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GENE DELIVERY TO BONE

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Abstract

Gene delivery to bone is useful both as an experimental tool and as a potential therapeutic strategy. Among its advantages over protein delivery are the potential for directed, sustained and regulated expression of authentically processed, nascent proteins. Although no clinical trials have been initiated, there is a substantial pre-clinical literature documenting the successful transfer of genes to bone, and their intraosseous expression. Recombinant vectors derived from adenovirus, retrovirus and lentivirus, as well as non-viral vectors, have been used for this purpose. Both *ex vivo* and *in vivo* strategies, including gene-activated matrices, have been explored. *Ex vivo* delivery has often employed mesenchymal stem cells (MSCs), partly because of their ability to differentiate into osteoblasts. MSCs also have the potential to home to bone after systemic administration, which could serve as a useful way to deliver transgenes in a disseminated fashion for the treatment of diseases affecting the whole skeleton, such as osteoporosis or *osteogenesis imperfecta*. Local delivery of osteogenic transgenes, particularly those encoding bone morphogenetic proteins, has shown great promise in a number of applications where it is necessary to regenerate bone. These include healing large segmental defects in long bones and the cranium, as well as spinal fusion and treating avascular necrosis.

Keywords

Gene therapy; research translation; bone healing; osteogenesis imperfecta; animal models; facilitated endogenous repair

1. INTRODUCTION

Bone is subject to a number of systemic and local disorders that may be, to a greater or lesser degree, genetic or environmental in origin. Gene therapy is being investigated as a way to treat or cure several of these (Table 1).

Gene therapy is an obvious strategy for treating Mendelian diseases, including those that affect bone. Its use in treating complex genetic or non-genetic disorders is less evident. In such cases, the intent is not to compensate for a genetic defect, but to serve as a delivery system for therapeutic gene products, be these RNA or protein. The advantages of gene delivery over protein delivery are several (Table 2), including the flexibility to express the protein locally and focally, or in a disseminated fashion, as needed. Gene transfer is

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particularly useful for the delivery of intracellular proteins, which are otherwise difficult to target into cells. Unlike its recombinant equivalent, the protein delivered via gene transfer will be nascent and uncontaminated by a variable percentage of incorrectly folded, and possibly antigenic, molecules; moreover, proteins delivered via gene transfer will have undergone authentic post-translational modification. Additional advantages of gene delivery include the ability to express proteins for extended periods of time and to regulate the level of transgene expression, both quantitatively and temporally. Moreover, the use of tissue specific promoters opens additional possibilities for controlling the geography of gene expression. Depending on the application, there may also be advantages of cost, as a gene therapy may only need to be delivered once and in a relatively small amount.

In addition to its therapeutic potential, gene delivery is a valuable experimental tool for laboratory research into the biology of bone.

This article reviews strategies for gene transfer to bone and summarizes progress towards clinical application.

2. STRATEGIES FOR GENE TRANSFER

2.1 Vectors

Successful gene therapy requires vectors that deliver transgenes to the nuclei of target cells in an efficient manner that ensures adequate levels and duration of transgene expression. The therapeutic transgene product may be a protein or non-coding RNA. Vectors have been the subjects of numerous extensive reviews (e.g. (1–4)), so they will be dealt with only briefly.

Viruses are frequently used as the basis for vectors because they naturally transfer their genetic material very efficiently into the cells they infect. For gene therapy, the viral genome is modified to remove sequences that contribute to pathogenicity and, in most cases, viral replication without eliminating infectivity. Therapeutic genes, usually in the form of their cDNA equivalents, are cloned into the modified viral genome to produce a recombinant, viral vector. Their experimental use for gene delivery to bone includes vectors derived from oncoretroviruses (often referred to just as retroviruses), lentiviruses (also members of the retrovirus family), adenovirus, and adeno-associated virus (AAV). Each of these vectors has its own idiosyncratic properties (Table 3). Moreover, progressive modification of each virus has led to the production of different generations of viral vectors with altered properties, making generalizations difficult. Of most relevance to the present discussion is the distinction between first generation adenoviral vectors, which continue to express viral proteins at low levels in the cells they transduce, and later generation vectors, which do not (5). These viral proteins trigger cell-mediated immune responses that eliminate the cells that express them. Gene transfer using a virus is known as transduction.

