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FEATURE ARTICLE

TERM

Gene Therapy for Bone Tissue Engineering

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Gene therapy holds a great promise and has been extensively investigated to improve bone formation and regeneration therapies in bone tissue engineering. A variety of osteogenic genes can be delivered by combining different vectors (viral or non-viral), scaffolds and delivery methodologies. *Ex vivo* & *in vivo* gene enhanced tissue engineering approaches have led to successful osteogenic differentiation and bone formation. In this article, we review recent advances of gene therapy-based bone tissue engineering discussing strengths and weaknesses of various strategies as well as general overview of gene therapy.
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Key Words: Gene therapy; Viral vector; Non-viral vector; Bone tissue engineering; Bone morphogenetic protein

INTRODUCTION

In many clinical conditions the bone growth takes place due to stimulation by proteins and osteogenic cytokines [1]. The *in vivo* studies over a decade have demonstrated that growth factors can stimulate bone formation and bone healing [2]. Osteogenic cytokines were investigated to check the osteoinductive capacity of demineralized bone matrix [3] and recently the role of muscle bone interactions by bone morphogenetic proteins (BMP) was explored [4]. The BMPs are secreted signal factors belonging to the transforming growth factor β (TGFβ) superfamily and exhibit an essential role during bone and cartilage formation and maintenance. Recently researchers demonstrated that the BMP pathway also has a role in controlling adult skeletal muscle mass. Thus, BMPs become essential regulators of both bone and muscle formation and homeostasis and BMP-2 and BMP-7 are already approved for the treatment of non-union fractures and spinal fusion [5]. The recombinant BMP's showed faster healing as well as less infections with reduced risk of failure [6] and they showed higher fusion rate as compared with autograft [7]. Although protein delivery has the great potential for bone tissue engineering, their application of these recombinant proteins are often arrested by challenges involved in delivery because this protein-based therapies do not find to be suitable due to short protein half-life as well as poor retention in defect site [8] and doses required for protein delivery are very high and expensive as compared with normal bone repair [9]. Furthermore, the use of recombinant BMP's delayed fracture healing and bone loss associated with trauma, revision joint arthroplasty, tumor resection and pseudarthrosis of the spine. Therefore it is of great interest for researchers to identify alternative approaches for stimulation of bone formation and repair. Recently gene therapy has attracted much attention because first gene therapy product (Glybera®, uniQure, Amsterdam, The Netherlands) in Europe has been approved and running successfully in post marketing surveillance phase (Phase IV Clinical trial) till now [10]. The Glybera® approved as a gene delivery brand was developed by Amsterdam-based uniQure for patients suffering from a rare lipid-processing disease, called lipoprotein lipase deficiency (LPLD) that affects only one or two people in a million by challenging regulatory procedure [11]. Gene therapy will be emerged as a promising approach for repairing bone-related diseases that overcomes limitations of protein-based delivery. Various animal studies showed the potential of gene transfer technology to deliver osteogenic drug molecules to precise anatomical locations at therapeutic levels for sustained time periods [12]. Genetic

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engineering of stem cells from different sources with osteogenic genes has led to repairing of advanced fractures, spinal fusion and rapid recovery from bone defects in preclinical models [13]. Also, gene delivery has attracted great attention due to endogenous synthesis of proteins by central dogma of life, a fundamental principle of all living things thus covering vast number of species. Furthermore, endogenously synthesized proteins may have greater biological effectiveness than exogenous and recombinant counterparts [14-16]. Delivering nucleic acids (NAs) induces osteogenic growth factors, which may provide high sustained concentration of growth factors locally for prolonged period of time [17-19]. The characteristics of protein and gene delivery are summarized in Table 1. This review discusses current viral and non-viral gene delivery strategies for bone tissue engineering and provides future perspectives as well as broader avenues for potential use of gene enhanced tissue engineering to enter in the widespread clinical use.

GENERAL OVERVIEW OF GENE THERAPY

Gene therapy requires transfer of genes into suitable cells and subsequently inside the nucleus. To carry and protect the genes from nucleases as well as negative charges of the genes, gene carriers should be required and these carriers are called as vectors. Broadly gene delivery can be classified into two categories based upon type of vectors used for delivering genes: viral and non-viral. Each type has its own pros and cons, for example, viral vectors show high transfection efficiency but show immunogenicity and toxicity putting a big question mark on safety of patients. However, non-viral vectors are non-immunogenic and quite safe although they show lower transfection efficiency when compared with viral vectors. The researchers have designed and synthesized non-viral vectors which can show high transfection like viral vectors with safety profile altogether in the single system. They require concrete and persistent determination to achieve the desired targeted delivery. The various types of gene delivery carriers are involved here which can become potential carrier source for bone tissue repair or in regenerative medicine.

TYPES OF GENE DELIVERY CARRIERS

Viral and non-viral vectors have shown immense potential to deliver gene at the numerous sites. While each vector possesses its own pros and cons, an ideal vector should have high transfection efficiency, low toxicity and consistent gene expression without immunogenicity.

VIRAL VECTORS

As we already stated, gene delivery can be done by both virally and non-virally. Both vectors have their own pros and cons and choice of suitability generally depends upon the targeting cell type and desired expression period. Viral delivery is considered as one of the most efficient gene delivery which relies on capsid (viral envelope) proteins to transfer the gene of interest into the cytoplasm and thereafter it is transported to the nucleus and subsequently expressed. Additionally they have highly evolved mechanism of introducing DNA into cells [20]. The viral vector may either integrate into the host genome thus leading to the stable expression of gene or remain present as an episomal vector, which is gradually lost after cell division. In this case, the viral vectors' genes which are responsible for pathogenicity (disease causing property) are replaced by the genes of our interest such as osteogenic genes [21]. As a result of this modification, the viral vector becomes safer and specifically enable osteogenesis. A large variety of viral vectors have been investigated due to the key natural property of viruses to enter into cells and integration by controlling the genetic material in the nucleus by removing virulence or disease causing property. Wide number of viruses have been engineered with this way and more than 1200 human gene therapy trials have been performed using viral vectors by far [22]. The most common viral vectors are adenovirus, retrovirus, lentivirus and adeno-associated viruses (AAVs) [23].

