone union in acute open tibial fractures [4] or long-bone nonunion fractures [8]. However, morbidity at the donor site, the low availability of bone grafts [6, 7, 8], and recent criticism regarding the use of rhBMPs [14, 15, 16] may increase the need for other means to enhance bone formation.

Another hurdle preventing the widespread use of gene therapy lies in the choice of vector used for gene delivery. Viral vectors carry the risk of an immune response, such as the one that caused the death of Jess Gelsinger in a phase I clinical trial [23]. They are also associated with insertional mutagenesis, which may cause malignancy, as occurred in a child suffering from X-linked severe combined immunodeficiency, who developed and died of leukemia after being treated by retrovirus-mediated *gamma(c)* gene transfer to autologous CD34 bone marrow cells [21, 22]. Nonviral vectors, while considered safer, are much less effective than viral vectors [25]. In fact, there has been only one published report documenting complete repair of a nonunion bone defect using nonviral direct gene delivery [35]. The development of novel, safer, and more efficient gene delivery vectors will improve the ability of gene therapy to compete with current therapeutic techniques.

Up-scaling gene therapy from small animal models to large, weight-bearing and functional models is not trivial. Although success has been reported in bone augmentation when direct gene delivery was used in rodent models [35, 47], experiments performed using large animal models so far have yielded modest successes. When the efficiency of adBMP-2 or adBMP-6 was tested in a pony model of an osteochondral defect (13×7 mm) in the femoral condyle, some bone and cartilage formed, but long-term monitoring revealed that this intervention was insufficient to provide proper repair [52]. When the ability of adBMP-2 to achieve bone regeneration in an equine rib model was compared to cell-mediated delivery of the same gene, a lower level of bone regeneration was noted [104]. Those results demonstrate that even after performing proof of concept in small-animal models, a long optimization stage is necessary before application of this method in humans.

Many genes, especially those encoding for growth and differentiation factors, such as the *BMP*s, *basic fibroblast growth factor (bFGF)*, *insulin-like growth factors (IGFs)*, *TGF-β*, *platelet-derived growth factor (PDGF)*, and *vascular endothelial growth factor (VEGF)*, have been used to induce bone formation in vivo [105, 106] with varying results. The use of upstream gene targets, such as *Runx2* [107], or the discovery of novel target genes involved in fracture healing could potentially promote this field. Targets like *leptin* and it receptors, which are up-regulated during the chondrogenic phase of fracture healing [108], or the use of MSCs overexpressing *hypoxia-inducible factor 1α* for the repair of critical-size rat calvarial defects [109] are examples of such genes. When ex vivo gene therapy has been used, genes like *HIF-1α* and *osterix* were successful in promoting bone formation [92, 110]. The combination of several genes, such as *VEGF* and *BMP-6* [111], or *BMP-2* and *BMP-7* [112], also yielded convincing results demonstrating the undiscovered potential of this field. The choice of gene for delivery should also take into account potential adverse effects of

gene overexpression and the effect of the formed protein. rhBMP-2, for example, has been shown to contribute to nerve root irritation in the central and peripheral nervous systems [15] and to osteolysis [16] in many clinical cases.

Another issue that will require much attention prior to clinical application of gene therapy for bone regeneration is control of the amount and shape of formed bone. The use of high doses of rhBMP-2 for cervical spinal fusion resulted in high rates of complications [113]. In several cases of lumbar spinal fusion, rhBMP-2 use resulted in delayed neural compression caused by ectopic bone formation [114], which eventually required surgical removal [115]. It is therefore obvious that strategies for controlling the amount and location of newly forming bone are required. Such a strategy is the use of scaffolds [35]. In this regard, cell-mediated gene delivery, which usually requires placement of scaffolds on which the cells are seeded, may have an advantage. Moreover, the use of injected scaffolds allows for better spatial control of bone formation [94, 95, 116].