Because viral vectors can be expensive and complicated to make, and continue to raise safety concerns, interest in the use of non-viral vectors remains high (3, 4). These can be simple plasmids. Often the efficiency of gene transfer is improved by associating the DNA with a carrier such as a liposome or other polymer. Efficiency can also be improved by physical means such as electroporation or sonication. In general, non-viral vectors, although simpler, are far less efficient than viral vectors. Gene transfer using a non-viral vector is known as transfection.

The choice of vector depends upon a number of issues. In the first instance, there is a need to achieve the required level and duration of transgene expression. These parameters will vary, depending upon the nature of the disease or condition to be treated. A genetic disease

is likely to require life-long expression of a transgene, while healing an osseous defect probably only requires transient expression of suitable osteogenic factors.

Long-term transgene expression is best achieved by the use of an integrating virus, such as retrovirus or lentivirus; certain non-viral constructs based upon transposons also integrate (6). Prolonged transgene expression can sometimes be obtained with non-integrating vectors if the host cells do not divide. As noted, cells transduced with first generation adenovirus vectors, unlike those transduced with retroviruses and AAV, continue to express antigenic viral proteins that trigger cell-mediated immune responses leading to elimination of the transduced cells. This need not be a disadvantage if long-term transgene expression is not necessary and success does not require persistence of the transduced cells. For certain indications it may be necessary to regulate the level and duration of transgene expression with more precision than is provided by the natural history of the virus and the cells it infects. In this case, it is possible to use a variety of native or engineered, inducible promoters that respond to exogenous or endogenous cues, such as a drug, inflammation or other local conditions (7–12). When clinical translation is intended, additional factors, such as safety, cost and intellectual property become very important (13).

AAV is widely perceived to be the safest of the commonly used viral vectors, as the wild-type virus causes no disease and cannot even replicate without a helper virus. The death in 2007 of a subject receiving intra-articular injections of AAV as part of an arthritis gene therapy trial was not attributed to AAV (14, 15). Clinical grade, recombinant AAV is, however, demanding to manufacture and it needs to be used at high multiplicities of infection; both of these factors increase costs. Retroviruses efficiently transduce dividing cells, but are known to cause insertional mutagenesis (16) and are unlikely to see clinical application in any but life-threatening diseases. Lentiviral vectors raise the same safety issues, but non-integrating lentiviral vectors have been developed and are very attractive, given the high transduction efficiency of lentiviruses and ability to transduce non-dividing cells (17) (Table 3).

Perceptions concerning the safety of adenoviral vectors have had to recover from the death of a subject in a gene therapy trial in 1999 (18). When delivered locally at moderate doses, there is no associated toxicity and when used in an *ex vivo* fashion, risk is reduced even more. The major issue surrounding the use of adenoviral vectors is their high immunogenicity, which may cause inflammation, curtail transgene expression and interfere with repeat dosing. Most humans have been infected naturally with adenovirus serotype 5, the one most commonly used for making vectors, and have circulating neutralizing antibodies that may interfere with even the first use of the vector (19). Using adenovirus in an *ex vivo* fashion reduces this problem (20), as does the use of an alternative serotype, such as adenovirus 35 (21). Use of first generation, serotype 5 adenovirus vectors is not constrained by issues of intellectual property, and their construction and manufacture are straightforward, thus reducing costs.

2.2 Modes of delivery

In general terms, the vector can be introduced into the patient directly (*in vivo* delivery) or indirectly, using cells that are first genetically modified outside the body and then injected, infused or implanted (*ex vivo* delivery). In the latter case it is usual to use autologous cells, although there is increasing interest in the use of allografted cells for certain purposes. These delivery modes can be used for systemic or local transgene delivery. Systemic delivery aims to disseminate and express the transgene widely in the skeleton and is particularly useful for applications such as osteoporosis or *osteogenesis imperfecta*, which affect the entire skeleton. Local delivery, in contrast, introduces and expresses the transgene in a limited, defined area such as a fracture or a tumor.

