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Review Article

Nonviral gene transfer strategies to promote bone regeneration

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Abstract: Despite the inherent ability of bone to regenerate itself, there are a number of clinical situations in which complete bone regeneration fails to occur. In view of shortcomings of conventional treatment, gene therapy may have a place in cases of critical-size bone loss that cannot be properly treated with current medical or surgical treatment. The purpose of this review is to provide an overview of gene therapy in general, nonviral techniques of gene transfer including physical and chemical methods, RNA-based therapy, therapeutic genes to be transferred for bone regeneration, route of application including *ex vivo* application, and direct gene therapy approaches to regenerate bone. © 2013 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 101A: 3009–3018, 2013.

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INTRODUCTION

Bone has the capacity for self-repair without scarring, which is a property uncommon in adult tissues. Therefore, most fractures heal spontaneously or with the help of surgical procedures.[1] However, despite the inherent ability of bone to regenerate itself, there are a number of clinical situations in which complete bone healing fails to occur.[2] These situations are commonly found in smokers, alcoholics, and diabetics.[3] Bone healing is also frequently hampered in the elderly[4] and patients with osteoporosis.[5] In addition, extreme conditions in patients with trauma or malignant tumor resection results in a critical-size bone defects (spanning $>$ 2 cm).[6] Failures in bone healing are also frequently seen after attempting to achieve posterolateral lumbar spinal fusion.[3]

The most commonly used surgical procedure to promote bone healing in these clinical situations is autogenous bone grafting, that is, surgical implantation of the patient's own living bone, usually taken from the iliac crest of the pelvis.[7] While this method has been thought to be the "gold standard" for treating bone defects or nonunion, there is a limit in the amount of autologous bone available from a patient. Autografting is also associated with side effects such as pain at the harvest site.[8] To solve the dilemma and limitations of autografting, allografts from dead donors have been used to replace or supplement autografts. While allografted bone is by and large unlimited in quantity, it is a dead bone that is less osteogenic than an autograft.[9] Because it does not undergo remodeling like living bone, allografts frequently fail if implanted into weight-bearing areas such as the long bone shaft. In addition, possible disease transmission and the risk of infection pose a challenge to the safety of this procedure.[7]

Growth factors that stimulate one or more of the steps involved in bone formation have been recently identified and investigated for clinical applications. The most extensively investigated growth factors are the bone morphogenetic proteins (BMPs).[10] BMPs are more effective for spinal fusion than for segmental long bone defects.[11] Commercially available recombinant human BMP-2 and BMP-7 have been used for spinal fusion in patients undergoing back surgery. However, the doses needed for spinal fusion are extremely high, and several milligrams are usually applied. That amount is several orders of magnitude greater than the physiological level at which BMPs exist naturally in bone. The use of such high doses also raises the cost of treatment enormously as well as causing a safety issue.[3]

In view of such shortcomings of conventional treatment, hitherto untried novel approaches to bone healing such as gene therapy may have a place treating patients with critical-size bone loss that cannot be properly treated with current medical or surgical methods.[12] The purpose of this review is to provide an overview of the gene therapy used to treat bone defects with particular emphasis on the

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nonviral method which is safer and more desirable for nonlethal conditions such as bone defects.

OVERVIEW OF GENE THERAPY

Gene therapy is defined as introducing genetic material into cells with the intent of altering cellular function or structure at the molecular level to improve a clinical outcome.[13] Gene therapy either adds genetic information to cells or alters their genome structure.[14] Gene transfer can be performed using either *in vivo* or *ex vivo* approaches. During *in vivo* gene transfer, a vector carrying the therapeutic gene is directly injected into the recipient patient. A substantial number of host cells must be available for gene transfer and subsequent protein production with the *in vivo* approach. In addition, there is a difficulty targeting gene delivery to a specific cell population, as cells of surrounding tissue are often transfected. During *ex vivo* gene transfer, isolated cells are first transfected *in vitro* and then implanted into the recipient. Although the *ex vivo* approach requires more time and cost, it allows gene delivery to a specific cell population and screening of the cells prior to implantation.[14]

