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Abstract

Bone, despite its strength, is a fragile tissue that often fails under a variety of conditions. While biological therapies for stimulation of bone formation and repair have advanced to clinical practice, gene delivery is alternatively explored to provide more cost-effective and efficacious therapies. Due to safety concerns associated with viral gene carriers, non-viral gene carriers are actively developed to realize the dream of safe and efficient bone repair based on gene therapy. This chapter will summarize the recent gene therapy attempts, focusing on outcomes in key studies involving animal models. Carriers derived from synthetic materials will be first reviewed, followed by efforts to utilize gene activated matrices. Direct injection of genes and gene-modified matrices will be subsequently summarized, followed by physical methods of forced gene expression, electroporation and sonoporation, with select examples. We conclude with the authors' perspective on the future of gene therapy for bone repair.

Introduction

The bone is an important tissue of the body that can physically protect internal organs, provide a space to support hematopoiesis, and serve as a reservoir to maintain blood calcium homeostasis. Structurally, bones can be classified into two categories, namely, *cortical* and *cancellous* bones [90]. While a compact structure and uniform microscopic channels are present in cortical bones, nonuniform convolutions of lamellae are the property of cancellous (or trabecular) bones [29, 30]. Developmentally, bone formation happens in two distinct processes. One process is the *endochondral ossification*, where a cartilaginous

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template precedes the formation of a calcified mature bone structure. Formation and elongation of most load-bearing bones in the body occurs by this process. The other process occurs without a cartilaginous template and is termed *intramembranous ossification*. Skull bones, for instance, are formed by this process.

Structural integrity and shape of the bone are constantly maintained by dissolution (or resorption) and removal of old matrix and in situ synthesis of new bone. The primary players in this process constitute two major cellular components of bone tissue, namely, the “bone-forming” *osteoblasts* and the “bone-resorbing” *osteoclasts*. Osteoblasts are derived from mesenchymal stem cells (MSCs) and are responsible for synthesizing organic extracellular matrix and controlling matrix mineralization [75]. Osteoclasts, on the other hand, are multinucleated cells derived from differentiation of hematopoietic precursor cells in the monocyte-macrophage lineage, under the influence by macrophage colony-stimulating factor and nuclear factor- κ B ligands. These cells continuously resorb the formed bone [107]. Although bone resorption and formation appear as two independent processes, in reality, there are closely connected within temporary anatomical structures termed *basic multicellular units* (BMUs). A developed BMU supports osteoblasts, osteoclasts, connective tissue, and a dedicated source of blood supply. As the osteoclasts resorb bone, the entire BMU is postulated to advance alongside the bone with the resorbed bone replaced by osteoblasts synthesizing the bone matrix. Osteoblasts directly regulate bone matrix synthesis and mineralization, in addition to indirectly regulating osteoclast-mediated bone resorption via paracrine control [95].

Bone tissue has regenerative capabilities that allow majority of fractures and/or tissue loss to heal on its own or recover after noncritical orthopedic procedures [26]. Following a fracture, the tissues surrounding the fracture site – the cortical bone, periosteum, external soft tissues, and bone marrow – contribute to the healing process by formation of a connective tissue *callus*. A combination of endochondral and intramembranous ossification followed by bone remodeling com-

pletes the process of fracture repair [25]. Despite the excellent repair potential of bone tissue, in situations where trauma results in a critical-sized bone defect, complete regeneration cannot occur [46]. Severe trauma may lead to permanent impairment of blood supply, ischemia, and osteonecrosis and ultimately result in bone loss. In addition, the process of bone healing is impaired in conditions such as excessive alcohol intake [16], diabetes [36], smoking [63], old age [43], and osteoporosis [41]. Fractures in these clinical settings do not heal properly or in time and often result in *nonunion* and/or *malunion*. Therapeutic stimulation of bone healing is expected to offer significant clinical benefit in such scenarios.