Adenoviruses are mostly useful as gene delivery vectors as

Table 1. The characteristics of protein and gene delivery

Protein delivery	Gene delivery
Low safety concerns	High safety concerns by viral vectors
Difficulty of sustained and local delivery	Possibility of sustained and local delivery
Difficulty of regulated delivery	Possibility of regulated gene expression temporally and quantitatively
Difficulty of intra-cellular delivery	Possibility of intra-cellular delivery
High costs	Less expensive
Repeated delivery	One delivery
Inauthentic protein	Authentic protein

they can be purified at high titers and have a potential to invade into broad range of cells. The gene delivery using adenoviruses showed high gene expression levels along with induction of bone formation [24-26]. For repairing the tissue, these vectors have several advantages such as easy preparation, non-integrating and high transduction efficiency. The ideal expression pattern for bone fracture healing was presented by Betz et al. [15] which expresses that cells transduced with first generation adenoviral vectors *in vivo* generally express transgenes at high levels for 2−3 weeks, after that expression level quickly falls to very low with complete loss of expression in about 6 weeks [27].

Retroviral and lentiviral vectors integrate similarly like adenoviruses and show sustained and long term gene expression. These carriers are considered as an ideal one due to low immunogenicity. The treatment of very large segmental defects after severe trauma is one of the example of utilizing osteogenic gene of BMP-2 for bone tissue repair [28]. While generating prolonged gene expression, the overproduction of bone or unregulated overexpression of gene may lead to major barrier for gene delivery. To overcome these problems, inducible expression systems have been developed. Here, an inducible promoter can be activated or inactivated by external chemical agents e.g., tetracycline to regulate the expression of osteogenic gene [29,30].

AAVs are safer than adeno, retro and lenti viral vectors. AAV is often the preferred method for delivering genes to target cells due to its high titer, mild immune response, ability to infect a broad range of cells, and overall safety. Compared with other viral vectors such as adenovirus, AAV elicit a mild immune response in animal models, making AAV an ideal virus for researchers delivering genes *in vivo* or who are concerned about safety. One concern when using other viruses, such as retrovirus or lentivirus, is the random integration events that can disrupt gene function. Because AAV do not integrate into the host cell genome, the risk of insertional mutagenesis is low. Since the AAV vector genome lacks viral coding sequences, the vector itself has not been associated with toxicity or any inflammatory response (except for the generation of neutralizing antibodies that may limit re-administration) [31]. AAV studies for bone tissue engineering have just begun and progressing soon [30]. Currently, AAV appear to be the most promising vector for gene therapy. In 2012, Glybera®, an AAV vector designed to treat LPLD, became the first gene therapy product approved in the western world [32]. However, the production of these vectors are challenging and if they become simplified in terms of cost and purity, these gene delivery vehicles may become a promising candidate for gene enhanced bone tissue engineering [33].

NON-VIRAL VECTORS

Non-viral vectors are lipid-based carriers, inorganic nanoparticles and polymers [34]. The non-viral gene transfer is generally performed using plasmid DNA which is small, circular, double stranded DNA to show stable chemistry and easily can be produced in bacteria and may contain variety of promoters and therapeutic copy DNAs (cDNAs) [35]. The pDNA was the starting approach of gene delivery and now it has been proved that all types of NAs which have obvious negative charges can be delivered. The underlying principle is nothing but the electrostatic interaction of negatively charged nucleic acid with positively charged materials, resulting in formation of polyplexes. These polyplexes need to cross cell and nuclear membrane to enter the nucleus and the NA has to be released from any possible transfection complexes [36]. The polymers used in gene delivery have been broadly classified into two types of natural and synthetic polymers.

Natural polymeric vectors

Natural polymer-based vectors have been used in gene therapy applications as they have biodegradability, biocompatibility, low/non-toxicity and they can be modified to increase the functionality of these vectors [37-40].

Among natural polymers, chitosan obtained by deacetylation of chitin has been most used as a gene delivery carrier because it has biodegradability, biocompatibility, low toxicity and immunogenicity [41]. The chitosan forms complexes with genes and can deliver the gene into cells although the transfection efficiency is low without cell specificity. Therefore, modification of chitosan has been tried to increase transfection efficiency and to get cell specificity by chemical modification of the chitosan using polyethylenimine (PEI) [38], urocanic acid [42] and lactobionic acid [43], galactose [44], dextran [45], folic acid [46], mannose [47,48] and spermine (SPE) [49]. The chemical modification of the chitosan has been reported as the one of the suitable methods to deliver gene showing synergism [50].

Alginate as an anionic linear copolymer of beta-D-mannuronic acid and alpha-L-glucouronic acid residues was also used for gene delivery [51] because it is non-toxic and biocompatible, and it gelates with bivalent cations like Ca^{2+} [52]. Therefore, polyplexes as nanoparticles can be loaded into the alginate hydrogel [53]. Alginate-mediated transfection with plasmid DNA recently showed slow release of biologically active BMP-2 and osteogenic differentiation *in vitro* and bone formation *in vivo* [54,55].

Gelatin obtained by denaturation of collagen was also used as a gene carrier because it is biocompatible and biodegradable. After treatment with glutaraldehyde, it can be crosslinked to control release of gene from the crosslinked gelatin [56-58].

Synthetic polymeric vectors

Synthetic polymers provide opportunities for improved safety, greater flexibility and more facile manufacturing. These polymers electrostatically bind with genes and condense the gene into particles of ten to several hundred nanometers in size, additionally protect genes and mediate cellular entry. Such complexes are also called as polyplexes [36].

Among synthetic polymers, PEI as a golden standard has been used to deliver genes because the PEI has a proton sponge effect and results in fast release of the genes from the endosome [59] although high molecular weight of PEI (25 kDa) has shown high cytotoxicity [60] and aggregation of nanoparticles occurs under high ionic strength conditions and results in reduced biological activity [61]. To overcome these limitations, many researchers have reported a number of degradable PEIs consisting of low molecular weight (LMW) PEIs and cross-linkers for intracellular degradation, such as simple hydrolysis, hydrolysis at low endosomal pH, enzymatic degradation, and cytosol-specific reductive degradation by glutathione [40]. These PEIs displayed high transfection efficiency and low cytotoxicity as a result of the rapid *in-situ* degradation of the polymer into small molecular weight water-soluble fragments, which are processed easily and removed by the cells [39]. The PEI also successfully delivered genes into a variety of tissues including central nervous system [62], kidney [63], lung [64], and tumors [65]. Among degradable PEIs, Cho's group recently reported a polysorbitol-based osmotically active transporter (PSOAT) synthesized from LMW PEI and sorbitol dimethacrylate (SDM) through a Michael addition reaction, exhibited accelerated transfection ability with an interesting cellular internalization mechanism. The hyperosmotic activity by polysorbitol and proton sponge effect by PEI also revealed a synergistic effect on the improvement of the transfection capacity of PSOAT [66,67]. Thereafter, when SDM was replaced by sorbitol diacrylate to reduce the cytotoxicity of the hydrophobic methyl groups in SDM, the polysorbitol-mediated transporter showed higher cell viability than PSOAT with a high gene transfection due to selective caveolae endocytic pathway [40,68-71]. Similarly, polymannitol- [72-74] and polyxylitol- [75,76] based hyperosmotic transporters were designed as transport carriers effectively in gene delivery as shown in the Figure 1.