Fracture healing and bone formation are complex processes, with specific spatial and temporal gene activities appearing at different stages [51, 108]. As a bone defect site goes through various steps—inflammatory stage, chondrogenic phase, bone formation, and bone remodeling [51, 108]—one must carefully choose the correct timing for gene delivery. This choice must also be guided by the cellular component in the defect site. Delivering a therapeutic gene during the inflammatory stage, for example, will probably not result in proper bone formation. On the other hand, when gene delivery (using viral or nonviral methods) has been postponed until 10 days after defect creation, high levels of bone regeneration have been noted [35, 50]. Moreover, cell-mediated gene therapy may offer a means to circumvent this hurdle, because in that method of gene delivery the cellular component is delivered at the same time as the therapeutic gene.

From a practical standpoint, rAAV-coated allografts possess several empirical advantages over other forms of biological, stem cell, and gene therapies for bone regeneration. An rAAV-coated allograft is a simple off-the-shelf item produced by coating the surface of demineralized bone with rAAV [61], freeze-drying and packaging the coated allograft, and storing it at room temperature for longer than 6 months without a loss in efficacy. In contrast to synthetic scaffolds, the rAAV-coated allograft takes advantage of more than 50 years of clinical experience with massive allografts, which have remained the gold standard because of their broad availability, biocompatibility, and ability to restore biomechanical integrity during the early postoperative period. Moreover, the knowledge that dosing is a function of the allograft's surface and not the size of the patient, which for a 5-cm critical defect in a long bone scales to 10^{12} scAAV2.5-BMP2 particles, abates common concerns about under- or overdosing with commercial biological agents, which are available at a fixed dose for use in all patients. For these reasons, investigators have subsequently evaluated rAAV coatings for soft tissue allografts [117] and stents [118].

The costs of the therapeutic method and optional marketing will also play a pivotal role in moving the aforementioned therapies from the bench to the bedside. In the next few years, we expect to witness a growing trend toward reducing the costs of such a therapy, based on the relative easiness of manufacturing plasmid DNA and viral vectors. Successful delivery of plasmid DNA into the porcine heart by means of in vivo electroporation, which was recently performed [119], demonstrates the high applicability of this method and its future clinical potential. The use of MSCs for ex vivo bone gene therapy may be attractive, because this strategy includes the cellular component that is much needed when massive tissue loss is encountered. Moreover, it allows for better control over gene expression, a better choice of target cells to receive the gene of interest, and usually better experimental results. Since MSCs can be isolated during bone marrow aspirations or liposuction procedures, and can be

easily cultured or frozen with no apparent effect on their differentiation or proliferation capacity [120], they may prove to be an adequate cell source for this application. However, given that differentiated MSCs transduced with adBMP-2 elicited a significant stimulation of the immune system [121] and cell-mediated gene therapy is predicted to be expensive because of the cell's prolonged culture period, the use of "same-day" gene delivery and nonviral vectors may prevail.

6. Summary

We have reviewed gene therapy approaches to regenerating bone, both ex vivo and in vivo, by using viral and nonviral vectors for gene delivery. We have also discussed clinical translation via animal models, clinical trials, and expected challenges and hurdles in this exciting arena. There is no doubt that the field of gene therapy for bone regeneration will expand in the coming years. We believe that by continuing research in this field—both basic and applied—novel pathways will be identified that will enable the clinical application of some of the techniques discussed in this paper.