The process of fracture healing is a specialized postnatal repair response that recapitulates embryological skeletal development [33]. Adequate bone repair comprises three essential elements: osteoconduction, osteoinduction, and vascularization [73]. Osteoconduction is the growth of bone from existing bone and can be facilitated by the application of a scaffold in the defect site. The implanted scaffold in this case offers a bridging structure to connect the fractured bone ends and support cell attachment [10]. Osteoinduction, on the other hand, is a more complex biological process (reviewed elaborately in [21, 39, 79]), involving (1) recruitment of mesenchymal osteoprogenitor cells (e.g., MSCs), (2) transformation of undifferentiated mesenchymal cells into “bone-forming” osteoblasts, and (3) formation of new mineralized bone tissue by the differentiated osteoblasts. Vascularization involves growth of new blood vessels to perfuse the regenerating tissue. Aside from nutrient transport and waste removal, the vasculature plays a crucial role in bone formation via production of growth factors that control the recruitment, proliferation, differentiation, function, and/or survival of osteoblasts, osteoclasts, and other supporting cells [96]. The objectives of therapeutic bone regenerative strategies are to provide these three elements in the appropriate order and proportion to promote bone repair. The readily available materials which accommodate these characteristics and used clinically for bone regeneration include autologous bone grafts and

allogeneic banked bone grafts [12]. However, limited availability of autologous transplantable bone tissue, donor site morbidity, shortage of allogeneic donors, and concerns associated with allogeneic tissue transplantation have imposed serious limitations on the sustained use of these materials [35]. Conversely, several synthetic bone substitutes have been developed for clinical application [105], but they are unable to match the autograft in osteogenic capacity [12]. This calls for closer understanding of the physiology of fracture healing and repair, in order to develop efficient therapeutic alternatives.

Ongoing research demonstrates that bone regeneration is initiated and controlled at the molecular level by the coordinated play of several factors broadly categorized into three types: (1) proinflammatory cytokines, (2) bone morphogenetic proteins (BMPs) and related growth factors, and (3) proangiogenic factors. The proinflammatory cytokines are derived from macrophages at the injury site and primarily include interleukin-1 (IL-1), IL-6, and tumor necrosis factor- α (TNF- α). These cytokines initiate the repair response by chemotactic recruitment of inflammatory and endogenous fibrogenic cells, promote extracellular matrix synthesis, and trigger angiogenesis [86]. Growth factors derived from degranulating platelets in the injury site such as platelet-derived growth factor (PDGF) and transforming growth factor- β (TGF- β) are chemotactic and/or mitogenic for macrophages and osteoblasts. The osteoblasts, chondrocytes, and a subset of mesenchymal cells secrete several structurally and functionally related protein factors termed BMPs. BMPs constitute a group of crucial morphogenetic signals controlling important steps in cartilage and bone formation, such as chemotaxis, mesenchymal and osteoprogenitor cell proliferation/differentiation, angiogenesis, and controlled synthesis of extracellular matrix [13, 21]. BMPs also stimulate the synthesis and secretion of other bone and angiogenic growth factors such as insulin-like growth factor (IGF) and vascular endothelial growth factor (VEGF), respectively [21]. Almost all of these growth factors have been studied as potential therapeutic agents to enhance the repair of bone [70].

Several growth factors in the form of recombinant proteins have been investigated as therapeutic agents for bone regeneration and fracture repair. However, a major concern is the adequacy of a single dose of exogenous protein to elicit a sustained clinical response in patients, especially in situations wherein viability of the host bone and surrounding soft tissues is compromised. To tackle this potential concern, a better strategy for therapeutic delivery is gene therapy. Gene therapy involves delivery of genetic information to particular cells, which then synthesize the protein in situ. Firstly, gene therapy can be applied either regionally or systemically. Secondly, the therapeutic gene can be introduced into cells in situ, at the specific anatomic site of interest. Alternatively, the therapeutic gene can be introduced ex vivo, in which cells are harvested from the patient, the deoxyribonucleic acid (DNA) is transferred to these cells in culture, and the genetically modified cells are then administered back to the patient [73, 98]. Based on the therapeutic application, both short-term and long-term protein expressions are desirable following gene therapy. For instance, chronic diseases, such as osteoporosis or rheumatoid arthritis, would probably require long-term expression. Fracture or small bone defect repair may only require short-term protein production. The duration of protein synthesis after gene therapy depends on the techniques used to deliver the gene to the cells (either ex vivo or in situ). The rest of the chapter discusses various gene therapy approaches for bone repair and regeneration.