In steady of LMW PEI, SPE has been used to prepare degradable poly (amino ester) (PAE) as a good alternative for cytotoxic PEI. The SPE-based PAE prepared by a Michael addition reaction between trimethylolpropane triacrylate and SPE was used to deliver small interfering RNA (siRNA). The polyplexes showed good intracellular uptake and had efficacious gene silencing effect with low toxicity compared to PEI 25K. Similarly, aerosol delivery of (glycerol propoxylate triacrylate) GPT–SPE/Akt1 shRNA complexes significantly suppressed lung tumorigenesis in K-*ras*^{LA1} lung cancer model mice [77,78].

Dendrimers are attracting great interest in researchers to deliver gene either alone or by chemical modification with other polymer or by modifying the surface of dendrimer [79]. Dendrimers are highly structurally controlled dendritic polymers built up from branched repeat units called "branch cell monomers". These are considered as a new class of polymeric gene vectors and until now various dendrimer [79] types have been evaluated for the potential of gene delivery. Among these dendrimers, poly (amidoamine) (PAMAM) and poly (prophylenimine) dendrimers are the most-investigated ones for delivering gene. The degraded PAMAM dendrimer-based material SuperFect is available in the market as a gene transfection reagent [80] thus showing a transfection potential although the further development has been arrested due to moderate transfection efficacy and serious cytotoxicity [80,81]. To minimize cytotoxicity and to optimize maximum safe transfection efficiency, dendrimers for gene delivery have been chemically modified. Hence, the structure—function relationships of these surface—engineered and chemically modified dendrimers still need in-depth investigations and can show the great potential for gene delivery.

Cyclodextrin (CD) have become the polymer of choice for gene delivery by several modifications on significant functional groups [82]. CD has been largely dominated by their unique ability to form inclusion complexes with guests fitting in their hydrophobic cavity. Chemical modification was soon recognized as powerful means of improving CD applications including gene delivery. Croyle et al. [83] observed some neutral and cationic CD derivatives enhanced adenoviral-mediated gene expression. Cationic CD derivatives interact with the negatively charged adenoviral surface preventing non-specific interactions and facilitating their access to intestinal epithelial cells. The CD shield improved viral dispersion and bioavailability thus facili-

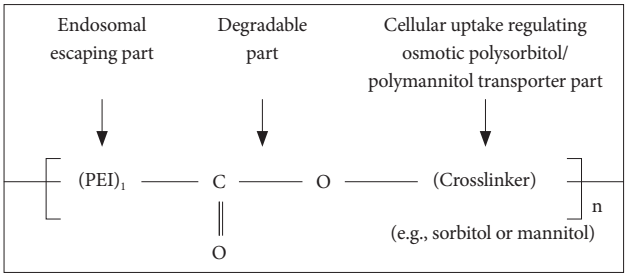

Figure 1. Schematic illustration on the design of polysorbitol or polymannitol gene transporter based on LMW PEI and sorbitol or mannitol backbone as a form of diacrylate or dimethacrylate cross-linking chain. Different parts of the transporters show different functional properties. Adapted from Islam MA, et al. J Control Release 2014;193:74-89, with permission from Elsevier [136]. LMW: low molecular weight, PEI: polyethylenimine.

tating cellular absorption of adenoviral vectors even after prolonged storage [84]. Polymeric CD-containing materials have been exploited for biomedical and pharmaceutical purposes, the application of CD-containing polymers to gene delivery was established by Gonzalz et al. [85] and the CD-based polymeric system is the first representative of polymeric gene carriers that entered clinical trials for siRNA delivery.

GENE THERAPY IN BONE TISSUE ENGINEERING

Bone tissue engineering via gene therapy has been evolving over the past decade with delivering various types of nucleotides. Generally, three factors are needed for effective the gene therapy-based tissue engineering as shown in Figure 2: 1) genes based on growth factor & transcriptional factor, 2) scaffolds, and 3) cells. This section is focused on the recent progresses of three key elements-based the gene therapy for bone regeneration, especially *in vivo* & *ex vivo*.

TARGET GENES IN BONE TISSUE ENGINEERING

As discussed above, gene therapy is a promising option to enhance bone repair in tissue engineering by controlling expression and regulation of bone formation related-genes, which are categorized into two groups: growth factor and transcriptional factor genes. Bone-related growth factors such as BMPs, platelet-derived growth factor (PDGF), fibroblast growth factor, insulin-like growth factor, transforming growth factor, and vascular endothelial growth factor (VEGF) have been used for bone formation and regeneration. Growth factors as proteins are secreted by cells. They act as signaling agents in the cellular network and control cell proliferation, matrix synthesis, and tissue differentiation in the osteogenic lineage.

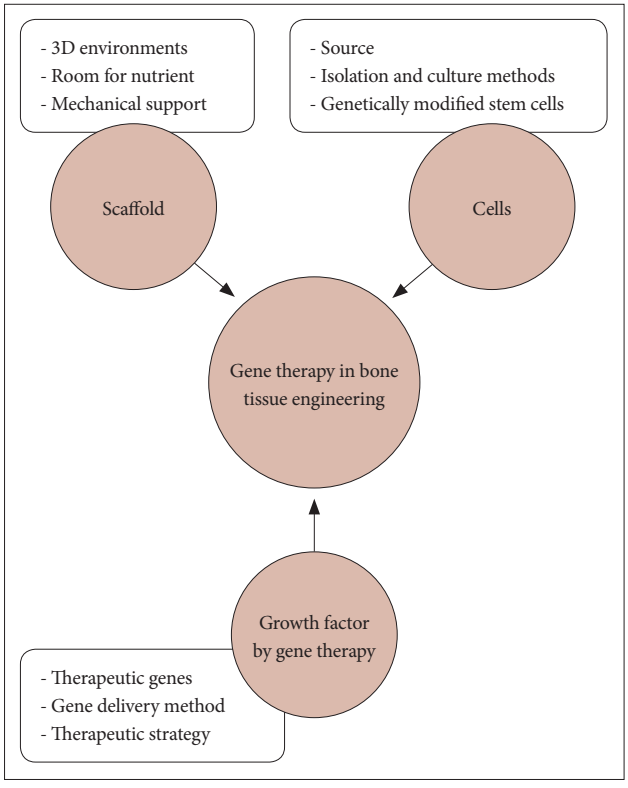

Figure 2. Key elements for gene therapy in bone tissue engineering.

