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Gene therapy for the regeneration of bone

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Abstract

Gene transfer technologies offer the prospect of enhancing bone regeneration by delivering osteogenic gene products locally to osseous defects. In most cases the gene product will be a protein, which will be synthesized endogenously within and around the lesion in a sustained fashion. It will have undergone authentic post-translational processing and lack the alterations that occur when recombinant proteins are synthesized in bioreactors and stored. Several different *ex vivo* and *in vivo* gene delivery strategies have been developed for this purpose, using viral and non-viral vectors. Proof of principle has been established in small animal models using a variety of different transgenes, including those encoding morphogens, growth factors, angiogenic factors, and transcription factors. A small number of studies demonstrate efficacy in large animal models. Developing these promising findings into clinical trials will be a long process, constrained by economic, regulatory and practical considerations. Nevertheless, the overall climate for gene therapy is improving, permitting optimism that applications in bone regeneration will eventually become available.

Keywords

vector; adenovirus; adeno-associated virus; animal model; osteoprogenitor cell; osteoblast; gene activated matrix; allograft revitalization; clinical trial

Introduction

Thanks to a considerable volume of research into the embryology of osteogenesis and the biology of fracture repair in adult organisms, we possess a large database on the ways in which nature forms bone^{16, 37}. This provides the opportunity to use such information to improve bone healing in the clinic. As the success of autograft bone demonstrates⁴⁹, new bone can be formed from pre-existing osteoblasts. However, most future strategies for improving bone regeneration will rely on the use progenitor cells¹⁹. These require, at a minimum, a source of these progenitors and morphogenetic stimuli to promote their differentiation into osteoblasts or, for endochondral ossification, chondrocytes. Additional requirements, including suitable scaffolds and appropriate mechanical environments, are beyond the scope of this review but have been dealt with extensively by other authors^{64, 68}.

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Gene transfer is a technology that allows the targeted, *in vivo* synthesis of useful gene products, both RNA and protein, in a controlled fashion. Because many important bone morphogenetic signals are proteins, transfer of their cognate genes can serve as the basis for an osteogenic reparative response. Unlike proteins delivered in recombinant form, those synthesized endogenously will have undergone authentic post-translational modification and be free from the altered molecules that can reduce activity and provoke immune responses. This article discusses the various strategies for developing these concepts into clinically useful gene therapy\ies for bone regeneration and reviews progress towards this end. Additional recent reviews of this area can be found in references ^{20, 48, 10, 52}.

Gene therapy basics

Vectors

Gene therapy requires the transfer of therapeutic genes, or more usually their complementary DNAs (cDNAs), into suitable cells. Vectors are used for this purpose. Because viruses transfer their own genetic material very efficiently to the cells they infect, they serve as the basis for many vectors ²⁸. For most purposes, the viral genome is modified to remove sequences that contribute to virulence, replication and other confounding properties. The therapeutic cDNA (transgene) is then cloned into the genetic space created by these modifications to form a recombinant virus that retains its infectivity and its ability to transfer genes to cells, but is non-replicating, much safer and less virulent than the original virus. Gene transfer using viral vectors is called transduction.

A number of different viruses have been modified in this way. Prominent viral vectors that have been used in human clinical trials are listed in Table 1. As noted in this Table, different vectors vary in their carrying capacity, immunogenicity, ease of manufacture, whether or not the target cells need to be dividing and their ability to integrate viral DNA into the host cell genome. Integration into the host cell genome is one way to prolong expression of the transgene, and is therefore useful for treating chronic genetic diseases such as X-linked severe combined immunodeficiency disease (SCID) ²⁷. Because successful bone regeneration will not require such long-term transgene expression, most research has used vectors which do not integrate their DNA and which are spontaneously cleared by the body. Among these, recombinant adenovirus vectors have been the most widely used. They are quite easy to construct and produce at high titers, they are highly infectious in many types of cells and usually express at high levels *in vivo* for a period of several weeks, after which they are cleared by cell turnover or the immune system. In many instances, this profile of gene expression is likely to match nicely the needs of bone regeneration. Concerns when using adenovirus vectors include its antigenicity and the fact that most of the population has circulating antibodies against adenovirus serotype 5, the serotype most often used by adenoviral vectors. Immune recognition of the virus can inhibit gene transfer.