Overview of Therapeutic Approaches

Gene therapy has become a promising research area for bone regeneration [115, 119]. Successful studies on modified osteogenic cells, which were delivered by using biomaterials for triggering bone repair, have been reported for years. Plasmid deoxyribonucleic acids (pDNA) encoding for BMPs and other growth factors were explored for accelerating the healing process and period. Administration of pDNA coding for “therapeutic

proteins” by the host cells is a process of *positive gene expression*, where the introduced factors are expected to stimulate bone formation. *Negatively regulated protein expression* refers to situation where application of genetic elements is intended for hindering osteogenesis. Ribonucleic acid interference (RNAi) mechanism can provide a means to suppress translation of desired proteins and prevent protein expression, therefore hindering osteogenesis usually [94]. The short interfering RNA (siRNA), in this case, can act as a pharmacological agent to suppress the expression of desired proteins. Most therapeutic approaches, however, are intended to stimulate bone formation directly, and negative regulation could be employed to inhibit the inhibitors of bone formation, so as to ultimately enhance the bone formation. Plasmids are the preferred expression vectors [73] due to reduced manufacturing expenditures and good safety properties when compared to viral vectors [109]. Storage conditions of pDNA are important for transfection efficiency [18, 47, 77, 102], and activity loss in gene delivery systems can be reduced by lyophilization or low storing temperature [18, 20, 47, 102]. Degradation rate of the scaffolds can effect pDNA release [58] and possibly influence the induction of new bone tissue.

MicroRNAs (miRNAs) are small single-stranded RNA molecules that are responsible for coordination of protein expression by reducing level of target messenger RNA (mRNA) or binding to 3' untranslated region of target mRNA and constraining mRNA translation [45]. Endogenous expression of multiple growth factors can be regulated by overexpression or inhibition of miRNAs [114]. For tailoring bone regeneration, select miRNAs may have a role by adjusting osteogenesis and/or endogenous angiogenesis [69]. Gene expression at the time of MSC differentiation to osteoblasts can be induced by miRNAs [106]. miRNAs can therefore serve as an alternative to gene delivery approaches for protein expression.

Tissue-engineered bone grafts that are derived from gene therapy approaches have been also used in curative bone regeneration [71, 72]. In this approach, the cells intended for the engineered tissue are modified by genetic means for

enhanced osteogenesis. In contrast to relying on *in situ gene uptake and expression*, this approach allows *ex vivo gene transfer* for better control of cell modification.

Nonviral Approaches to Gene Delivery in Animal Models

Multiple approaches have been used to induce bone regeneration by using gene medicines. A common model used to evaluate gene medicines, for example, is calvarial osteotomy model (Table 20.1), where different approaches for gene-based bone repair can be readily seen; one can find the use of gene expression vectors with and without a carrier, as well as direct *in situ* administration, and indirect delivery via modified cells. Below, the bone tissue gene therapy paradigm is presented in detail.

Synthetic Carriers Used in Gene Delivery

Gene therapies are intended to maintain optimal doses and local concentration of therapeutic proteins over a period of time under a minimal side effect which demonstrates the superiority of gene delivery over conventional protein delivery [81]. Significant achievements in gene delivery for bone regeneration and their intraosseous expression using both viral vectors, derived from adenovirus, retrovirus, lentivirus, and synthetic vectors, liposomes, and cationic polymers/dendrimers, have been reported elsewhere [4, 6, 40, 49]. Both *ex vivo* and *in vivo* approaches to delivery have been attempted with both types of vectors (Fig. 20.1) [92].

Nonviral gene delivery has been recently emphasized in the field due to facile chemical strategies, stability for long-term storage and reconstitution, safe toxicity profiles, and unlimited capacity of gene sizes (cargo). In gene therapy of the bone, two fundamental (indispensable) aspects of the therapy are *carrier vectors* (i.e., cationic molecules with enough binding capacity to protect nucleic acids and facilitate intracellular trafficking) and

Table 20.1 Selected studies using gene therapy to promote bone regeneration in calvarial critical-sized bone defect models