Among growth factors, BMPs are important proteins for bone regeneration as members of the TGF-β superfamily and have been the most widely investigated using recombinant human BMPs. They regulate cartilage and bone formation during embryonic development and regeneration [86]. Recently, VEGF has been also widely used in bone tissue-engineering studies and plays essential role in the angiogenesis, endothelial cell growth, and inhibition of apoptosis. Gene regulation of VEGF by gene therapy is crucial for bone formation because this secreted-cytokine regulates multiple biological functions in the endochondral ossification of mandibular condylar growth [87]. However, examination of the effect of VEGF alone needs to achieve a better understanding of its function in bone formation. Therefore, nowadays, gene delivery strategies using the combination of various growth factor genes have been investigated to enhance bone fracture healing via their unveiled synergistic effect.

Another candidate genes in bone tissue engineering are the transcription factors, which are proteins that induce the expression of new genes, as well as several growth factors by regulation of transcription via binding the specific promoter site of DNA. In osteogenic transcription factors, runt-related transcription factor 2 (Runx2) is the most widely investigated transcription factor, which is crucial for osteogenic differentiation and endochondral bone development during the early stages of embryogenesis by binding to specific promoters of osteoblast-related genes [88]. Zhao et al. [89] examined *ex vivo* & *in vivo* osteogenic activity in MSCs via Runx2 gene transfer with adenoviral vectors. According to their results, Runx2 gene delivery increased in alkaline phosphatase activity and mineralization of osteoblast differentiation, indicating that Runx2 gene transfer selectively redirected the differentiation fate of multipotent MSCs to the osteoblast lineage. Furthermore, Lee et al. [90] confirmed significant mineralization & bone formation by implantation of Runx2 gene transfected adipose stem cells (ASCs)-scaffold [poly (lactic-co-glycolic acid) (PLGA)] hybrids despite lower osteogenic potential of ASCs than BMSCs in nude mice. Also, they tested another type of transcrip-

tion factor, osterix (osx), which is a downstream gene of Runx2 and important factor for osteoblast differentiation [91]. Although there is no obvious differences *in vivo* data between runx2 and osx, osx gene transfected ASCs also promoted enormous bone formation after 6 weeks. Therefore, MSC-dependent osteogenesis via transfection transcription factors may have therapeutic effects for bone regeneration.

In followed sections, we will cover gene delivery research using various growth factors and/or transcriptional factors gene in the field of bone tissue engineering, and particular emphasis will be on the *in vivo* & *ex vivo* gene delivery methodologies: direct injection, gene-activated matrix (GAM), and cell-based therapy. Schematic illustration of methods *in vivo* and *ex vivo* gene delivery for bone tissue engineering is shown in Figure 3.

IN VIVO GENE DELIVERY FOR BONE TISSUE ENGINEERING

Direct injection

Direct injection of gene provides simplicity and a reduction of risks associated with viruses, such as immune response, viral infection, and insertional mutagenesis for bone tissue regeneration via physical methods through electroporation, sonoporation, and microinjection. These are the simplest ways of delivering genes to the cells. Also, these methods enhance the permeability of cell membrane for putting naked DNA depending upon the origin of physical forces, which increase gene entering efficacy into the cytoplasm. Although some manipulations can cause damage to the cells or tissues, physical methods are an effective way for the transfection of hard-to-transfect cell lines, primary cells, and MSCs.

Among physical force-based direct gene transfer method, electroporation is most widely used since it is safe and inexpensive. Kishimoto et al. [92] transferred plasmid vector via *in vivo* electroporation for bone regeneration. They injected solution of plasmid DNA containing mouse BMP-4 (pMiw-BMP4) into the gastrocnemius of BALB/cA mice, and applied electric pulses. As their results, 28 days after electroporation, new ectopic bone formation was observed by electroporation of pMiw-BMP4 (ectopic bone: 44% and bone marrow-like cells: 22%). Following their subsequent study, the rate of bone formation was significantly enhanced from 67% at 14 days after electroporation in BALB/cA mice to 100% bone formation in C57BL/6J by transfer of plasmid vector containing mouse BMP4 gene (pCAGGS-BMP4) [93]. They suggested that C57BL/6J strain's better dependence against BMPs than the BALB/cA strain, higher activity of the CAG promoter in muscle, and induction of muscle necrosis by pretreatment with bupivacaine may improve induction of ectopic bone formation. Other group, Kimelman-Bleich et al. [94] adopted *in-vivo* DNA electroporation

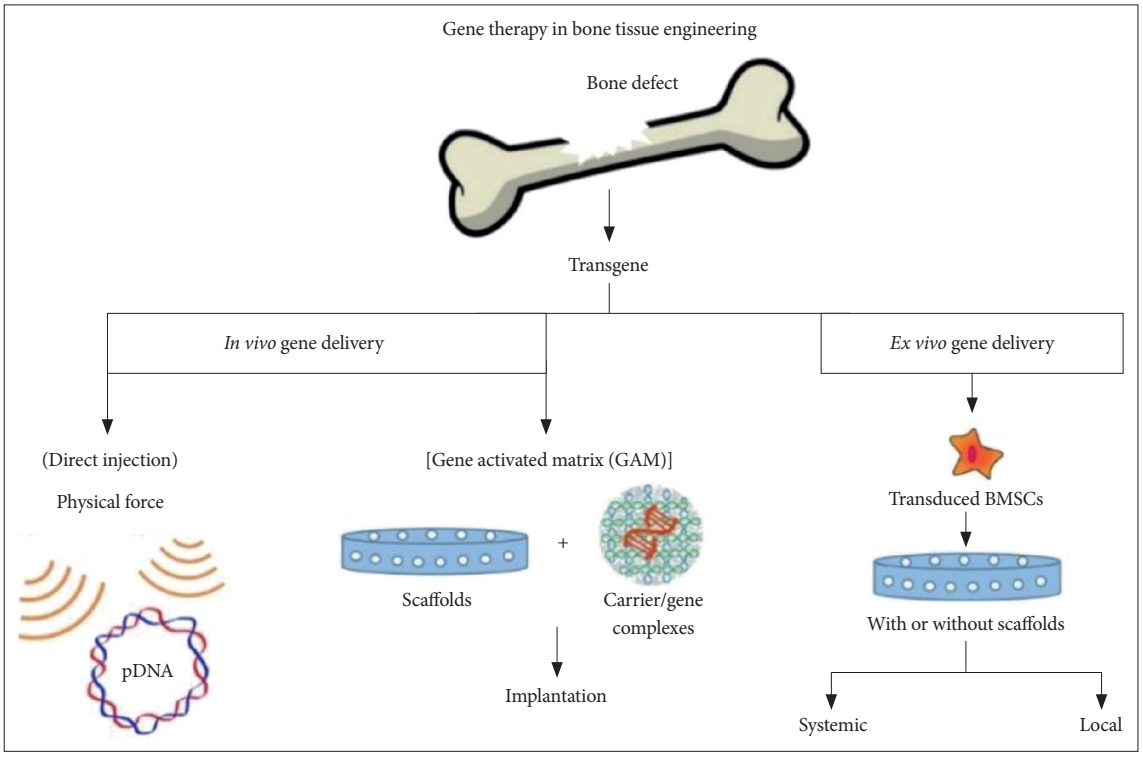

Figure 3. Schematic overview of gene therapy for bone tissue engineering. BMSC: bone marrow stromal cell.