Because of residual safety concerns when using viral vectors, as well as their cost and complexity, there is sustained interest in using non-viral vectors to deliver therapeutic DNA ⁵¹. Gene transfer using non-viral vectors is called transfection. This may be accomplished with naked DNA, but it is more usual to facilitate cellular uptake of the DNA by associating it with a carrier, such as a liposome or other polymer, or by using a physical stimulus, such as sonication or an electric pulse (electroporation). In general terms, non-viral vectors tend to be less effective than viral vectors, both in terms of the level and duration of transgene expression.

Strategies

Cells can be genetically modified *in situ* or by their removal, genetic modification and reimplantation. The former process, also known as *in vivo* gene therapy, obviates the need to

recover and manipulate cells, which greatly simplifies the process. However, not all vectors are suitable for *in vivo* gene delivery and the introduction of extraneous genetic material directly into the body raises considerable safety issues. Genetic modification of cells outside the body, known as *ex vivo* gene therapy, obviates many of these safety concerns but is cumbersome and expensive, especially when using autologous cells that need to be cultured *in vitro*. Based upon these principles, four strategies have emerged for promoting bone regeneration by gene transfer (figure 1). Two of them are *ex vivo* (traditional and expedited) and two are *in vivo* (direct injection and gene-activated matrix (GAM))²⁰.

Choice of transgenes

Table 2 lists the transgenes that have been used in pre-clinical studies of gene therapy for bone healing. Not surprisingly, cDNAs encoding osteogenic bone morphogenetic proteins (BMPs) have been the most widely used. BMP-2 and BMP-7 have the additional attraction that their recombinant proteins are already in clinical use. This greatly aids the translatability of a gene therapy based on these molecules. As well as growth factors that enhance osteoblast differentiation and matrix deposition, there is interest in using factors, such as vascular endothelial growth factor (VEGF), that promote angiogenesis¹⁴. This reflects the increasing recognition of the vital need for a blood supply when forming bone. In this context it is interesting that, although most research has focused on forming new bone by stimulating direct osteogenesis of precursor cells, there is a growing appreciation of the advantages of the endochondral route to bone formation⁵⁸. One attraction of this pathway is the spontaneous secretion of angiogenic factors by the cartilage as it hypertrophies and prepares to ossify. This means that the endogenous biology of the system relieves the clinician of having to manipulate the system exogenously to ensure adequate blood supply to the healing bone. This is important, because large osseous lesions are likely to be highly hypoxic, a circumstance that strongly favors the chondrogenic differentiation of progenitor cells¹⁴.

As well as morphogens and growth factors, there is literature on the use of cDNAs encoding transcription factors, such as Runx2 and Osterix, that are associated with osteogenesis (Table 2). Because these are intracellular proteins, as are the osteogenic LIM mineralization (LMP) proteins, they are difficult to deliver by other means. The same is true for the constitutively active form of the BMP receptor Alk-2, which requires endogenous synthesis to insert itself correctly into the cell membrane.

Rather than promoting osteogenesis directly, an additional strategy is to reduce the synthesis of BMP inhibitors using a technology known as RNA interference. Encouraging results have been published when chordin³⁸ or noggin⁷¹ are reduced in this manner. In future, it may be worthwhile to investigate the effects of transgenes that reduce inflammation or inhibit osteoclast activity.

It seems likely that a combination of different transgene products would be more osteogenic than any single factor delivered alone. While there is experimental evidence that this is indeed the case^{36, 41, 53, 75}, the delivery of multiple transgenes would greatly complicate the regulatory issues surrounding clinical translation.

Experimental findings

Traditional *ex vivo*

Lieberman and colleagues were the first to use this approach^{42, 43}. Using a rat model, they removed bone marrow, expanded the stromal cell population by monolayer culture, and introduced a cDNA encoding bone morphogenetic protein-2 (BMP-2) using an adenovirus

vector (Ad.BMP-2). The transduced cells, secreting high levels of BMP-2, were seeded onto a collagenous scaffold and placed into critical sized defects in the femora of rats. This procedure healed the defects within 12 weeks, with evidence of superior new bone quality as compared to the use of recombinant (r) BMP-2. This group of investigators has subsequently demonstrated the efficacy of this method for achieving spinal fusion in a rat model⁵⁴. In later work, they used a lentivirus for delivery of BMP-2 cDNA for both long bone healing⁷⁰ and spinal fusion^{46,47}. This vector enabled the *in vivo* expression of BMP-2 for much longer than the adenovirus vector and produced bone with greater mechanical strength. Gazit et al²⁵, in contrast, were able to heal osseous defects in mice effectively using transfected cells transiently expressing low levels of BMP-2.