Gene therapy requires several components for successful production of the desired protein: the cDNA that codes for the protein, a vector that carries the genetic material into the cells, and the target cells or tissue capable of transcribing and translating the genetic information into the protein.[15] Vectors used to deliver genetic information into cells are generally divided into nonviral and viral vectors. Nonviral gene transfer is also called transfection, and viral gene transfer is known as transduction.[3] Viruses transfer their own genetic material very efficiently to the cells they infect. This property of viruses has been utilized to develop vectors for gene transfer after modifications.[16]

Some viral vectors may only infect dividing cells while others can infect both dividing and nondividing cells. Some vectors incorporate into the genome of host cells while others vectors remains episomal.[17] Gene sequences of an adenoviral vector that code for virulence, replication, and other useless or deleterious properties are removed from the viral genome. The therapeutic cDNA (transgene) is then cloned into the genetic space created to form a recombinant virus that is not pathogenic but retains its infectivity and ability to transfer genes to host cells. In contrast, retroviruses integrate into the host cell genome and show prolonged transgene expression. The property makes a retroviral vector useful for treating chronic genetic diseases.[18]

The specific type of gene transfer is determined by the clinical problem to be solved, either systemic or local. The clinical situation may require life-long or transient expression of a transgene. Musculoskeletal problems such as segmental bone defects require local expression of a transgene for weeks to months.[19] Therefore, a vector that has transient, short duration expression is needed for gene therapy to enhance bone repair. The local increase in osteogenic growth factors at the site of a bone defect is likely to speed healing in difficult clinical settings with poor osteogenic potential. Viral vectors are much more efficient for gene transfer than nonviral vectors. However, safety issues inevitably arise with viral gene transfer even though viruses are engineered to reduce pathogenicity while retaining the ability to infect target cells.[3] Adenoviral vectors elicit an immune response that can limit their clinical efficacy.[15] Retroviral vectors carry the risk of oncogenesis because of their integration into the host genome. These risks arising from the use of viruses are not justified for nonlethal musculoskeletal diseases.

Physical or chemical methods are used to facilitate cellular uptake of DNA during nonviral gene transfer. The former include physical stimuli such as ultrasound or an electric pulse (electroporation) as well as a gene gun, and the latter comprise associating DNA with a carrier, such as a liposome or other polymer. Generally, nonviral vectors are less effective than viral vectors, both in magnitude and duration of transgene expression. However, recent advances in the nonviral gene transfer technology have markedly enhanced the transfer efficiency of nonviral genes.

NONVIRAL GENE TRANSFER TECHNIQUES

An ideal vector should have a high efficiency of transfection, low toxicity, and reliable gene expression. Nonviral gene transfer techniques possess several advantages, as nonviral vectors are usually easy to manufacture, less expensive, and less toxic than viral vectors. Furthermore, immunogenicity and oncogenesis are not issues with nonviral vectors. Nonviral gene transfer usually demonstrates transient gene expression, which is advantageous in clinical settings such as wound healing or bone regeneration. Physical or chemical methods are employed to insert the therapeutic gene into the cells during nonviral gene transfers.

Physical methods

Transfection using naked DNA is the safest method for gene delivery. Successful delivery and gene expression has been achieved in several different tissues by hypodermic needle injection. Although transfection efficiency was very low, and the DNA is more susceptible to degradation, a clinical effect was observed nevertheless.[20,21] particularly for transfection of skeletal and cardiac muscle cells. Superior transfection of wounds has been achieved using a gene gun in which DNA-coated gold particles are accelerated[22] or by micro-seeding in which DNA is delivered through a set of oscillating needles via an infusion pump.[23] These methods improve transfection efficiency by increasing surface area and inducing a micro-trauma in the treated tissue, thereby improving DNA uptake. However, a disadvantage of the gene gun is that foreign particles, that is, gold, are introduced into the tissue and microseeding experience is very limited so far.[24]

Electroporation employs brief electric pulses to cells, which transiently create pores in the plasma membrane and allow DNA to enter the cell. The technique was originally developed to introduce genes into plants and was later adapted to transfect mammalian cells.[24] *In vitro* and *in vivo* gene delivery using electroporation has been reported.[25] This method shows good transfection efficiency of human embryonic stem cells and adult stem cells including bone marrow-derived cells and mesenchymal stem cells (MSCs),

as transfection efficiency reached 90%.[26,27] In addition, electroporation does not seem to affect MSC potential for multi-differentiation.[28]