For many purposes with *ex vivo* delivery there would be advantages to using cells that can home to bone after systemic administration. If the intent is to promote osteogenesis, it may be a further advantage to use cells that can also differentiate into osteoblasts. A number of types of cells are being investigated in this regard, particularly so-called mesenchymal stem cells (MSCs). These are able to differentiate into osteoblasts, but they do not seem to home to bone very efficiently after intra-venous injection, as approximately 98% of the injected cells are lost to the liver and spleen (22). Homing to bone seems to require expression of CXCR4 and about 30% of murine MSCs express this molecule (23). Restoration of bone mass and mechanical strength has been achieved in a murine model of osteoporosis by the injection of transduced MSCs expressing CXCR4 and Cbfa-1 (24). Incorporation into bone is increased in areas of bone turnover (25) and in sites of injury (26). Homing to bone is also enhanced by expression of CD49d (27).

Niyibizi and colleagues have succeeded in grafting MSCs by suspending the cells in collagen and injecting them in an intra-medullary fashion (28). This appears to be a useful technique for the regional treatment of an individual bone. Because large numbers of autologous MSCs may be necessary for the treatment of *osteogenesis imperfecta*, Li et al. have suggested using induced pleuripotent stem (iPS) cells that are differentiated into MSCs prior to use (29). MSCs have the potential not only to serve as convenient, osteogenic cells with the possibility to home to bone, but also to secrete helpful trophic factors (30). Other types of circulating osteoprogenitor cells, reviewed recently by Pignolo and Kassem (31), may also be useful for systemic transgene delivery to bone, but remain to be evaluated.

Although systemic delivery holds promise for treating disseminated diseases of bone, most of the literature describes local gene delivery to accelerate the healing of fractures and large segmental defects.

3. LOCAL GENE DELIVERY TO BONE

Based upon the principles described above, four different strategies have been described in the pre-clinical literature (figure 1). Two of these are *ex vivo* techniques and two of them are *in vivo.*

3.1. In vivo delivery

3.1.1. Gene Activated Matrices (GAMs)—Gene activated matrices (GAMs) consist of a matrix with associated vectors that are released into surrounding tissues after implantation (Table 4). They are usually designed to be stable and “off-the-shelf”.

The first *in vivo* strategy to be described for bone used a GAM comprising a collagen sponge impregnated with plasmid DNA encoding parathyroid hormone (PTH) 1–34 or bone morphogenetic protein (BMP) - 4. It was designed to deliver DNA to infiltrating reparative cells when implanted into an osseous defect. By expressing the transgene, the infiltrating cells create an autocrine and paracrine osteogenic environment. Very promising results were demonstrated in segmental defect models in rats (32) and dogs (33). Improvements to this system use viral vectors instead of plasmid DNA, and a construct incorporating an adenovirus vector expressing platelet-derived growth factor (PDGF) has shown promise in peridontal lesions (34, 35). As the same construct has given encouraging results in human clinical trials for healing diabetic foot ulcers (36, 37), it is well positioned to enter clinical trials for bone healing.

Schwarz’s group has developed a different type of GAM. This comprises allograft bone coated with recombinant AAV expressing one or more genes associated with osteogenesis or bone remodeling. Compelling data have been reported using a murine, segmental defect

model with AAV carrying cDNAs encoding BMP-2 (38), constitutively active Alk-2 (a BMP receptor) (39), or a combination of receptor activator of NF-κB ligand (RANKL) and vascular endothelial growth factor (VEGF) (40). When freeze-dried onto allograft bone, AAV is stable enough for "off-the-shelf" use.

Reference (41) provides a comprehensive, recent review of viral gene delivery from scaffolds.

3.1.2. Direct Injection—Direct injection provides an alternative way to introduce transgenes into osseous lesions. Success has been reported in experimental animals using several different genes delivered by recombinant adenoviral, retroviral or plasmid vectors (Table 5). Success seems to vary by the test species, at least when delivering BMP-2 with an adenovirus. Encouraging results have been reported with rabbits (42), rats (43) and horses (44), but not sheep (45). An immune response to both the adenovirus and the human BMP-2 by the sheep seemed to play a role in the lack of success in this species (45, 46). Efficacy in the horse is noteworthy, as successful treatment in large animal models is normally a requirement before clinical trials can start.

One safety concern with *in vivo* delivery is dissemination of the vector and adverse side effects in non-target sites. However, when using adenovirus in a rabbit segmental defect model little transgene was expressed outside of the osseous lesion (47). Indeed, most of the transgene was expressed in the muscle surrounding the defect. However, heterotopic or ectopic bone formation was not been observed in a rat model (43). It is interesting that, although direct adenoviral delivery of BMP-2 formed copious bone in femoral segmental defects in rats, intra-muscular injection of the same vector formed no bone in immunocompetent rats (43). The immune response is probably important in this dichotomy, because this vector formed far more bone after intramuscular injection into SCID mice than immunocompetent mice (48).