Subsequent investigators have confirmed the utility of this approach with Ad.BMP-2 and osteoprogenitor cells derived from fat⁵⁵, periosteum⁸, muscle⁴⁰ and other such sites. However, it is unclear whether osteoprogenitor cells are necessary for this method to work efficiently, and success has been achieved using fibroblasts obtained from skin³² and gingiva⁶⁰. Moreover, data from experiments in regenerative medicine using so-called mesenchymal stem cells (MSCs) suggest that the MSCs do not survive long in the defect and do not form part of the regenerated tissue. Instead, they may serve to secrete morphogens that stimulate endogenous regeneration by host cells³⁰. This matter remains the subject of considerable research.

MSCs are of additional interest because they may have the ability to home to sites of osseous injury⁵⁹ which would greatly simplify delivery, especially when there are multiple sites needing repair. There is also the possibility that MSCs can be successfully allografted¹³. If so, the establishment of genetically modified, universal donor MSCs would greatly reduce the cost and complexity of *ex vivo* gene delivery to bone. However, Tsuchida et al⁶⁶ were unable to heal osseous defects in rats with allograft marrow cells expressing BMP-2 in the absence of immunosuppression. Nevertheless, Yi et al⁷³ reported success in healing fractures in osteoporotic rats using xenogeneic, irradiated, transduced chondrocytes expressing BMP-2.

Because the bones of small animals used by laboratory researchers tend to heal well, it is important and necessary to perform experiments in large animals. The traditional *ex vivo* approach using Ad.BMP-2 has given highly encouraging results in horses³², goats^{15, 65, 72} and pigs¹².

Expedited *ex vivo*

Because traditional *ex vivo* approaches are cumbersome and very expensive, there is interest in developing expedited methods that can be achieved in the operating room²³. The aim is to biopsy tissue, genetically modify the cells and return them to an osseous lesion within a single operative period. There are two examples of this approach.

Using a rabbit spinal fusion model, Vigneswarapu et al⁶⁹ withdrew blood, isolated “buffy coat” cells, transduced them with a recombinant adenovirus carrying cDNA encoding LMP-1, and implanted them within a single operation. All animals went on to successful spinal fusion. In a second approach known as “facilitated endogenous repair”²³, critical sized defects in the femora of rats were healed using genetically modified muscle and fat²². These tissues were selected because they contain osteoprogenitor cells, can be readily biopsied and have intrinsic scaffolding properties. They were transduced with Ad.BMP-2 and inserted into the osseous lesions. Healing of the critical sized defects was uniform and rapid²².

Further study of bone regeneration using gene-activated muscle suggested that the implant rapidly undergoes chondrogenesis, leading to efficient healing by endochondral ossification²². At least some of the newly formed lining osteoblasts were of donor origin. The speed and efficiency of this method probably reflects the fact that the implantation of fat or muscle expressing BMP-2 provides simultaneously progenitor cells, a morphogenetic signal and an osteoconductive scaffold. Unlike the case when Ad.BMP-2 was injected directly into the rat, abbreviated *ex vivo* delivery using fat and muscle did not increase the levels of circulating anti-adenovirus antibody²². Clotted bone marrow provides an additional vehicle for this approach to tissue regeneration⁵⁰.

One advantage of *ex vivo* approaches, whether expedited or not, is the provision of cells to the lesion. This will be of major importance when the soft tissue surrounding a defect has been compromised through irradiation, injury or disease.

In vivo delivery

The most direct way to deliver genes to osseous lesions is to inject the vector directly; in many cases, this could be achieved percutaneously³⁵. In most instances adenovirus has been used for this purpose, but success has been reported using retrovirus vectors^{56, 57, 63} suggesting that there is sufficient cell division in the area of experimental defects to support retroviral transduction. Most investigators have used BMP-2 as the transgene, but success has been reported with BMPs -2, -4, -6, -7 and -9, cyclooxygenase-2 and LMP-1. In the rat segmental defect model, healing in response to the intralesional injection of Ad.BMP-2 was improved by delaying the injection until 10 days after creation of the defect⁴. This may reflect the time needed for osteoprogenitor cells to enter the lesion, as well as the time of optimum expression of the coxsackievirus adenovirus receptor (CAR)³⁴. Bone formation in response to Ad.BMP-2 was dose-dependent⁶, but as high doses of adenovirus are inflammatory this needs to be carefully titrated. The inflammatory response to adenovirus in the rat may explain why the mechanical strength of the healed bone was only about 25% of normal after eight weeks⁵. Studies in rabbits suggested very limited transduction of cells occurred beyond the osseous lesion into which the recombinant adenovirus was injected², indicating that adverse systemic sequelae are unlikely.