Animal	Gene	Scaffold	Vector	Outcome	Reference
No carrier					
Rat	BMP-2	Triacrylate/ amine-gelatin	Free plasmid	Minimal to no effect on bone formation	Chew et al. [19]
Synthetic carrier					
Rat	PDGF-B	Collagen	PEI	14- and 44-fold higher new bone volume/total volume in treated calvarial defects compared to empty defects or empty scaffolds after 4 weeks of implantation	Elangovan et al. [27]
Rabbit	BMP-2	Porous HA	Cationic liposomes (SuperFect™, QIAGEN GmbH)	Robust bone formation in the cranial defect in animals treated with BMP-2 gene without HA; complete ossification observed at 9 weeks	Ono et al. [83]
Mouse	caALK6 and Runx2	PEG-block cationomer	Linear PEI (Fermentas) and FuGENE6 (Roche)	Bone formation covering entire lower surface of the implant at 4 weeks	Itaka et al. [50]
Rat	BMP-2	PFF	TAPP complexed with GMP	No enhanced bone formation (via micro-CT and histology) at 12 weeks postimplantation	Chew et al. [19]
Rat	BMP-4	PLGA	PEI	Enhanced bone regeneration at defect edges measured via histological and micro-CT evaluation; increase in osteoid and mineralized tissue density	Huang et al. [48]

HA hydroxyapatite, PEG poly(ethylene glycol), PEI polyethylenimine, caALK6 activin receptor-like kinase 6, Runx2 runt-related transcription factor 2, PFF 1/4 poly(propylene fumarate), TAPP triacrylate/amine polycationic polymer, GMP gelatin microparticles, MSC mesenchymal stem cell

biomaterial scaffolds (i.e., a three-dimensional construct to deliver complexes to repair sites, control release kinetics of complexes, and provide a milieu for osteoinduction). Effective nonviral vectors are generally constructed from cationic polymers, dendrimers, or lipids, but cationic polymers are the most attractive candidate [78, 80, 85, 89]. The common cationic polymers employed in these approaches comprise of polyethylenimine (PEI), poly(L-lysine) (PLL), poly[2-(dimethylamino)ethyl methacrylate] (PDMAEMA), poly(amidoamine) (PAMAM), and chitosan (CS) (Fig. 20.2a) [78, 80, 85].

Electrostatic interaction between cationic primary amines of polymers and anionic phosphate of polynucleotides forms the foundation of nonviral

gene delivery. It leads to formation of condensed polyionic complexes (polyplexes) and protects the encapsulated cargo from enzymatic and nonenzymatic degradation, avoids the clearance through the reticuloendothelial system (RES), enhances cellular uptake via interactions with anionic cell surface proteoglycans, and finally increases half-life in the cytoplasm [3, 24, 99]. Many studies have shown that factors such as size, surface charge, chemical composition, degradability, and stimulus sensitivity affect cellular uptake and intracellular trafficking [17, 56]. The widely accepted benefit of cationic polymers in gene delivery is thought to be derived from their extraordinary cationic charge density and buffering capacity. Buffering capacity

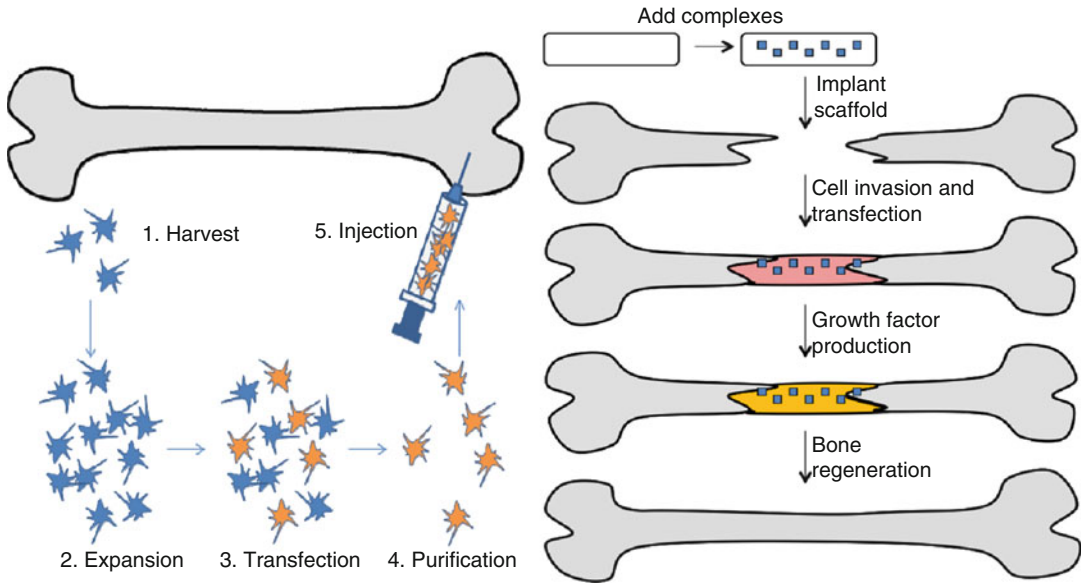


Fig. 20.1 Gene delivery approaches to the bone. In vivo approach (*right*) relies on the introduction of the therapeutic genes directly in suspension form or via gene-activated

matrix (as shown). Ex vivo approach (*left*) relies on modified primary cells and subsequent implantation to the repair site (Adapted from Rose et al. [92] with permission)