Sonoporation utilizes the tissue-permeabilizing effect of ultrasound. Ultrasound allows for the controlled deposition of the gene after systemic delivery from outside the patient's body using suitable force fields.[29] Low-intensity ultrasound in combination with microbubbles has attracted much attention as a gene delivery method.[30] The use of microbubbles as gene vectors is based on the hypothesis that destruction of DNA-loaded microbubbles by a focused ultrasound beam during their microvascular transit through the target area will result in localized transduction upon disruption of the microbubble shell while sparing nontargeted areas.[30] The properties of DNA binding microbubbles can be improved using stabilizing them with lipids, such as combined poly-(ethylene glycol)-modified bubble liposomes.[31,32]

Chemical methods

Organic vectors. The organic vectors used as gene delivery materials carry cationic charge and condense the anionic DNA through electrostatic attraction.[33] The surface groups of polymers can also be modified with specific signaling factors targeted for cellular delivery via receptor-mediated endocytosis.[34] Positively charged lipid vesicles complex with negatively charged DNA to form particles with diameters of about 100 nm. Transfer of DNA across the cell membrane occurs through an endocytosis-like process. Large amount of DNA containing transgenes can be incorporated using this method.

While both synthetic and naturally occurring polymers facilitate gene transfection,[34] the degradation products of natural polymers including collagen, gelatin, and chitosan, are less cytotoxic.[35] However, natural polymers show inferior transfection efficiency compared to most synthetic polymers. Polymers can also be used impregnated in a scaffold for tissue engineering.[34] The fast degradation of a scaffold with rapid release of organic vectors is beneficial for certain applications. However, these conditions are not suitable for applications in which the scaffold needs to maintain its structural integrity for a prolonged period.

Plasmids adsorbed to a collagen sponge to form a gene activated matrix (GAM) enhance fracture healing.[36] The GAM increases stability of the gene complexes and provides a means for sustained DNA release. The transfection efficiency of DNA condensed with liposomes or polyethylenimine improves when the complexes are incorporated in a GAM.[37]

Systemic delivery of organic nonviral vectors is associated with additional hurdles, including strong interactions between blood components and cationic delivery vehicles, kidney filtration, toxicity, uptake by the reticuloendothelial system (RES), and the targeting ability of the carriers to the cells of interest.[30,38,39]

Surface modification of the cationic vectors can reduce their interaction with blood components, decrease their toxicity and RES uptake, and increase their binding affinity with target cells. Binding of plasma proteins (opsonization) is the primary mechanism for RES to recognize circulating nanoparticles.[30] Modifying the surface with poly(ethylene glycol) reduces opsonization and aggregation of nonviral vectors and minimizes clearance by the RES, leading to prolonged circulation lifetime after intravenous administration.[38,39]

Nonviral vectors of different classes can be converged to produce a novel nonviral vector that combines features of different classes.[30] Designing and synthesizing novel cationic lipids and polymers and covalently or noncovalently binding genes with peptides, targeting ligands, polymers, or environmentally sensitive moieties can resolve the problems encountered by nonviral vectors.[40,41]

Inorganic vectors. The application of inorganic nanoparticles for gene delivery is an emerging field that holds great promise because they can be prepared and surface-functionalized in many different ways.[42–44] The inorganic materials that carry DNA are calcium phosphates, gold nanoparticles, silica, magnesium phosphates, and iron oxides.[45,46] The benefit of inorganic vectors includes stability during storage, manufacturing cost-effectiveness, low immunogenicity, and resistance to microbial attack.[45,47] Inorganics are superior to organics for attached cell lines because of their increased density.[48] Because of gravitational forces, the inorganics settle onto the region containing the cells or tissues at the base of the tissue culture flask and thereby greatly enhance their gene transfer efficiency by increasing adherence to the cell membrane.[48,49]

Complexes of organic and inorganic materials such as collagen-hydroxyapatite scaffolds have been investigated for nonviral gene delivery, although transfection efficiency is low.[50] Polyethyleneimine (PEI)-pDNA polyplexes have more prolonged and elevated levels of transgene expression when loaded onto collagen-nanohydroxyapatite scaffolds compared with collagen or collagen-glycosaminoglycan when transfected into MSCs.[51]