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Table 1

In Vivo Gene Therapy for Bone Regeneration

Vector and Gene	Target Site	Main Results	Reference
Adenoviral *BMP-7*	Ectopic site (subcutaneous and intramuscular) in a mouse model	Induced bone formation 4 weeks following implantation	Franceschi et al., 2000 [45]
Adenoviral *BMP-2*	Ectopic site (intramuscular) in a mouse model	Bone was formed 2 weeks following gene delivery in immunodeficient mice and 3 weeks following gene delivery in immunocompetent mice	Musgrave et al., 1999 [42]
Adenoviral *BMP-9*	Injection into thigh muscles of athymic rats or Sprague-Dawley rats	Bone volume obtained in athymic rats was 3 times higher than that noted in immunocompetent animals	Li et al., 2003 [41]
Adenoviral *BMP-2*	Rabbit femur segmental defect model	After 7 weeks, robust bone formation was noted in the defect sites and some defects were bridged by new bone	Baltzer et al., 2000 [48]
Adenoviral *BMP-2*	Femur segmental defect regeneration in a rat model	50% of defects were bridged with mature bone that did not contain cartilage islands 8 weeks after gene delivery	Betz et al., 2006 [49]
Adenoviral *BMP-2*	Femur segmental defect regeneration in a rat model	When the adBMP-2 injection was delayed until 10 days after defect formation, 86% of defects were bridged with bone, compared with no defects when gene delivery occurred during defect formation and 50% of defects when the adenovector was injected 1 day later	Betz et al., 2007 [50]
Adenoviral *BMP-2*	High dose of adBMP-2 injected 5 days after defect formation in a rat femur segmental defect model	100% bridging of defects 8 weeks following gene delivery	Betz et al., 2007 [47]
Adenoviral *BMP-2* or *BMP-6*	Osteochondral defects in a femoral condyle in a pony model	Bone formation failed to provide long-term healing	Menendez et al., 2011 [52]
Adenoviral *BMP-2*	Tibia bone defects in an osteoporotic sheep model	Induced faster defect healing, higher callus stiffness during the initial stages of the healing process	Egermann et al., 2006 [53]
AAV *rhBMP-2* under TetON regulation	Ectopic site (intramuscular) and critically sized calvarial defects in a mouse model	Mice that were given Dox demonstrated bone formation in both in vivo models compared to none in mice prevented from receiving Dox	Gafni et al., 2004 [56]
rAAV-caAlk2	Mouse femoral allograft Model	Complete bridging of bone around a cortical allograft was possible	Koefoed et al., 2005 [58]
scAAV2.5-BMP2	Mouse femoral allograft model	scAAV2.5-BMP2 allografts formed a new cortical shell that was indistinguishable from that formed by live autografts	Yazici et al., 2011 [59]
Retroviral fused *BMP-2/4*	Femoral fracture in a rat model	Healing was achieved in a similar rate to untreated controls and was followed by production of massive amounts of ectopic bone that eventually remodeled	Rundle et al., 2003 [44]
Retroviral *COX-2*	Femoral fracture in a rat model	Faster healing (3 vs. 5 weeks in the control group) and avoided ectopic bone formation	Rundle et al., 2008 [63]
naked DNA, *BMP-2*	Repeated injections (1 –8 times) into the skeletal muscle of mice at a divided dose	Bone formation was more frequent when more injections were used	Osawa et al., 2010 [40]
GAM, *BMP-4*	Femoral osteotomy model in rats	Bridging of the gap observed after 9 weeks, while healing was achieved after 18 weeks	Fang et al., 1996 [39]
GAM, *BMP-4 & PTH1–34*		Bridging was observed at 4 weeks and healing at 12 weeks	

Vector and Gene	Target Site	Main Results	Reference
GAM, *PTH1–34*	Canine tibia osteotomy model	Connection found between bone formation and both the dose of plasmid DNA delivered and defect size	Bonadio et al., 1999 [64]
Sonoporation, *BMP-9*	Ectopic (intramuscular) bone formation in a mouse model	Gene activity was limited for several weeks and no tissue damage was found	Sheyn et al., 2008 [30]
Electroporation, *BMP-9*	Nonunion radial defect regeneration in a mouse model	Complete healing of the bone defect 5 weeks following gene delivery	Kimelman-Bleich et al., 2011 [35]
Liposome-mediated *BMP-2*	Peri-implant bone defects in a porcine calvaria model	New bone formation was enhanced compared with control groups	Park et al., 2006 [69]