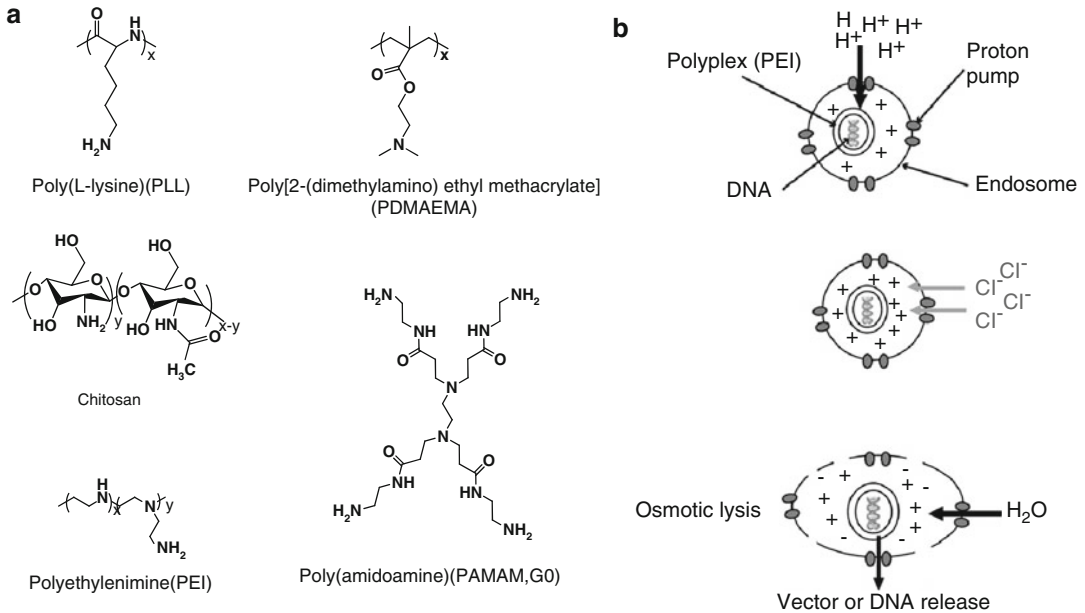


Fig. 20.2 (a) Chemical structure of common synthetic cationic polymers/dendrimers used in nonviral gene delivery. (b) Schematic representation of proton-sponge effect

of the cationic polyplexes (The figure is adapted from Ref. [80] with permission)

is a specific feature of cationic polymers that enables “proton-sponge” effect (Fig. 20.2b) [108]. In PEIs particularly, secondary and tertiary amines generate buffering capacity over a wide range of pH values and facilitate endosomal escape. It has been reported that the gene delivery efficiency of cationic polymers depends on degree of polymerization (molecular weight), branching (topology), and the buffering capacity, which is a function of cationic charge density. As an example, Godbey et al. reported that, under in vitro condition, transfection efficiency of PEIs increases with molecular weight (MW) (70 kDa PEI >10 kDa PEI >1.8 kDa PEI) [42]. However, in vivo efficiency decreases with MW (25 kDa PEI >50 kDa PEI >800 kDa PEI) [1]. Despite intensive activity, however, concrete relationships among structure-property-functional performance remain incompletely described [37]. In the last few years, PEIs and its derivatives are investigated in gene delivery for bone regeneration studies in both ex vivo and in vivo models [27, 93]. The efficacy of native polymers is generally improved by hydrophobic modification using aliphatic lipid molecules [93]. The effect of MW in transfection efficiency was also observed in PLL, a widely used biodegradable polypeptide. Low MW PLL (<3 kDa) cannot even form complexes with DNA, whereas the efficacy of PLL 211 kDa/DNA complexes was 20-fold higher than PEI-20 kDa/DNA complexes, but the intolerable toxicity of higher molecular weight PLL limits its frequent application [64]. The amines of PLLs are completely protonated at physiological pH indicating inefficient buffering capacity, an essential mechanism for endosomal escape [2]. Dendrimers are another class of synthetic polymers with spherical highly branched geometry that comprises of primary amines on the surface to participate in DNA binding and buried tertiary amines to generate the proton-sponge effect. The particular interest in PAMAM is due to their customizable structure with reasonable functionality, which provides enough space for tailoring of appropriate ligand [23]. Like linear polymers, transfection efficacy of PAMAM dendrimers is also proportional to MW (i.e., the generation number) [61]. The CS, on the other hand, is a natural cationic polysaccharide polymer with a good biocompatibility and muco-