Results for large animal models have been mixed. Both Ad.BMP-2 and Ad.BMP-6 have been used successfully to heal experimentally created osseous defects in horses^{31, 32}. However, responses in sheep models were muted, possibly because of immune responses by the sheep to adenovirus and human BMP-2^{17, 18}.

Gene activated matrices (GAMs)

In their original formulation GAMs consist of a collagenous scaffold impregnated with plasmid DNA encoding osteogenic products²⁴. When the GAM is inserted into an osseous lesion, host cells enter the scaffold, become transfected by the DNA and secrete the osteogenic gene product, leading to repair. Because DNA is chemically stable, GAMs have the potential to become “off-the-shelf” products – a major practical advantage.

Although promising initial results were published using rat²⁴ and dog⁷ models, the original plasmid-based technology did not prove efficient enough for further development. However, scaffolds containing viral vectors have proved much more promising and a GAM containing adenovirus carrying platelet-derived growth factor (PDGF) cDNA is in clinical trials for healing diabetic skin ulcers (www.t-r-co.com). The same GAM has shown promise in rat model of periodontal bone loss¹¹. There is evidence that a collagenous scaffold can reduce the immune response to adenovirus⁶².

An intriguing extension of the GAM concept uses allograft bone as the scaffold and recombinant adeno-associated virus (AAV) as the vector. This technology was primarily developed to heal large segmental defects that would normally receive a structural allograft. Because these allografts do not remodel, they integrate poorly with the host bone and have a high failure rate. Schwarz's group has developed a "revitalized" allograft coated with AAV. In their first experiments they used two AAV vectors, one of which encoded receptor activator of NF- κ B ligand (RANKL) to promote osteoclastogenesis and the other encoded VEGF to promote angiogenesis³³. Allograft coated with these two vectors was inserted into segmental defects in mouse femora. Cells were locally transduced by the AAV vectors, leading to invasion of the allograft by osteoclasts and its replacement with host bone. Similar data³⁵ were subsequently obtained using as the transgene a cDNA encoding a constitutively active form of the Alk-2 type I receptor for BMP which bears the same mutation as found in patients with the disease *fibrodysplasia ossificans progressiva*⁶¹. Because AAV is quite stable, especially when freeze-dried onto a solid support, this construct could also serve as an "off-the-shelf" product.

Progress towards clinical trials

Getting gene therapy protocols into human clinical trials is a lengthy, expensive and frustrating process, especially for conditions that are not lethal²¹. Clinical trials require Investigational New Drug (IND) permission from the FDA. Before gaining approval, the efficacy of any potential gene therapy for bone regeneration would need to be confirmed in large animals, such as sheep, goats, pigs or horses. Safety is of especial concern for any gene therapy and safety testing requires extensive and exacting pharmacology and toxicology studies, including biodistribution analysis of the transferred genomes, conducted under Good Laboratory Practice (GLP) conditions. Production of the relatively large amounts of clinical grade vector needed for trials is another complicated and expensive requirement. Outcome measures are presently inadequate for clinical trial purposes and novel, non-invasive techniques are being developed to determine bone quality and mechanical strength.

Gene therapy for bone regeneration has applications in veterinary, as well as human, medicine and the regulatory route is less burdensome. Success in veterinary applications would facilitate their adoption into human clinical use.

All things considered, it is likely that gene-based modalities for regenerating bone will enter clinical trials within the next 5–10 years. As noted, a GAM containing adenovirus encoding PDGF is already in the clinic for wound healing and has shown promise in stimulating periodontal bone formation. Moreover a number of additional genetic strategies have already proved effective in large animal models. Moving such technologies into the clinic requires considerable sums of money that are unlikely to be found in academia. Other sources of funding are thus required, and future development may depend to a large degree on the level of enthusiasm generated within industry for gene-based products. Recent clinical successes of gene therapy for the treatment of several diseases, including X-linked SCID²⁷, Leber's congenital amaurosis^{1, 45}, and lipoprotein lipase deficiency⁹ should be helpful in this regard.