Less promising results were seen when the healing environment was more challenging, as in an infected, sclerotized, non-union in rabbits (49), and an atrophic non-union in rats (Kelly and Simpson, personal communication) although marker gene transfer to cells within an experimental, atrophic non-union in rabbits has been confirmed (50).

In vivo gene delivery to bone has recently been reviewed by Pelled et al (51).

3. 2. Ex vivo delivery

The direct introduction of vectors into the body always raises safety concerns and, as already discussed, the effectiveness of strongly antigenic viruses, such as adenovirus, can be reduced by the immune system. *Ex vivo* technologies help obviate these concerns as all genetic manipulations are performed outside the body.

3.2.1. Traditional Ex Vivo approaches—Traditional *ex vivo* strategies enable the use of expanded cultures of particular cell types, such as MSCs. Recombinant adenovirus (52) has been most commonly used in such studies, although retrovirus (53), lentivirus (54) and non-viral vectors (55) have also been evaluated (Table 6). Human and rat bone marrow MSCs have recently been reported to resist efficient transduction by AAV serotype 2 (56), although previous authors have not noted this problem (12, 57, 58). MSCs have been obtained most frequently from bone marrow, but similar cells derived from fat (59) and muscle (53) have also proved effective. When using transduced cells in this way it is usually necessary to attach the cells to a scaffold. Discussion of such scaffolds is beyond the scope of this paper; see Zippel et al (60) for recent review. The *ex vivo* delivery of genetically modified cells using a variety of different transgenes has given promising results in the

healing of segmental defects in long bones, cranium and mandible, as well as in spinal fusion. In some cases, efficacy has been demonstrated in large animal models (Table 6).

3.2.2. Expedited *Ex Vivo* Delivery—One disadvantage of the traditional *ex vivo* approach is the time, laboriousness and expense of establishing cultures of autologous cells. For clinical use, cells would need to be grown under Good Manufacturing Practice (GMP) conditions, which is very expensive. Moreover, the patient is subject to two invasive procedures, one to harvest cells and the other to reimplant their expanded and genetically modified progeny (61). One way to address these issues is to use allograft cells, and there is presently much interest in this possibility. Interest in allografted MSCs has been enhanced by the recent perception that these cells need not persist in the host to effect tissue repair and regeneration (30). Allografted chondrocytes expressing TGF-β_1 have been used to improve the healing of fractures in osteoporotic rats (62). Nevertheless Tsuchida et al. (63), using a rat femoral defect model, were only able to achieve healing with allogeneic MSCs expressing BMP-2 in the presence of immunosuppression.

As an alternative approach, expedited *ex vivo* techniques are being developed where tissue is harvested, genetically modified and reimplanted within a single operative session. Our group is very interested in the use of autologous muscle, fat and marrow in this regard, because these tissues contain progenitor cells that can be genetically modified *in situ* (61). Moreover, they have intrinsic scaffolding properties and thus do not require matrices for reimplantation. In preliminary experiments, samples of fat and muscle were genetically modified with adenovirus expressing BMP-2 and implanted into critical size femoral defects in rat femora. The data confirmed rapid healing of the defects in all animals (20). We have also used clotted bone marrow for gene delivery to osteochondral lesions (64).

In a slightly different approach, Viggeswarapu et al. (65) isolated buffy coat cells from the peripheral blood of rabbits, transduced them intra-operatively with adenovirus carrying LMP-1 cDNA, and implanted the cells on a collagen-ceramic sponge in a spine fusion model. All animals that underwent this procedure achieved solid spinal fusion. Following a similar logic, Lieberman's group have also obtained very good results using buffy coat cells obtained from the bone marrow of rats and transduced intraoperatively with a lentivirus expressing BMP-2 (66). All rats receiving the genetically modified cells healed a critical sized, femoral defect.

4. PROPECTS FOR CLINICAL APPLICATION

Of the candidate indications listed in Table 1, two areas of application offer the greatest potential for clinical application: *osteogenesis imperfecta* and bone healing. Although osteoporosis is a tempting target several new, non-genetic drugs have recently become available and these seem very effective.