adhesive and immunogenic properties that are obtained by partial deacetylation of chitin derived from crustacean shells [7].

Calcium phosphate (CaP) is one of the most studied inorganic materials employed to fabricate gene-activated matrix (GAM) for bone tissue engineering [9, 62, 113]. CaP/DNA coprecipitation technique has been used since 1970 for in vitro gene delivery due to its simplicity and nontoxic profiles [22]. CaP complexes of DNAs are tight and compact that likely keep DNA intact at transplanted or injected site; this increases its bioavailability, which is greater than common polymeric carriers [34]. These complexes display enough resistance against serum DNases that is the cause for higher efficacy [62].

In recent years, multiple strategies are being actively pursued in designing second-generation polymeric carriers via chemical modification [56, 118] and by engineering prefabricated nanostructured carriers [101]. Chemical modification alters the physicochemical properties of native polymers and generates new chemical functionalities that can further alter the physical and biological properties of native polymers. Despite extensive work in cell culture, the potential of the vast array of second-generation polymers remains to be fully explored in bone regeneration.

Gene-Activated Matrices (GAMs) for Gene Delivery

Controlled release of an expression vector (pDNA) to surrounding tissues can be achieved by a GAM. Diverse materials such as collagen, CS, silk, synthetic polymers, minerals, and their composites can be utilized as the basis of GAM. The specific examples from published studies were provided below. For enhancing GAM’s transfection effectiveness in cells and triggering more effective bone regeneration in vivo, researchers have utilized PEI [48], CaP precipitates [28], or liposomes [74] as the pDNA carriers, although pDNA without any carriers were also attempted [94].

In a recent study, collagen scaffolds were used to deliver PEI/pDNA complexes encoding PDGF-B for bone regeneration in a rat calvarial

model [27]. Bone bridges were established at critical-sized defects in rats that were healed with scaffolds involving PEI/pDNA complexes alone. Trabecular bone volume fraction, degree of trabecular connectivity, and connectivity density were significantly higher in complex-enclosed scaffolds than the control interventions. A collagen sponge incorporating pDNA encoding parathyroid hormone (PTH) 1–34 or BMP-4 was explored by Fang et al. [31]. Osteogenic stimulation was obtained in segmental defect models in rats with desired consequences. In another study, the osteogenic potential of BMP-2 gene/fibronectin/apatite composite layer on hydroxyapatite (HA) ceramic scaffolds was investigated. The scaffolds were implanted into rats subcutaneously [110], and gene expressions for BMP-2 and alkaline phosphatase (ALP) were found to be increased upon application of composite layer-coated scaffolds. Administration of PEI-condensed pDNA encoding for BMP-4 from poly(lactic-co-glycolic acid) scaffolds was studied in rat cranial critical-sized defect models [48]. Bone regeneration was significantly enhanced at defect edges according to histological and microcomputed tomography evaluation in this approach. Increase in osteoid and mineralized tissue density was also obtained. Bone defects in a very large animal (horses) were repaired with collagen matrix containing human PTH (1–34) expression vector [5]. Enhanced bone formation in cortical defects was observed in human PTH-collagen matrix group.