of a BMP-9 gene to treat a nonunion bone fracture. Nonunion radius bone defect site was recruited by host progenitor cells in C3H/HeN mice. After electroporation of BMP-9 plasmid, bridging the bone gap via bone formation was detected by microcomputed tomography (μCT) and histological analysis whereas the control groups (the genes luciferase; pLuc) remained unbridged.

Another promising and emerging physical gene transfer method is sonoporation, which transiently increase the permeability of molecules to vessels and tissues by combining the ultrasound waves and the intravascular or intratissue administration of gas microbubbles [95]. Although electroporation is very well established and studied as a gene delivery method and has better efficiency than the sonoporation technique, it can induce tissue damage. Therefore, sonoporation is considered as a safe alternative technique. Furthermore, according to previous report [96], low-intensity ultrasound stimulation promoted proliferation of alveolar bone marrow stem cells at optimized condition (intensity: 100 mW/cm^2, duty cycle: 30% and duration time: 10 min), which may act as synergistic effect of gene delivery for bone regeneration. In 2008, Sheyn et al. [97] firstly adopted *in vivo* ultrasound-based osteogenic gene delivery for bone tissue formation. They mixed naked DNA (recombinant human bone morphogenetic protein-9, rh-BMP-9) with microbubbles injected into the thigh muscles of mice, and subsequently applied noninvasive sonoporation. Although formation of bone tissue was considerably detected from results of μCT and histology by ultrasound-based rh-BMP-9 delivery, electroporation method had superior efficiency of gene delivery in their comparison data. However, adoption of ultrasound-based gene transfer is first in tissue formation *de novo* approach, which suggested potentials for ultrasound-based gene delivery in bone-tissue engineering and need for further development of sonoporation to increase efficiency. Recently, Feichtinger et al. [95] also used sonoporation for *in vivo* gene therapy in ectopic models. Gene transfection efficiency and rate of ectopic bone formation were confirmed by comparison between passive (intramuscular injection) and ultrasound power-based gene (doxycycline-inducible BMP-2/7 co-expression plasmids) transfer. According to their results, sonoporation showed 100% of gene transfer efficacy and ectopic bone formation whereas passive gene delivery showed 41% and 46%, respectively, indicating that sonoporation is minimally invasive and possible osteogenic gene technique for bone regeneration.

Although the above mentioned methods of gene transfer via direct injection have advantages and potential, it should be further developed to overcome concerns about undesirable distribution of the vectors to non-target sites, which may induce side effects such as heterotopic ossification and fusion of adjacent joints [98]. The characteristics of *in vivo* gene therapy for bone tissue engineering by direct injection are summarized in Table 2.

GAM

Since the concept of GAMs was established in 1999 [99], the most widely investigated *in vivo* non-viral gene delivery systems for bone tissue regeneration are GAM-mediated gene transfer methods. GAMs can incorporate genes as well as nucleic acid/non-viral vector complexes, which can be implanted into the body to promote cell differentiation and tissue development under controllable prolonged releasing time of growth/transcription factor depending on the material composition of the scaffold. Also GAMs have advantages such as low immunogenicity, prevention of distribution of vectors into other organs, and relative ease of large-scale production [100]. The gene activated-scaffolds were composed of natural polymers including collagen, chitosan, and silk. And also synthetic polymers can themselves act as gene delivery vectors by condensing and protecting pDNA [101,102]. Among natural polymers, firstly, collagen has high biodegradability, low antigenicity, and cell growth potential [103]. Therefore, it has been widely used in bone tissue engineering as structurally and mechanically superior scaffolds and sponge type are often used to engineer skeletal tissues [104]. Geiger et al. [100] used pVEGF$_{165}$-GAM (commercially available collagen sponges) to investigate whether their collagen-based GAM system could produce a sufficient level of angiogenesis and bone healing in a critical size defect in the rabbit radius by producing active VEGF protein. Osteoneogenesis and angiogenesis were quantified by μCT and histomorphometry of CD31$^+$ vessels, respectively. By treating

Table 2. *In vivo* gene therapy for bone tissue engineering by direct injection

Method	Vector	Gene	Model	Reference
Electroporation	Plasmid	BMP-4	The gastrocnemius of BALB/cA mice	[92]
Electroporation	Plasmid	BMP-4	The gastrocnemius of C57BL/6J	[93]
Electroporation	Plasmid	BMP-9	Nonunion radius bone defect in C3H/HeN mice	[94]
Sonoporation	Plasmid	rhBMP-9	The thigh muscles of mice	[97]
Sonoporation	Plasmid	BMP-2/BMP-7	A rat femur segmental defect	[95]

BMP: bone morphogenetic protein

$pVEGF_{165}$-GAM, partial or total bone regeneration was detected in most of the animals, also 2–3 times more the number of vessels were counted, compared to the respective control groups after 6 weeks. Similarly, Endo et al. [105] modified collagen as a GAM with calcium-phosphate precipitates (CaP) to overcome the low gene transfection efficiency. They transplanted GAM consisting of bovine atelocollagen and BMP-2 plasmid with CaP into bone defects of rat tibiae. The results demonstrated that CaP modification showed a perfect bridge in the bone defect using a significantly low dose (12 μg plasmid DNA) and induced a superior bone regeneration compared to the other treatments (BMP2-collagen, collagen, and vacant vector-CaP-collagen), because CaP effectively stabilizes plasmid DNA [106].