Table 2

Ex Vivo Gene Therapy for Bone Regeneration

Vector and Gene	Cells	Target site	Main Results	Reference
Adenoviral *BMP-2*	Autologous bone marrow cells	Femur segmental defect in syngeneic rats	Superior bone properties to those treated BMP-2 protein alone	Lieberman et al., 1999 [37]
Adenoviral *BMP-2*	Human bone marrow MSCs	Subcutaneously implantation and radius bone segmental defect in nude mice	Bone formation in ectopic sites, and radius bone regeneration. Similar results were obtained with hMSCs isolated from a patient suffering from osteoporosis	Turgeman et al. 2001 [70]
Adenoviral *BMP-2*	Rabbit bone marrow MSCs	Subcutaneously implantation in nude mice	Robust ectopic bone formation after 4 weeks	Han et al., 2008 [73]
Adenoviral *BMP-2*	Human bone marrow vs. adipose tissue–derived MSCs	Athymic rat spinal fusion	No difference in bone formation was noted	Miyazaki et al., 2008 [74]
Adenoviral *BMP-2*	Human bone marrow MSCs	Mandible bone defect regeneration in NOD/SCID mice	Complete defect regeneration achieved	Steinhardt et al., 2008 [75]
Adenoviral *BMP-2*	Porcine bone marrow MSCs	Large-scale skull defect in a porcine model	Defects were completely repaired after 6 months. The bone formed was significantly thicker and stiffer.	Chang et al., 2009 [76]
Adenoviral/retroviral/cationic lipid, *human BMP-2*	Rat bone marrow MSCs	Ectopic (subcutaneous) bone formation or orthotopic critical-size defect in a rat cranium	adBMP-2 MSCs showed statistically significant increase in bone formation relative to the other vectors	Blum et al., 2003 [78]
Lentiviral *BMP-4*	Rat bone marrow MSCs	Segmental defect in rat calvaria	Complete defect healing	Gysin et al., 2002 [79]
Retroviral *osterix*	Mouse bone marrow MSCs	Calvaria bone defects in a mouse model	MSCs induced 85% healing	Tu et al., 2007 [80]
Lentiviral *BMP-2*	Rat bone marrow buffy-coat cells	"Same-day" implantation in a rat femoral defect	Radiographic evidence of bone healing and higher bone volume	Virk et al., 2011 [36]
Plasmid DNA, *BMP-4* and *VEGF*	Human bone marrow MSCs	Implanted subcutaneously in NOD/SCID mice	Significant increase in the quantity of bone formed	Huang et al., 2005 [81]
Plasmid DNA, *BMP-2*	Rat bone marrow MSCs	Culture in a perfusion bioreactor and then implanted subcutaneously in a rat model	Homogeneous bone formation was histologically observed	Hosseinkhani et al., 2006 [82]
Liposome-mediated and adenoviral, *BMP-2*	Rat bone marrow MSCs	Healing of critical size defects in the rat mandible.	Defects were healed at 6 weeks after gene transfer when liposomes were used, and within 4 weeks when adBMP-2 was used	Park et al., 2003 [89]
Liposome mediated, *bFGF*	Rabbit periosteum MSCs	Critical-size segmental bone defect in the rabbit radius	Elevation in bone formation and capillary regeneration	Guo et al., 2006 [90]
Liposome mediated, *BMP-7*	Rat bone marrow MSCs	Mandible distraction osteogenesis site in rat model	Higher bone formation and earlier mineralization in the distracted callus	Hu et al., 2007 [91]
Liposome mediated, *osterix*	Rabbit bone marrow MSCs	Rabbit model of mandibular lengthening	Higher bone mineral density, thickness of new trabeculae, and volume of the newly	Lai et al., 2011 [92].

Vector and Gene	Cells	Target site	Main Results	Reference
			formed bone in the distraction zones	
Nucleofection, *BMP-2* or *BMP-9*	Human bone marrow MSCs	Implantation to ectopic (intramuscular)site s in NOD/SCID mice	Bone formation was induced 4 weeks after cell implantation	Aslan et al., 2006 [93]
Nucleofection, *BMP-6*	Porcine adipose tissue–deriv ed MSCs.	Posterior spinal fusion in a immunodeficient mouse model	Large bone mass was formed adjacent to the lumbar area.	Sheyn et al., 2008 [94]
Nucleofection, *BMP-6*	Porcine adipose tissue–derived MSCs	Repair of vertebral bone defects in a rat model	The rate of bone formation was two times faster than that in the no cells treated group	Sheyn et al., 2011 [95]
Nucleofection, *BMP2/Runx2*	Human adipose SCs	Dorsal Subcutaneous implantation to BALB/c-nu mice	Superior bone formation compared with cells nucleofected with *BMP-2* alone	Lee et al., 2010 [96]