RNA TRANSFECTION

RNA-based therapies have recently received increased attention and are effective for gene transfer.[52–54] Transfer of mRNA also induces transgene expression.[52,55] In contrast, small interfering or silencing RNA (siRNA) suppress proteins related to the pathogenesis of a disease[56] as demonstrated by *in vitro* models of osteoporosis and osteosarcoma.[57] While a gene in DNA enters the nucleus to function, mRNA operates in the cytoplasm.[58] This means that mRNA has less cellular obstacles to overcome than a DNA molecule. As an example, mRNA transfection efficiency during electroporation into dendritic cells reaches 95% with mRNA versus 1–10% with DNA.[52] The fundamental difference in the therapeutic location within the cell can lead to a significant difference between the two therapies.[59] In addition, RNAs are biologically safer because they does not integrate into the host genome.[52] As the effect of the mRNA is transient compared to DNA transfection,[59] mRNA may be more beneficial than DNA segments in applications such as bone

regeneration where therapeutic action is required only for short durations.

siRNA has been extensively studied to identify a possible therapeutic option for intractable diseases such as genetic diseases and cancer.[60,61] siRNA molecules bind to the specific pathogenic mRNA molecules in the cytoplasm, blocking translation to the pathogenic protein.[62–64] Similar to DNAs, siRNAs also require a vector to be transported into the cell so that they can reach the RNA-induced silencing complex in the cytoplasm. A variety of materials such as polymers, liposomes, and inorganic materials such as calcium phosphate are effective vectors for siRNA delivery.[60,65]

Despite the potential advantages of RNA-based therapy, there also are several obstacles that need to be overcome for clinical applications. The main challenge is susceptibility to degradation.[66,67] An mRNA molecule must be protected from the cytoplasmic enzymes until it reaches the ribosomes to exert its desired therapeutic function. mRNA also has a net negative charge that renders it unable to penetrate the cell membrane without the use of a carrier molecule. Factors that are important for stable complexation between the vector and DNA are also critical for RNA-vector complexation. These factors are hydrophobicity/hydrophilicity, molecular weight, pH of the solution during condensation, charge density, and electrostatic binding.[68] Several vectors that are effective for RNA delivery are cationic lipids and cationic polymers, which have been used as DNA vectors.[69–73] As RNA delivery is an emerging technique, *in vivo* effectiveness for bone regeneration has not been investigated in numbers and this technique awaits further preclinical evaluation through animal models.

THERAPEUTIC GENES TRANSFERRED FOR BONE REGENERATION

A large number of growth factors stimulate osteogenesis. Most of these factors either stimulate differentiation of progenitor cells into chondrocytes or osteoblasts or promote bone-forming activities of mature osteoblasts. In addition, angiogenic factors including vascular endothelial growth factor (VEGF) are also important for osteogenesis, particularly for intramembranous ossification.[3,74] Gene transfer of VEGF by stem cells enhances repair of cranial defects in mice, whereas that of the antagonist (sFlt1) inhibits the repair.[74] Table I summarizes therapeutic gene transferred for bone regeneration in animal models.

BMPs are the most commonly used genes for nonviral gene transfer to promote bone healing. High transfection efficiencies of up to 95% are obtained in human MSCs cells using naked DNA *in vitro* by delivering BMP-2 cDNA in an alginate hydrogel. An increasing amount of biologically active BMP-2 was released from the cells over 5 weeks, and transfected cells were found after 2 and 6 weeks of implantation in naked mice.[75] pBMP-2 transfection mediated by GenEscort II, a PEI derivative, enhances the osteogenic differentiation of canine bMSCs and promotes new ectopic bone formation in nude mice.[76] Constructs consisting of an alginate hydrogel and BMP-2 cDNA together act as a nonviral gene-activated matrix and promote osteogenic differentiation and subsequent bone formation in cell-free constructs at an orthotopic location in a goat model.[77] Collagen-hydroxyapatite scaffolds for nonviral delivery of a plasmid encoding BMP-7 have also been developed. Although transfection efficiency was low, significant levels of BMP-7 were expressed and were associated with an increase in cell proliferation.[50] Hepatocyte growth factor (HGF) has also been investigated for possible bone regeneration applications. Percutaneous nonviral delivery of HGF in an osteotomy gap promotes bone repair in rabbits.[78]

Considering that individual growth factors act at different stages of osteogenesis, a combination of factors may promote bone healing more potently than a single factor. This concept has been demonstrated in animal models using gene delivery of BMP-2 and VEGF,[79] BMP-4 and VEGF,[74] BMP-2 and BMP-7,[80,81] basic fibroblast growth factor and BMP-2,[82] BMP-4 and transforming growth factor (TGF)-β.[83]