Most GAM explored the delivery of a BMP-2 expression vector by using a carrier (or transfection agent). HA ceramic buttons with a layer containing BMP-2 gene and fibronectin were utilized to treat bone defects on cranium of rats [116]. Results showed that expressions of BMP-2, ALP activity, and osteocalcin were elevated in the HA-BMP-fibronectin group, with increased bone formation. An alginate hydrogel containing BMP-2 expression vector and goat multipotent stromal cells was implanted intramuscularly in goats [112]. Bone induction was observed at the implanted ectopic sites, and goat multipotent stromal cells/BMP-2 pDNA treatment resulted with higher collagen deposition and bone formation. A BMP-2 expression vector was also implanted in dorsal muscles of rats by using HA

fibers [82]. The HA fibers incorporating high doses (50 or 100 μg) of BMP-2 expression vector induced more bone mineral content than other implant groups at 4 weeks. The HA fiber containing 50 μg dose of BMP-2 expression vector led to higher osteogenesis as compared to other groups according to radiographic analyses at 8 and 12 weeks. Alternatively, a pDNA encoding BMP-2 in triacrylate/amine polycationic polymer (TAPP) was applied with gelatin microparticles buried within scaffolds in a critical-sized rat cranial defect model [19]. Unlike other studies, the TAPP/pDNA polyplexes did not cause higher bone formation rate than other groups. The reason for this lack of effect could be due to polycationic polymers that had slow degradation rate to trigger sustained pDNA release from scaffolds. Also, in situ cytotoxicity of the polymers might have prevented bone induction, which should be evaluated in conjunction with osteogenesis studies. Bovine atelocollagen and pDNA that encodes human BMP-2 with CaP combination were investigated for treatment of critical-sized segmental bone defects in rats [28]. Bone defects were started to be healed and improved bone strength was obtained in BMP2/CaP-collagen group. Finally, a unique gene formulation was developed by coating preformed cationic PEI/pDNA polyplex with anionic peptide-PEG copolymers for development of so-called copolymer-protected gene vector (COPROG) [100]. Kirschner wires were then coated with COPROG/BMP-2 pDNA and applied in rat tibias intramedullary. Based on biomechanical analysis, the highest load was obtained with the BMP-2 gene delivery group, indicating the feasibility of turning a fracture stabilization device into a plasmid (gene) delivery system.

In addition to these osteogenic genes that were intended to directly stimulate osteogenic events, genes for cytokines involved in angiogenesis were also delivered for indirect stimulation of bone induction. A pDNA coding for human VEGF-165 was coated on collagen sponges (using no carriers or transfection agents) and applied to critical-sized defects in rabbits [38]. More bone formation and endothelial area were observed in the VEGF gene-delivered group, presumably linking enhancing angiogenic activity to bone deposition ultimately.

The synthetic siRNA has been alternatively used to silence specific targets and stimulate bone formation. Dioleoyl trimethylammonium propane (DOTAP)-based cationic liposomes were used in rats to deliver a siRNA that targeted casein kinase-2 interacting protein-1 (encoded by *Plekho1*) [117]. The mass (given by bone mineral density) and micro-architecture of bone were augmented in treated groups. Implantation of silk fibroin-CS scaffolds with a siRNA against guanine nucleotide-binding protein alpha-stimulating activity polypeptide 1 and prolyl hydroxylase domain-containing protein 2 was studied in the periosteum of sheep [91]. An increase in induced bone volumes was observed in the siRNA treatment groups.

In other studies, transfected cells/scaffold combinations were investigated which functions differently from the principle of GAMs. The basic fibroblast growth factor (bFGF)-transfected MSC containing beta-tricalcium phosphate ceramics (beta-TCP) were applied to critical-sized segmental bone defects of rabbits [44]. The results demonstrated that capillary-bone regeneration was higher in bFGF-transfected MSC/beta-TCP group. Arthrodesis at the dorsal spine of rats was treated with devitalized bone matrix which was soaked with bone marrow cells [8]. In a controlled design, one defect site had marrow cells transfected with complementary DNA (cDNA) encoding LIM mineralization protein-1 (LMP-1), and the other site had cells transfected with a reverse copy of the cDNA (as a control). Spine fusion and bone formation were significantly induced by marrow cells transfected with LMP-1 group, but not in the control group.

Direct Injection of Genes and Gene-Modified Cells

Direct injection of expression vectors to wound sites was proposed as a simple, clinically convenient way for bone induction and repair [73]. A significant concern with direct injection approach is the dissemination of the expression vectors to neighboring tissues and haphazard ossification [87]. The optimal timing of injection needs to be

evaluated for each gene, that is, the timing of injection after a bone defect might be evaluated. The persistence of gene expression might be additionally difficult to control, but these are important variables to understand for an efficacious therapy [54]. Examples of studies that employed direct injection of pDNA or cells modified with therapeutic genes are below.