Zhang et al. [107] firstly prepared porous chitosan/coral composites combined with plasmid encoding PDGF-B gene and demonstrated that gene-activated coral scaffolds had a great potential to enhance periodontal tissue regeneration. In their further study [108], chitosan/collagen scaffolds were prepared and combined with adenoviruses expressing both BMP-7 and PDGF-B to support the regeneration of alveolar bone at dental implant sites because PDGF-B promotes proliferation and differentiation of mesenchymal cells, and BMP-7 is a powerful growth factor. Interestingly, new bone formation by GAM expressing the combined both BMP7 and PDGF-B was greater than the all other scaffolds at the longer term (8 weeks and 12 weeks). During the later stages of bone regeneration, synergistic effect of both of cytokines had a great potential for bone healing by acting as autocrine and paracrine agents. Subsequently, same group, Luo et al. [109] designed two groups of GAM (1. chitosan/collagen scaffold both Ad-BMP-2 and Ad-VEGF & 2. chitosan/collagen scaffold VEGF protein and Ad-BMP-2) to demonstrate the synergistic effect of BMP-2 and VEGF on the healing bone defects around dental implant. Unlike their previous study, bone formation decreased at 4 weeks and 8 weeks by treating group 1 in dog's mesial bone defects. They hypothesized that the long-lasting expression of VEGF gene was not optimal for bone healing, because it can induce vessel leakage and the formation of massive non-physiological endothelial cells, maintaining high level of VEGF [109]. As they expected, combination of 1 μg of VEGF protein and Ad-BMP-2 using chitosan/collagen scaffold significantly improved bone regeneration showing synergistic effect of two different types of growth factor.

Another biocompatible natural polymer for tissue engineering is silk fibroin, which has water solubility, tunable architecture, superior mechanical strength, and gradual degradability *in vivo*. Although there are various kinds of silk-based scaffolds for biomedical applications, porous sponge scaffolds are the best option for bone tissue formation, because their high porosity (92–98%) facilitates nutrient and waste transport into and out of the scaffolds [110]. Using porous 3D silk fibroin scaffolds, Zhang et al. [111] constructed GAM with BMP-7 encoding adenoviruses and implanted critical-sized skull defect in a mice model. In their results of H&E staining and immunohistological staining, not only significant new bone formation was confirmed in the Ad-BMP-7 silks fibroin scaffolds, but also noticeable expression of markers of new bone formation (COL1, ALP, and OPN) were detected. Although clinical safety concern remain about the usage of viral vector, it is clear that silk scaffold-based GAM is cost-effective, biocompatible, and easily preparable with aqueous solution for bone tissue engineering.

Like the natural polymers, synthetic polymers are also highly useful since porosity, degradation time, uniformity, and mechanical strength can be produced by controlled conditions [112]. Among various synthetic polymers, PLGA is the most widely used as an osteoconductive material. Huang et al. [113] employed PLGA scaffold for bone regeneration and evaluated the bone repair potential in a rat cranial defect. Also, they used non-viral vector (PEI) to condense BMP-4 expressing plasmid DNA and to enhance transfection efficiency. Their results through μCT analysis and histomorphometric analysis consistently indicated that incorporation PLGA scaffold with PEI/DNA (BMP-4) significantly increased bone formation, osteoid, and mineralized tissue density. Interestingly, Chew et al. [114] delivered pDNA encoding BMP-2 in critical-size rat cranial defect model as the form of polyplexes with biodegradable branched triacrylate/amine polycationic polymer that were complexed with gelatin microparticles loaded within a porous tissue engineering scaffold. However, there are no significance on bone formation *in vivo* results because the premature degradation of cationic polymers may trigger the release of naked pDNA. Also, their results strongly indicated the degradation rate of non-viral carrier is most important in both transfection capability and therapeutic effect *in vivo*.

Collectively, GAM development has made a noteworthy contribution to bridge between gene therapy and bone tissue engineering and also holds much promise as therapeutic applications in the repairing of various bone defects. The characteristics of *in vivo* gene therapy for bone tissue engineering by GAM are summarized in Table 3.

EX VIVO CELL-BASED GENE THERAPY FOR BONE TISSUE ENGINEERING

Mesenchymal stem cells (MSCs) such as BMSCs, ASCs, muscle-derived stem cells, etc. have the capability to differentiate into a variety of cell types including osteoblasts and can be easily isolated and expanded by tissue culture techniques. For these

reasons, MSCs have been widely used in tissue-engineering applications [115]. Especially, genetically modified BMSCs via nucleofection of various growth factors encoding genes have been commonly utilized in bone regeneration by two methods: systemic & local delivery of BMSCs with or without various material-based scaffolds.

For bone healing by systemic delivery of BMSCs transduced with recombinant adeno-associated virus 6 encoding both osteogenic (BMP-2) and angiogenic (VEGF) factors, Kumar et al. [116] intravenously injected in right tibiae of mice model of segmental tibial defect. A robust bone growth was detected in their X-ray imaging of mice after 5 weeks & end-point μCT image of healed bones as shown in Figure 4. Although, the potential of MSC expressing BMP2 and VEGF for enhancement of bone healing was demonstrated, considerable bone growth was observed in control mice. Because used mice was young, and self-healing capacities reached its peak. Also, they detected BMSCs homed in both other organs and the fracture site. This low homing efficiency was previously reported by Gao et al. [117]. Rat marrow-derived MSCs were labeled with 111In-oxine and delivered via intravenous (i.v.) injection for real-time imaging biodistribution of MSCs using a gamma camera. After 48 h, most of the BMSCs were detected in the lung and liver. This undesirable distribution can induce a broad spectrum of disorders [118]. Therefore, cell trafficking-related molecules such as chemokines, adhesion molecules, and matrix metalloproteinases were adopted to enhance homing efficiency of MSCs using migration of MSCs specifically into damaged tissue sites with inflammation [119].

Engineered BMSC expressing CXC chemokine receptor-4 (CXCR-4) is commonly employed to enhance bone homing [120]. On the other hand, Kumar et al. [121] transduced the rAAV2 encoding α-4 integrin (CD49d) into mouse BMSC, and also heterodimerized the α4 integrin with endogenous β1 integrin (CD29) retaining their original stem cell property. Subsequently, the modified BMSCs were systemically injected through the tail vein in C57BL/6 mice. They demonstrated that ectopic expression of α4 integrin on BMSCs significantly increased bone homing & retention. And also forming osteoblasts and osteocytes were found in the growth plate of recipient mouse limb bones (femur/tibia). Interestingly, Guan et al. [122] coupled specific peptidomimetic ligand (LLP2A), against activated α4β1 integrin with bone-seeking component, a bisphosphonate (alendronate, Ale). By i.v. injection of huMSCs/LLP2A-Ale, not only the bone homing and retention of BMSCs were significantly enhanced, but also bone formation was increased in the endosteal and periosteal bone surfaces. Interestingly, in both xenotransplantation studies, a single i.v. injection of LLP2A-Ale also induced and increased trabecular bone formation and bone mass. Recently, Zwingenberger et al. [123] demonstrated synergistic effects of the cytokine stromal cell-derived factor 1 alpha (SDF-1α) and BMP-2 in a murine critical size segmental bone defect model. The SDF-1α enhanced low concentration of BMP-2-mediated bone healing and the attraction of MSCs.