An alternative approach to deliver a gene coding for growth factors includes the delivery of key osteogenic transcription factors such as Runx2[84] and osterix (transcription factor Sp7).[24] As these transcription factors reside and act inside cells, traditional protein delivery methods are not available for these factors. Because they are not secreted and do not enter systemic circulation, they may be more safely used for gene transfer than growth factors. Electroporation-mediated transfer of Runx2, osterix, or both genes enhances *in vitro* and *in vivo* osteogenesis from adipose stem cells (ASCs) *in vitro* and also in a mouse ectopic model.[85] Delivery of caALK6, a BMP receptor, and Runx2 genes from nanomicelles incorporated into a scaffold induces substantial bone formation covering the entire lower surface of the implant with no sign of inflammation at 4 weeks using a bone defect model in a mouse skull bone.[86] Cotransfection of BMP-2 and Runx2 by electroporation also significantly increases *in vivo* ectopic formation of ASCs compared with untransfected ASCs or ASCs transfected with the BMP-2 gene only.[87]

LIM mineralization protein-1 (LMP-1) is another intracellular, osteogenic molecule that was discovered while screening transcripts induced during osteogenesis.[88] Although its function is largely unknown, some of its activity is related to its ability to prevent degradation of Smad signaling molecules.[89] Dramatic results were reported in rat spinal fusion models using plasmid DNA liposomes as the vector to deliver LMP-1.[90] Because LMP-1 is an intracellular protein, it may offer strategic advantages over extracellular proteins such as BMPs, whose action may be limited by the low prevalence of specific BMP receptors on the surface of resting osteoprogenitor cells.[90] A related protein, LMP-3, also promotes osteogenesis in animal models.[91–93]

Knockdown of the BMP inhibitors noggin[94] and chordin[95] promotes bone regeneration. The delivery of siRNA molecules by RNA transfection or delivery of a vector that codes for the inhibitory RNA molecule stimulates osteogenesis. These approaches are novel concepts and the clinical applicability is not widely investigated. Experimental attempts to enhance fracture repair by inhibiting osteoclast function using gene transfer have not been reported,

TABLE I. Therapeutic Genes Transferred for Bone Regeneration

Gene	Gene carrier	Animal model	Key results	Ref.No
VEGF	Retrovirus	Mouse ectopic ossification	The beneficial effect of VEGF on bone healing elicited by BMP-4. Flt1 inhibited bone formation elicited by BMP-4.	74
BMP-2	cDNA in alginate	Mouse ectopic ossification	The protein levels were effective in inducing osteogenic differentiation *in vivo*.	75
BMP-2	PEI derivative (GenEscort™ II)	Mouse ectopic ossification	Enhanced osteogenic differentiation of canine MSCs and promotion of the ectopic new bone formation.	76
BMP-2	cDNA in alginate and ceramic granules	Goat ectopic ossification	Bone formation in cell-seeded constructs at an ectopic location.	77
HGF	HVJ-E virus	Rabbit tibia osteotomy	hHGF plasmid promoted bone repair in the osteotomy gap.	78
BMP-2+ VEGF	Lipofectamine™ 2000 (Invitrogen)	Mouse ectopic ossification	The combination of BMP-2 and VEGF formed significantly more bone at 4 weeks, and VEGF transfection resulted in more blood vessels relative to the conditions without VEGF.	79
BMP-2+ BMP-7	Adenovirus	Mouse calvarial defect	AdBMP-2/7-transduced cells were more effective in healing cranial defects than were cells individually transduced with AdBMP2 or BMP7.	80
BMP-2+ BMP-7	Adenovirus	Rat spine fusion	Combined BMP-2 and BMP-7 gene transfer was significantly more effective in inducing osteoblastic differentiation and spine fusion than individual BMP gene transfer.	81
BMP-2 +bFGF	PEI with linoleic acid (PEI-LA) and 25 kDa PEI (PEI25)	Rat ectopic ossification	The BMP-2 secretion from PEI-LA delivered expression vector was equivalent and/or superior to PEI25 depending on the plasmid DNA implant dose.	82
BMP-4+TGF-β1	DOTAP liposome (Roche)	Rabbit femoral defect	Osteogenetic speed was prominently accelerated, and the quality was improved after the treatment with BMP-4 gene combined with TGF-beta1.	83
Runx2	Adenovirus	Mouse calvarial defect	Runx2-expressing cells dramatically enhanced the healing of critical-sized calvarial defects and increased both bone volume fraction and bone mineral density.	84
Runx2, osterix	Electroporation+PLGA	Mouse ectopic ossification	Runx2- or Osterix-transfected-PLGA hybrid promoted bone formation in nude mice after 6 weeks of *in vivo* implantation.	85
caALK6+Runx2	Poly(ethyleneglycol) (PEG)-block-catiomer (PEG-b-P[Asp-(DET)])	Mouse calvarial defect	Substantial bone formation covering the entire lower surface of the implant was induced with no sign of inflammation at 4 weeks.	86
BMP-2+ Runx2	Electroporation+PLGA	Mouse ectopic ossification	BMP-2/Runx2-transfected ASCs showed a significant increase in bone formation compared to ASCs and BMP-2-ASCs.	87
LMP-1	Superfect transfection reagent (Quiagen)	Rat spine fusion	Controlled new bone formation in the carrier and marrow transfected with the active LMP-1 cDNA.	90
LMP-3	Adenovirus	Mouse ectopic ossification	Dermal fibroblasts expressing Ad.LMP-3 were able to induce ectopic bone formation following implantation into the mouse muscles.	91
LMP-3	Adenovirus	Rat mandible defect	Efficient neoosteogenesis was observed in animals treated with LMP-3-expressing skin fibroblast.	92