4.1. Osteogenesis imperfecta

After a long delay and many setbacks, the field of gene therapy is finally registering some major successes (67, 68). These are all in the treatment of rare, genetic diseases that are difficult to treat by conventional means. Some of these gene therapies have a persistence that suggests a possible cure. With such encouraging outcomes, it might be worth investing in the most common Mendelian disease of bone, *osteogenesis imperfecta,* even though the pre-clinical work in this area is still evolving (69, 70).

Most, but not all, cases of *osteogenesis imperfecta* result from mutations in type I collagen genes. Because they are predominantly dominant negative disorders, in most cases the mutant gene will need to be silenced before the beneficial effects of a wild-type gene can be

realized. This is complicated because the primary amino acid sequence of each alpha chain of type I collagen largely consists of repeating triplets with glycine at every third residue. Thus gene silencing with anti-sense RNA affects expression of wild-type, as well as mutant, alpha chains (71). Ribozymes and RNA interference have greater precision and offer better prospects in this regard (72, 73). However, because many different mutations can cause *osteogenesis imperfecta*, specific RNA molecules will need to be designed for each mutation.

A more generalized approach is suggested by the work of Chamberlain et al (74, 75) using a novel strategy in which AAV is targeted to the collagen alpha chain gene, where it inserts and disrupts expression. Although this does not discriminate between mutant and wild-type genes, it is possible to select for the appropriately modified cells, which can then be expanded and used for transplant. As noted (29), the resistance of iPS cells to replicative senescence is an advantage when generating modified MSCs for repeated use in *osteogenesis imperfecta*. In this context, Deyle et al (76) have isolated MSCs from patients with *osteogenesis imperfecta*, inactivated mutation collagen genes with AAV and generated iPS cells which were then re-differentiated into MSCs. These cells formed normal collagen and bone in immunodeficient mice.

Because *osteogenesis imperfecta* affects the entire skeleton, *ex vivo* gene therapy using osteoprogenitor cells is an attractive strategy. This has received a boost from reports claiming that allo-transplants of bone marrow or MSCs from normal donors improves certain clinical measures in patients with *osteogenesis imperfecta* (77). Such studies are, however, controversial because the engraftment rate of the MSCs is very low and the cells are cleared with time, possibly as a result of an allograft response. As discussed earlier in this review, the ability of MSCs to home to bone is an issue that remains to be resolved. Nevertheless, if the patient's own cells were used and engraftment of MSCs improved, there would be considerable potential for clinical application in the more severe forms of *osteogenesis imperfecta*, a rare and troubling condition with orphan drug status. Because *osteogenesis imperfecta* can be diagnosed soon after birth, there is the possibility to administer cell-based gene therapy in very young individuals in whom the ability of MSCs to engraft in bone after injection seems much higher than in adults (78). It is also possible to contemplate *in utero* delivery, where engraftment rates seem particularly high (79, 80).

4.2. Long Bone Healing

As noted above, there is convincing proof of principle in animal models that gene transfer can be successfully used to heal segmental defects in bone (81). Most experimenters have used BMP-2. The significance of this has recently achieved additional prominence with the revelation that when used clinically to promote spine fusion, recombinant human BMP-2 causes a number of previously unreported adverse side effects, including osteolysis, infections, boney overgrowth, radiculitis, malignancy, and retrograde ejaculation and other urogenital events (82, 83).

Because of the problems associated with achieving the slow release of recombinant proteins at sites of bone healing, Infuse™ deposits milligram quantities of rhBMP-2 directly into the lesion. Most of this diffuses away within hours. Gene transfer, in contrast, achieves the sustained secretion of nanogram quantities of newly synthesized BMP-2 in a local and focal manner. It is highly likely that all the nascent BMP-2 is consumed locally, suggesting that the adverse side-effects seen at distant anatomical locations when using Infuse™ will not occur with gene transfer. Ironically, gene therapy thus may turn out to be safer than protein therapy in this application. The major impediments to clinical application of gene therapy are the lack of large animal studies, which are expensive and take a long time, and the restrictive regulatory environment (13, 84).