A pDNA encoding for osteogenic protein-1 (OP-1; BMP-7) with a collagen carrier was injected to rats as a model for posterolateral lumbar interbody arthrodesis [11]. Based on the radiological and histological results, bone formation was evident in pDNA/collagen-applied groups. A calcium phosphate/pDNA nanoparticle formulation encoding for BMP-2 was also found to be functional when injected subcutaneously in alginate hydrogels in mice [60]; bone formation was evident when pre-osteoblast cells were injected along with such a BMP-2 gene expression system. Beyond the delivery of BMP genes, the effect of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) gene delivery on osteosarcoma and Ewing's sarcoma was investigated in mice [88]. Bone fractures are usual consequences of tumor expansion and broad bone disruption of Ewing's sarcoma [66], so that gene therapy could be beneficial at sites of localized, excessive bone resorption. Lipophosphoramidate/DNA injections were undertaken as TRAIL therapy into retro-orbital veins of osteosarcoma and Ewing's sarcoma models. Overexpression of TRAIL was confirmed in plasmid construct-applied groups, and tumor incidence and osteolytic lesions were decreased in TRAIL-applied groups.

Gene-modified cells were also applied directly for bone regeneration. Healing of bone defects in the rabbit tibia was explored with fibroblasts that were transfected with VEGF [67]. More ossification as bone bridges and vessel formation were observed in VEGF fibroblast group. Bone marrow-derived MSCs (BM-MSCs) that were transfected with osterix (OSX) gene were also injected to distraction gaps in the mandibles of rabbits [65]. Increased bone development in distracted callus and also higher bone sialoprotein expression were observed in OSX-modified BM-MSCs treatment groups.

Electroporation and Sonoporation in Gene Expression

Utilization of electrical pulses for creating pores across plasma membrane is the process of *electroporation* [97], which can enable pDNA transfer into a cell without the need for a carrier. The operational parameters of pulses are important for effectiveness of transfection in this approach. Similarly, ultrasound-applied gene transfer (*sonoporation*) can serve for the same end by relying on microbubble-induced cell permeabilization for pDNA uptake [67, 68, 76]. Easier clinical translation and decreased invasiveness of sonoporation make it superior to electroporation [15]. Assimilating pDNA by electroporation or sonoporation percutaneously can be hard because of restriction by dense tissues enclosing human bones [94]. Studies involving electroporation and sonoporation showed that bone formation could occur especially in ectopic sites of bone in mice and rats following gene delivery [51–53, 57, 59, 84, 103]. A collagen sponge with pDNA encoding BMP-9 was injected into nonunion fractures of mice in one study [55]. After implantation, electroporation was undertaken and bone bridging was observed in electroporated BMP-9 group by histological analysis and microcomputed tomography. Feichtinger et al. investigated injection of BMP2/7 co-expressing pDNA with ultrasound application transcutaneously for bone regeneration in mice and rats [32]. Assessment of gene transfer effectiveness was performed with bioluminescence activity, which resulted with success rates of 85 % at 2 W/cm² and 100 % at 4 W/cm². In addition, sonoporation caused higher bone regeneration percentages according to microcomputed tomography consequences.

Perspective

Obtaining convenient means for harmless and effective gene delivery is difficult in functional bone regeneration, despite the availability of a spectrum of delivery agents. The spectrum of carriers functional for gene delivery is encourag-

ing, but concerted efforts to understand and overcome factors limiting gene expression are continuously needed. The biocompatibility and mechanical properties of materials need to be evaluated on one hand [111], while the in situ residence of pDNA (or other expression vectors) at the defect site needs to be ensured in a functional state [60]. Animal models are essential to explore the proof of principle for the newly developed therapies, but investigation of therapeutic features beyond functional outcomes (e.g., biocompatibility, immune response, etc.) must be also incorporated in such studies. Minimal immunogenicity associated with nonviral approaches of gene delivery makes it safer than the viral approaches of gene delivery [14]. Delivering growth factors in gene form may become more practical over utilization of high doses of protein, to better sustain protein presence at the site [104] and possibly overcome adverse effects associated with high protein doses locally. Since most clinical cases requiring bone repair are non-life threatening, it is likely that the nonviral approach will gain the upper hand on the long run (i.e., viral delivery is difficult to justify in such clinical scenarios). Studies in larger animal models (as compared to rodents) will be required to better realize the potential of gene-induced bone repair in slower-growing or nongrowing organisms reminiscent of humans.

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