although this might offer a promising short-term approach to increase bone mass.[3]

While a lot of therapeutic genes have been mostly tested using ectopic models in rodents as described above (Table I), reliable results from orthotopic models in large animals are required to assess the possibility for clinical trial. In this regard, preclinical evaluations in critical-size bone defect in larger animals are recommended for genes which demonstrated favorable results in rodent studies.

GENE TRANSFER APPLICATIONS TO PROMOTE BONE HEALING

Ex vivo applications

Traditional *ex vivo* approaches have been extensively studied in animal models using viral vectors carrying the BMP-2 gene for segmental defects[96–98] or for spinal fusion in a rat model.[99–101] The utility of BMP-2 transferred to osteoprogenitor cells from fat,[102] periosteum,[103] and muscle[104] has also been demonstrated. Furthermore, fibroblasts obtained from skin[105] and gingival tissue have been used successfully.[106] Because small animals have very good bone healing properties without intervention, it is important to confirm the results of a procedure in large animals.[17] *Ex vivo* approaches using BMP-2 have produced encouraging results in horses,[105] goats,[107–109] and pigs.[110]

An expedited *ex vivo* approach has been developed to avoid the drawbacks of conventional *ex vivo* approaches: being time-consuming and expensive.[111] The concept is to biopsy tissue in the operating room, genetically modify the cells, and return them to a bone defect within a single operative setting.[112,113]

One marked advantage of *ex vivo* approaches is presentation of cells in the lesion. This makes a difference when the soft tissue surrounding the defect has been compromised by irradiation, injury, or the disease process. The implanted MSCs may contribute to bone healing by secreting morphogens that stimulate endogenous regeneration rather than surviving in the defect and forming part of the regenerated tissue.[114] Establishing genetically modified, universal MSCs would greatly reduce the cost and complexity of *ex vivo* gene delivery to bone.

Direct gene therapy approaches

Therapeutic DNA can be delivered to cells *in vivo* by direct placement. Examples of direct gene therapy approaches are shown in Table II. Localization of the vector within the bone