Although systemic delivery of MSCs has potential to treat bone defects, most of the *ex vivo* studies preferably selected the local delivery using engineered stem cells with incorporation of scaffolds. Byers et al. [124] seeded BMSCs (transduced with Runx2 using retroviral vector) onto 3D-fused deposition-modeled polycaprolactone scaffolds. Runx2-modified cells were cultured during 21 days *in vitro* for mineralization, and implanted into critical size calvaria defects in syngeneic rats. Runx2-modified constructs precultured for 21 days showed nearly 2-folds higher new bone formation compared to control in μCT quantification of mineral deposition. Xu et al. [125] assessed osteoinductivity of Adv-hBMP-2-transduced BMSCs in the tibial bone defects of goats. At 16–24 weeks after implantation of BMSCs, defects completely healed by forming new bone whereas Adv-ßgal and non-transduced BMSC groups had not healed in radiographic examination although they reported that temporal cellular (4 weeks) and persistent humoral (more than 120 days) immune responses against usage of adenovirus were detected. It was the first report about the duration of cellular and humoral immune responses against adenovirus-me-

Table 3. *In vivo* gene therapy for bone tissue engineering by GAM

GAM	Vector	Gene	Model	Reference
Collagen sponges	Plasmid	VEGF165	Critical size defect in the rabbit radius	[100]
Chitosan/coral	Chitosan	PDGF-8	Athymic mice	[107]
Chitosan/collagen	Adenovirus	BMP-7/PDGF-B	Dog mandible	[108]
Chitosan/collagen	Adenovirus	BMP-2/VEGF	Dog's mesial bone defects	[109]
Porous 3D silk fibroin	Adenovirus	BMP-7	Critical-sized skull defect in mice	[111]
PLGA	PEI	BMP-4	Rat cranial defect	[113]
Gelatin micro-particles	Poly (triacrylate-co-amine)	BMP-2	Rabbit cranial defect	[114]

GAM: gene-activated matrix, VEGF: vascular endothelial growth factor, PDGF: platelet-derived growth factor, BMP: bone morphogenetic protein, PLGA: poly(lactic-co-glycolic acid), PEI: polyethylenimine

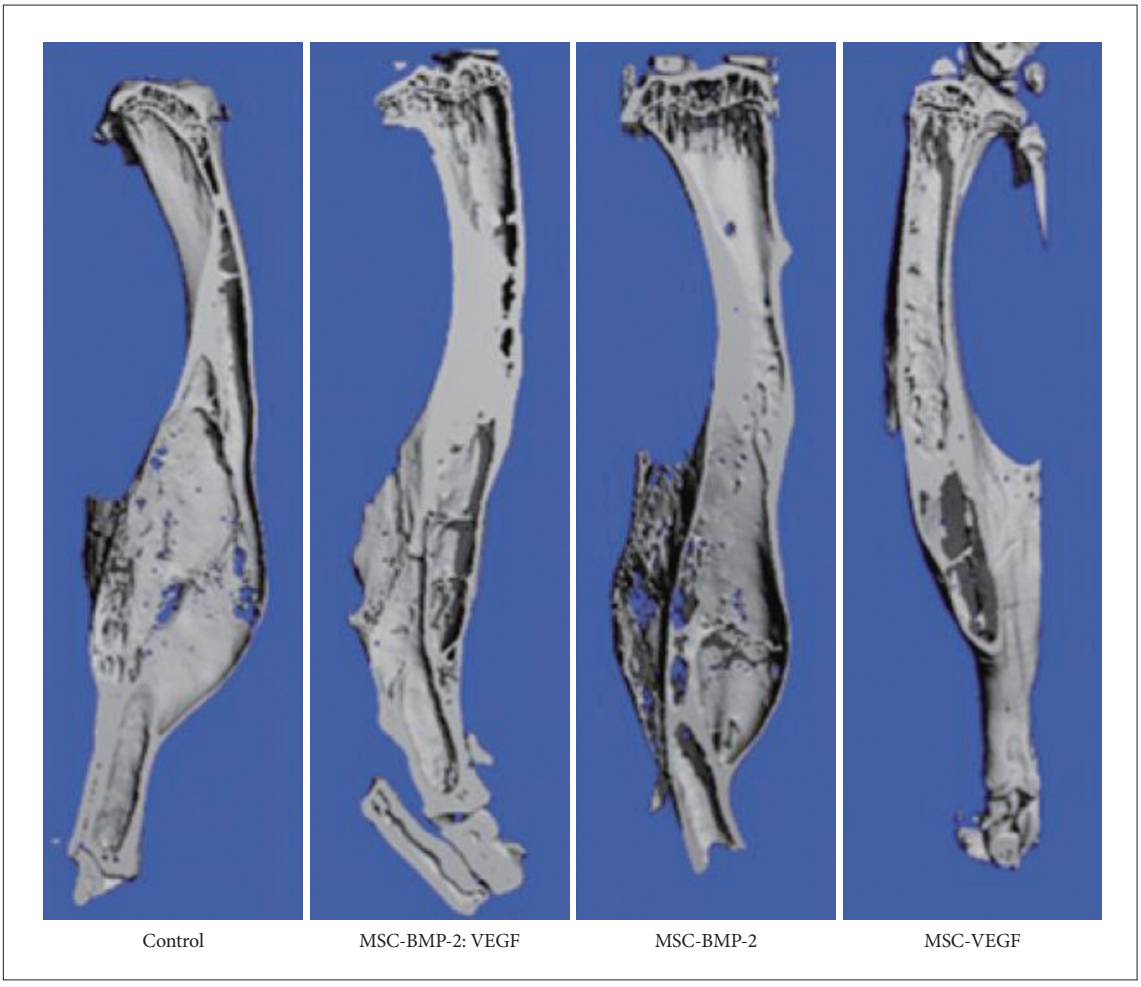

Figure 4. μCT analysis of fixed tibia after MSC therapy. Tibiae from MSC-transplanted cohorts of mice were used for μCT analysis. Representative images from indicated groups show three-dimensional images of tibiae extracted from reconstructed bone volume, 16 weeks after the treatment [116]. Adapted from Kumar S, et al. Mol Ther 2010;18:1026-1034, with permission from The American Society of Gene & Cell Therapy [116]. μCT: microcomputed tomography, MSC: mesenchymal stem cell, BMP: bone morphogenetic protein, VEGF: vascular endothelial growth factor.