5. SAFETY

Clinical application of gene therapy, especially for non-lethal diseases, is hindered by its pervasive reputation for being unsafe. This is more perception than reality. Table 7 lists the known cases of the death of a subject in a gene therapy trial (14). Of these 4 instances, only 2 were attributable to gene transfer. Given that over 1,700 human gene therapy trials have been initiated, this is a remarkably low mortality.

The use of viral vectors makes a major psychological contribution to the belief that gene therapy is inherently more risky than non-genetic therapy. This is especially the case for lentiviral vectors derived from HIV. It is often overlooked that non-genetic medicines can have fatal side effects too. For example, at least 16,500 NSAID-related deaths occur each year in the US among arthritis patients alone. It is likely that the first human gene therapy trials in the context of this article will be for bone healing. Adenovirus is a probable vector, which suffers from the epithet of being the vector that killed Jesse Gelsinger. However, in this case an enormous amount of virus was infused directly into the liver. For bone healing, a relatively small dose will be delivered locally and, if an *ex vivo* protocol is used, no viral particles will enter the body.

6. CONCLUSIONS

Genes can be transferred to the bones of experimental animals with a number of different viral and non-viral vectors using *in vivo* and *ex vivo* strategies. The choice of vector and strategy are largely determined by the biology of the indication. For systemic, disseminated diseases *ex vivo* strategies using modified osteoprogenitor cells that home to bone hold advantages; for local application, *in vivo* or expedited *ex vivo* methods may be preferred. Recombinant adenoviral vectors usually provide a high level of transgene expression for up to 3 weeks *in vivo* and so are appropriate for short term need, such as providing a growth factor to heal a fracture or supplying an agent to kill a tumor. Integrating vectors, such as retrovirus and lentivirus, provide the potential for the long-term transgene expression needed to treat a genetic disease. For clinical translation safety issues also determine the choice of vector, with AAV and non-viral vectors favored in this regard. Given the widespread, general concern about the safety of gene therapy it is worth noting that genetic delivery of BMP-2 has the potential to be much safer than delivery of the large amounts of recombinant protein presently administered in the clinic. However, gene therapy approaches to bone healing are still hindered by incomplete understanding of how much BMP-2 is needed to be produced and at what time(s) during the healing process.

Clinical application of gene transfer to bone is not yet on the near horizon, but certain applications hold potential. *Osteogenesis imperfecta* is a rare Mendelian condition that could benefit from gene therapy, especially as genetically unmodified MSCs have already been used experimentally to treat patients. Bone healing, another possible application, has an impressive body of supportive pre-clinical data in animal models using simple, readily available technologies. In this case, the route to the clinic is less likely to be retarded by science and technology, than by funding and regulatory issues. These are large, but under-appreciated barriers to progress (13).

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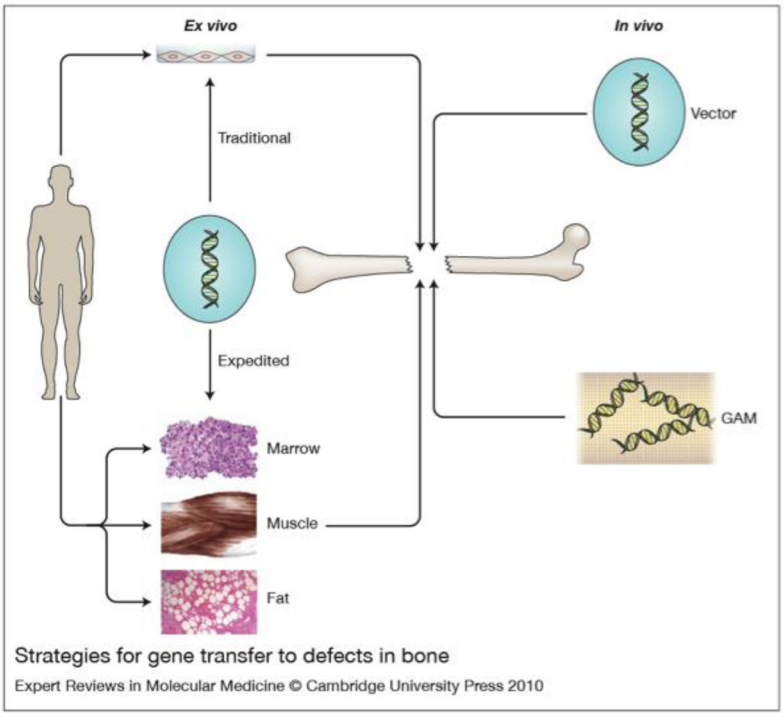

Figure 1. Strategies for gene transfer to bone

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 for the genetic modification of 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.
From reference (81) with permission.