TABLE II. Direct Gene Therapy Approaches

Gene	Transfer method	Animal model	Key results	Ref. No
BMP-9	Sonoporation with microbubble	Mouse ectopic ossification	Bone tissue was formed in the site of BMP-9 delivery. The sonoporation method was significantly inferior in its efficiency of gene delivery.	116
BMP-2	Sonoporation with microbubble	Mouse ectopic ossification	Transcutaneous sonoporation with pCAGGS-BMP-2 caused osteoinduction in the skeletal muscle of mice.	117
BMP-4	Electroporation	Mouse ectopic ossification	BMP-4 transferred by electroporation can induce *in vivo* and in situ ectopic bone formation in skeletal muscle	118
BMP-2	Electroporation	Rat ectopic ossification	Transcutaneous electroporation with pCAGGS-BMP-2 induced ectopic bone formation in the skeletal muscle of rats.	119
BMP-2	Electroporation	Rat ectopic ossification	BMP-2 gene transfer using *in vivo* electroporation induced not only endochondral ossification but also intramembranous ossification.	120
BMP-2+BMP-7	Electroporation	Rat ectopic ossification	Simultaneous transfer of BMP-2 and BMP-7 genes using *in vivo* electroporation induced more rapid bone formation than the transfer of either gene alone.	121
BMP-4	Electroporation	Mouse ectopic ossification	Ectopic bone formation by BMP-4 gene transfer into the muscle induced endochondral ossification that corresponded well with that by implantation of demineralized bone matrix.	122
BMP-4	PEI/PLGA	Rat calvarial defect	Scaffold delivery system encapsulating PEI-condensed DNA encoding for BMP-4 was capable of enhancing bone formation.	125
BMP-2	Liposome with collagen carrier	Pig calvarial peiimplant defect	A significantly positive effect of liposomal vector/BMP-2 on bone regeneration and osseointegration in bony circumferential peri-implant defects.	126
BMP-2	Atelocollagen and calcium-phosphate precipitates (CaP)	Rat tibial defect	Implantation of bmp2-CaP-collagen bridged the bone defect at 4 weeks, and the strength of the bone was comparable to that of an intact tibia at 6 weeks.	127

defect can be performed either by physical placement of the vector at the target site or by release of the gene from a three-dimensional scaffold implanted in the defect.[115] Physical placement means directly injecting the transgene into the lesion site.[115] An electric pulse or ultrasound is used to drive the genes into cells. Both *in vivo* electroporation or sonoporation are used to permeabilize cell membranes and translocate naked DNA into the nucleus.[115] An osteogenic gene has been directly injected into bone defect either trans- or percutaneously using these methods. Electroporation and sonoporation result in bone formation at ectopic sites when applied after delivery of genes from the BMP family.[116–122] The electroporation approach induces more bone formation than that of a sonoporation approach.[116] A careful pulse application is necessary to avoid tissue damage when using electroporation for regenerative medicine. Ultrasound-mediated osteogenic gene delivery could serve as a therapeutic solution for conditions requiring bone tissue regeneration, although it is significantly inferior in its gene delivery efficiency compared to that of electroporation.[116] Protocols or systems for sonoporation need further updating to achieve clinically relevant bone formation.

One major disadvantage of injecting an osteogenic gene into a bone defect is the difficulty in localizing transfection to a specific region.[115] Potential adverse effects are heterotopic ossification of adjacent muscle tissue and fusion of one bone to an adjacent bone as well as ossification of cartilaginous and ligamentous tissues, which may cause joint dysfunction.[115] Electrical impedance tomography can be used to monitor electroporation in real time and thereby control the extent of gene delivery by measuring conductivity of electroporated tissues.[123]

Implanting a biomaterial impregnated with DNA offers an alternative mode for direct gene transfer. This technique allows transfection of only cells surrounding or penetrating the biomaterial. A GAM, in which naked plasmid DNA is physically entrapped in a polymer matrix sponge, represents the first method of localized gene therapy for bone repair.[36,124] While a very low transfection efficiency was reported for this approach, condensed DNA with chemical vectors such as PEI,[125] liposomes,[126] and calcium-phosphate precipitates[127] increase transfection efficiency of the GAM. However, most of biomaterials used to produce GAMs lack the mechanical properties usually required to sustain loads present in a long-bone fracture site, which creates a challenge to use this technique for a lesion requiring structural support such as a segmental bone defect.

Although direct gene transfer is an attractive concept, the effectiveness and safety issues are not firmly established at this time and need further elaboration to improve its clinical applicability.

CONCLUSIONS

Recent advancement in the nonviral gene transfer technology has brought the gene therapy-based bone regeneration into clinical possibility. Either physical method such as electoporation and sonoporation or chemical methods using organic and inorganic vectors may be employed to promote bone healing. The nonviral gene therapy can be combined with stem cell therapy to further enhance the results in patients who have clinical situation intactable to conventional treatment. Well-controlled, proof-of-concept preclinical trials in large animals are warranted to choose a best method that can be carried over to a clinical trial.

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