diated gene therapy, especially immune defense leaded to shorten the duration of hBMP-2 expression and damage the adenovirus-transduced BMSCs [125]. Therefore, Lin et al. [126] engineered New Zealand White rabbit BMSCs by BMP2-expressing baculovirus or hVEGF-expressing baculovirus. After co-implantation using PLGA scaffolds into critical-sized femoral segmental defects, complete healing was detected at 8 weeks without no evident immune responses and infiltration of immune cells into the defects. Their data indicate that baculovirus is an alternative as a promising gene vector against adenovirus-mediated immune response. For a faster induced defect healing, He et al. [127] developed the injectable porous calcium sulfate/alginate (nCS/10%A, nCS/10%A, the average pore diameter: 70−80 mm) paste for support implantation of BMP2 gene-modified BMSCs (B2BMSC). nCS was used to increase surface area for MSCs & soft tissue attachment, and alginate was employed to produce pores and enhance strength of the paste. In their results, nCS/A+B2BMSCs induced dramatic perfect bone healing at the rat critical-sized calvarial defect model in a short time (7 weeks after surgery).

Other group, Zou et al. [128] genetically modified using hypoxia-inducible factor-1a (HIF-1a) lentivirus-mediated gene delivery into BMSCs. HIF-1a is a major mediator of the adaptive cell response to hypoxia and plays a crucial role in angiogenesis-osteogenesis coupling during bone regeneration [129]. After seeding transduced BMSCs on a gelatin sponge, it was implanted in rat calvarial defect. Gene delivery of HIF-1a significantly improved angiogenesis and osteogenesis *in vivo*.

Besides the use of genes for growth or transcription factors, in a recent study, Li's group [130] adopted miRNAs, which are small non-coding RNAs and act as repressors of gene expression at the level of post-transcriptional regulation. Therapeutic

potential of miRNAs such as miRNA-218 [131] (a pro-osteoblastic factors) and miRNA-148a [132] (a pro-osteoclastic factor) for bone regeneration has been reported. In order to regulate the angiogenesis-osteogenesis coupling, an miRNA, miR-26a mimics were transfected in human bone marrow-derived mesenchymal stem cells using siPORT NeoFX transfection agent, which upregulated genes associated with Runx2 & BMP-2 (osteogenesis) and VEGF & Ang1 (angiogenesis) *in vivo*. As the results, hydrogel/genetically modified BMSCs were implanted into calvarial bone defect in nude mice and significantly enhanced both vascularization and bone formation. Deng et al. [133] seeded miR-31-modified BMSCs on poly (glycerol sebacate) scaffolds and implanted them into critical-sized calvarial defects in rats. Knocking down of miR-31 promoted the osteogenesis *in vitro*, and also robust new bone formation was observed *in vivo*. Interestingly, Jia et al. [134] demonstrated two small interfering RNAs (siRNAs: casein kinase 2 interaction protein 1 targeted siCkip-1 & VEGF receptor 1 targeted si-Flt-1)-loaded chitosan sponge scaffold promoted osteogenesis and angiogenesis *in vitro*. Suppression of target genes led to significant upregulation of osteocalcin, alkaline phosphatase, and VEGF in BMSCs. Also, transduced BMSCs effectively regenerated bones in a skull critical-size defect rat model. Nowadays, much attention has been given to miRNA or siRNA-based gene therapy that has high potentials as new therapeutic agents for treating bone defects. The characteristics of in *ex vivo* cell-based gene therapy for bone tissue engineering are summarized in Table 4.

CONCLUSION

We presented recent advances of gene therapy-based bone tissue engineering focusing on three strategy: 1) direct injection of gene using physical forces, 2) GAM-mediated gene delivery, and 3) transduced stem cell-based gene delivery. All these approaches are highly efficient and has been demonstrated to have their therapeutic potential. However, the most important issue is safety concern. Generally, bone defect or bone loss is not fatal but gene therapy may induce lethal problems such as immune responses and mutagenesis via viral gene transfer. Although most of gene therapy-based bone regeneration studies are conducted by viral vectors until now since its high transfection efficiency, non-viral vectors also has been investigated in the bone tissue engineering. Because, as explained above, it is safer compared to viral vector and is recently advanced in transfection efficiency, specificity, gene expression duration. For this reason, number of non-viral vector products entering clinical trials is increasing now [135] for treating various dis-

Table 4. *Ex vivo* cell-based gene therapy for bone tissue engineering

Delivery method	Cells/scaffolds	Vector	Gene	Model	Reference
Systemic delivery (i.v. injection)	BMSC	AAV6	BMP-2/VEGF	Segmental defect in right tibiae of mice	[116]
	BMSC	rAAV2	α-4 integrin (CD49d)	C57BL/6 mice	[121]
	BMSC	Lentiviral vectors	SDF-1α/BMP-2	Murine critical size segmental bone defect model	[123]
Local delivery by implantation	BMSC/polycapro-lactone	Retroviral vector	Runx2	Critical size calvaria defects in syngeneic rats	[124]
	BMSC	Adenovirus	hBMP-2	Goat tibial defect	[125]
	BMSC/PLGA	Baculovirus	BMP-2/hVEGF	Rabbit calvarial defect	[126]
	BMSC/ nano-scale calcium sulfate-lginate (nCS/A)	Adenovirus	BMP-2	Calvarial bone defects of rat	[127]
	BMSC/gelatin sponge (GS)	Lentivirus	Hypoxia-inducible factor-1a (HIF-1a)	Rat calvarial defect	[128]
	BMSC/hydrogel	miRNA mimics (agomer)	miR-26a	Mouse calvarial defect	[130]
	Poly(glycerol sebacate) (PGS)	Lentivirus	miR-31	Critical-sized calvarial defects in rats	[133]
	Chitosan sponge	Lipofectamine™ 2000	siCkip-1/siFlt-1	Rat skull critical-size defect model	[134]

BMSC: bone marrow stromal cell, AAV: adeno-associated viruse, BMP: bone morphogenetic protein, VEGF: vascular endothelial growth factor, SDF-1α: stromal cell-derived factor 1 alpha, PLGA: poly(lactic-co-glycolic acid), VEGF: vascular endothelial growth factor

eases, also they will be exploited in bone tissue engineering. We expect that gene-based modalities for bone regeneration also will enter clinical trials in the near future.

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

There are no animal experiments carried out for this article.

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