TABLE 1

Disorders affecting bone addressed by gene therapy approaches

INDICATION	COMMENT	REPRESENTATIVE REFERENCES
Osteogenesis imperfecta	A monogenic, dominant negative, genetic disease. Some progress in silencing the mutant allele and restoring function. Lack of efficient cell homing to bone is a barrier to progress.	(69)
Osteoporosis	Some encouraging pre-clinical data in animal models. Transgenes delivered to sites where secreted factors will influence bone, or via MSCs. Gene therapy superseded by advances with more conventional therapies.	(24, 85–87)
Bone healing Fractures Osteoporotic fractures Non-unions Segmental defects Spine fusion Avascular necrosis	Considerable body of encouraging pre- clinical data, especially in segmental defects and spine fusion.	(51, 81, 88, 89)
Tumors Osteosarcoma Ewing's	Research in this area is at an early stage. The translocations in Ewing's provide targets for siRNA.	(90–92)
Osteolysis Cancer induced Aseptic loosening	Transgene not delivered to bone, but to sites where secreted factors will influence bone formation/lysis or inhibit reaction to wear debris. There has been one clinical trial in aseptic loosening, targeting the pseudosynovium around the implant.	(93–96)

TABLE 2

Gene and protein therapy compared

Gene Delivery	Protein Delivery
Use of viral vectors raises safety concerns	Safety concerns are psychologically lower
Sustained, local delivery is possible	Sustained, local delivery is difficult to achieve
Regulated gene expression, both temporally and quantitatively, is possible	Very difficult to achieve regulated delivery
Protein is nascent and authentically processed	Protein is usually stored and processing often inauthentic
Suitable for intra-cellular gene products (RNA and protein)	Difficult to deliver proteins to intra- cellular compartments
Therapy may only need to be delivered once	For many indications, therapy needs repeated delivery
Potentially less expensive, because one gene can give rise to large number of protein molecules	
Ultimately, the method of choice for Mendelian disorders	

TABLE 3

Salient properties of the main viral vectors used in studies of gene transfer to bone

Parent virus	Key properties of w.t. virus	Advantages	Disadvantages	Comment
Adenovirus	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		
	Genome episomal in infected cells			
Adeno-associated virus	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 complementing) DNA viruses
Oncoretrovirus	RNA genome ~ 8–10 Kb	Straightforward production of recombinant vectors at moderate titers	Require host cell division	Usually used *ex vivo*
	Enveloped	Pseudotyped vectors have wide host range	Risk of insertional mutagenesis	2 genomes per virion, reverse transcribed into DNA
Lentivirus	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		

w.t. = wild-type

Adapted from reference (97)

TABLE 4

Different types of GAMs and their use for bone formation in experimental animals

Scaffold	Vector	Transgene	Application	Species	Ref
Allograft bone	AAV	BMP-2; caALK2; VEGF and RANKL	Femoral defect	Mouse	(38–40)
Demineralized bone	Adenovirus	Nell-1	Spinal fusion	Rat	(98)
Fibronectin-apatite	Plasmid	BMP-2	Subcutaneous	Rat	(99)
Silk	Adenovirus	BMP-2	Cranial defect	SCID Mouse	(100)
Chitosan/collagen	Adenovirus	BMP-2 and VEGF	Dental implant	Dog	(101)
Triacrylate/amine- gelatin	Plasmid	BMP-2	Cranial defect	Rat	(102)
Collagen/calcium phosphate	Plasmid	VEGF-165	Intra-femoral	Mouse	(103)
Porous polymer	Adenovirus	RunX2	Osteonecrosis	Rat	(104)
Chitosan/collagen	Adenovirus	BMP-7	Mandible	Dog	(105)
PEG-block polycation	Plasmid	caALK6 and RunX2	Cranial defect	Mouse	(106)
PLGA	Plasmid condensed with poly(ethylenimine)	BMP-4	Cranial defect	Rat	(107)
Collagen	Plasmid/Calcium phosphate	BMP-2	Tibial defect	Rat	(108)
Collagen	Plasmid	VEGF-165	Radial defect	Rabbit	(109)
Collagen	Plasmid	PTH 1–34	Metacarpal defect	Horse	(110)
Collagen	Plasmid	BMP-4 or PTH 1–34	Femoral defect	Rat	(32)
Collagen	Plasmid	PTH 1–34	Tibial defect	Dog	(33)