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Figure 1. Strategies for gene transfer to defects in bone

There are two general strategies: *in vivo* (right hand side) and *ex vivo* (left hand side). For *in vivo* gene delivery, the vector is introduced directly into the site of the osseous lesion, either as a free suspension (top, right hand side) or incorporated into a gene activated matrix (GAM) (bottom, right hand side). For *ex vivo* delivery, vectors are not introduced directly into the defect. Instead they are used to genetically modify cells, which are subsequently implanted. Traditional *ex vivo* methods (top, left hand side) usually involve the establishment of cell cultures, which are genetically modified *in vitro*. The modified cells are then introduced into the lesion, often after seeding onto an appropriate scaffold. Expedited *ex vivo* methods (bottom, left hand side) avoid the need for cell culture by genetically modifying tissues such as marrow, muscle and fat, intraoperatively and inserting them into the defect during a single operative session. Reproduced from reference [20] with permission.

Table 1

Salient properties of the main viral vectors used for human gene therapy

Parent virus	Key properties of wild-type virus	Advantages	Disadvantages	Comment
<i>Adenovirus</i>	Double stranded DNA genome, ~ 35Kb	Straightforward production of recombinant vectors at high titers	Inflammatory and antigenic	Various generations with increasingly deleted genomes "Gutted" vectors have no viral coding sequences and large carrying capacity but are difficult to produce.
	Non-enveloped	Transduces non-dividing cells		Tropism can be modified by altering coat proteins
	Over 50 serotypes	Wide choice of serotypes		
	~ 100nm in size			
	Genome remains episomal in infected cells			
<i>Herpes simplex virus</i>	Double stranded DNA genome, ~ 150 Kb	Transduces non-dividing cells	Complex genome -Difficult to produce recombinant virus	HSV 1 and 2 most widely used as vectors. Herpes family includes Epstein Barr virus, CMV, etc
	Enveloped	Very efficient transduction of dividing and non-dividing cells	Cytotoxicity	
	~ 200nm in size	Has a natural latency in neurons		
	Genome remains episomal in infected cells	Very large carrying capacity		
<i>Adeno-associated virus</i>	Single-stranded DNA genome, 4.8 Kb	Perceived to be safe (w.t. virus causes no known disease)	Difficult to produce	W.t. virus cannot replicate without helper virus
	Non-enveloped	Transduces non-dividing cells	Carrying capacity is insufficient for certain applications	W.t. virus integrates in a site-specific manner; recombinant virus remains as a stable, concatameric plasmid
	Growing number of serotypes identified	Thought to have low immunogenicity, but this is being re-evaluated	Transduction efficiency sometimes low	Limitations of single stranded genome now overcome by development of double copy (self complementary) DNA viruses
	~20 nm in size			
<i>Oncoretrovirus</i>	RNA genome ~ 8–10 Kb	Straightforward production of recombinant vectors at moderate titers	Require host cell division	Usually used <i>ex vivo</i>
	Enveloped	Pseudotyped vectors have wide host range	Risk of insertional mutagenesis	2 genomes per virion, reverse transcribed into DNA

Parent virus	Key properties of wild-type virus	Advantages	Disadvantages	Comment
	~100nm in size			
<i>Lentivirus</i>	RNA genome ~ 8–10 Kb	Straightforward production of recombinant vectors at moderate titers	Risk of insertional mutagenesis, but non-integrating vectors being developed	2 genomes per virion, reverse transcribed into DNA
	Enveloped	Pseudotyped vectors have wide host range and are often very efficient		
	~100nm in size	Transduces non-dividing cells		

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Table 2

Transgenes used in experimental models of bone healing by gene transfer

Gene	Representative reference
BMP-2	42
BMP-4	56
BMP-6	31
BMP-7	8
BMP-9	29
BMP-2 plus BMP-7	36
TGF- β plus BMP-4	41
IGF-1	59
VEGF	53
VEGF plus BMP-4	53
VEGF plus RANKL	33
PDGF	11
FGF-2	26
LMP-1	69
LMP-3	39
ca Alk-2	35
PTH 1–34	24
PTH 1–34 plus BMP-4	24
COX-2	57
Nell-1	44
Osterix	67
Runx2	74

BMP: Bone Morphogenetic Protein, TGF: Transforming Growth Factor, IGF-1: Insulin-like Growth Factor-1, VEGF: Vascular Endothelial Growth Factor, PDGF: Platelet-derived growth factor, FGF: Fibroblast Growth Factor, RANKL: Receptor Activator of NF- κ B Ligand, LMP: LIM Mineralization Protein, Ca Alk-2: Constitutively active Alk-2 receptor, PTH: Parathyroid Hormone, COX-2: Cyclooxygenase 2, Nell-1: Nel-like molecule 1