PEG-block polycation: poly(ethyleneglycol) (PEG)-block-polycation (PEG-b-P[Asp-(DET)]): PEG-b-polyasparagine carrying the N-(2-aminoethyl)aminoethyl group (CH2)2NH(CH2)2NH2 as the side chain)

PLGA: poly(lactic-co-glycolic) acid

TABLE 5

In vivo gene delivery to bone

Transgene	Vector	Model	References
BMP-2	Adenovirus	Rabbit femur segmental defect	(42)
BMP-2	Adenovirus	Infected rabbit femur segmental defect	(49)
BMP-2	Adenovirus	Rat femur segmental defect	(43, 111, 112)
BMP-2	Adenovirus	Distraction osteogenesis–rat mandible	(113)
BMP-6	Adenovirus	Rabbit ulna, horse metatarsal	(44, 114)
BMP-2, BMP-9	Adenovirus	Rat mandible	(115)
BMP-4; COX-2; LMP-1	Retrovirus	Rat femoral fracture	(116–118)
VEGF	Adenovirus	Rat femur drilling	(119)
VEGF	Adenovirus	Rabbit avascular necrosis	(120)
BMP-9; BMP-2 BMP-2 + BMP-7	Adenovirus	Rat spine fusion	(121, 122)
BMP-6	Adenovirus	Rabbit spine fusion	(123)
BMP-7	Plasmid	Rat spine fusion	(124)
BMP-2	Adenovirus	Sheep tibial defect	(45, 46)
BMP-9	Plasmid (electroporation)	Mouse radial non-union	(125)

COX-2: cyclooxygenase-2

TABLE 6

Gene delivery to bone using traditional *ex vivo* methods

Transgene	Vector	Model	References
BMP-2	Adenovirus	Rodent long bone	(52, 59, 126)
IGF-1 *	Plasmid *		(26)
VEGF	Plasmid		(55)
BMP-4	Retrovirus		(53)
BMP-2	Lentivirus		(54, 66)
FGF-2	Plasmid	Rabbit radial defect	(127)
BMP-2, -7	Retrovirus, Adenovirus	Rodent cranial defect	(128) (129)
BMP-4 + VEGF	Adenovirus		(130)
Cbfa1	Adenovirus		(131)
Osterix	Retrovirus		(132)
LMP-1	Plasmid – liposomes	Rat spine fusion	(133)
BMP-7	Adenovirus		(134)
BMP-2	Adenovirus		(135)
BMP-2	Adenovirus; lentivirus		(136)
BMP-2	Adenovirus	Mouse spine fusion	(137)
BMP-2	Adenovirus	Rabbit spine fusion	(135)
BMP-2	Liposomes, Adenovirus	Rat mandibular defect	(138)
LMP-3	Adenovirus		(139)
Hepatocyte growth factor	Adenovirus	Avascular necrosis	(140)
BMP-2	Adenovirus	Goat Tibial defect	(141, 142)
BMP-2	Adenovirus	Horse Metatarsus/metacarpus Rib	(143, 144)
BMP-2	Adenovirus	Goat, avascular necrosis	(145)
BMP-2	Adenovirus	Pig, cranial defect	(146)
BMP-6	Plasmid (nucleofection)	Rat, vertebral defect	(147)

* stably transfected cell line

Table 7

Published deaths of subjects in human gene therapy trials

Year	Disease target	Vector	Comment	Death related to gene transfer?	Ref
1999	Ornithine transcarbamylase deficiency	Adenovirus	Patient died within 4 days from cytokine storm	Yes	(18)
2002	X-linked severe combined immunodeficiency disease	Retrovirus	Leukemia developed, linked to insertion of retrovirus adjacent to oncogene	Yes	(16)
2006	X-linked chronic granulomatous disease	Retrovirus	Loss of transgene expression led to death from underlying disease	No	(148)
2007	Rheumatoid arthritis	AAV	Histoplasmosis and retroperitoneal aneurysm	No	(15)

Adapted from Evans et al., 2008(14)

AAV: Adeno-associated virus