Gene therapy for bone healing

INVITED REVIEW ARTICLE

Gene therapy for bone healing: lessons learned and new approaches

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Although gene therapy has its conceptual origins in the treatment of Mendelian disorders, it has potential applications in regenerative medicine, including bone healing. Research into the use of gene therapy for bone healing began in the 1990s. Prior to this period, the highly osteogenic proteins bone morphogenetic protein (BMP)-2 and -7 were cloned, produced in their recombinant forms and approved for clinical use. Despite their promising osteogenic properties, the clinical usefulness of recombinant BMPs is hindered by delivery problems that necessitate their application in vastly supraphysiological amounts. This generates adverse side effects, some of them severe, and raises costs; moreover, the clinical efficacy of the recombinant proteins is modest. Gene delivery offers a potential strategy for overcoming these limitations. Our research has focused on delivering a cDNA encoding human BMP-2, because the recombinant protein is Food and Drug Administration approved and there is a large body of data on its effects in people with broken bones. However, there is also a sizeable literature describing experimental results obtained with other transgenes that may directly or indirectly promote bone formation. Data from experiments in small animal models confirm that intralesional delivery of BMP-2 cDNA is able to heal defects efficiently and safely while generating transient, local BMP-2 concentrations 2–3 log orders less than those needed by recombinant BMP-2. The next challenge is to translate this information into a clinically expedient technology for bone healing. Our present research focuses on the use of genetically modified, allografted cells and chemically modified messenger RNA. (Translational Research 2021; 236:1–16)

INTRODUCTION

Gene therapy arose as a strategy for treating genetic diseases, especially recessive Mendelian disorders.[1,2] Conceptually, it is simple. A wild-type version of the mutated gene is introduced into target cells where its sustained expression at an appropriate level will

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compensate for the genetic defect. If transgene expression persists for life the treatment becomes a cure.

Among the impediments to implementing this strategy have been safety, achieving efficient gene transfer, overcoming immune barriers and obtaining the appropriate level and duration of transgene expression. After half a century of fitful progress punctuated by several serious reversals, fourteen gene therapies were approved by various jurisdictions around the world by the end of 2020.[3] Present progress is rapid, with approximately 1,000 gene therapies in various phase clinical trials and the FDA expects to be approving 10 to 20 new gene and cell therapeutics a year by 2025 (https://www.fda.gov/news-events/press-announcements/statement-fda-commissioner-scott-gottlieb-md-and-peter-marks-md-phd-director-center-biologics).

The potential scope of gene therapy was expanded by the realization that gene therapy need not be restricted to monogenic diseases. Gene transfer could also be used to deliver therapeutic gene products to patients with complex diseases, such as cancer[4] and arthritis[5], as well as serving to provide gene products that stimulate the regeneration of damaged tissues, including bone.[6,7] Because Mendelian disorders are so rare, this additional scope vastly increases the number of patients who could potentially benefit from gene therapy.

Research into applying gene transfer to the problem of bone healing began in the mid-1990s[8,9] at a time when potent osteogenic morphogens had been identified and cloned[10], but their deployment was compromised by delivery problems. This review describes subsequent progress towards developing a gene therapy for bone healing and illustrates how the pre-clinical research involved in such an endeavor provides new biological insights that inform the development of novel therapeutic approaches. Given the focus of our laboratories' research much of the discussion involves the treatment of large segmental defects, although the findings are likely to be relevant to other settings where it is necessary to form bone. Emphasis is placed on those avenues most relevant to clinical translation.

THE CLINICAL NEED

Although long bone fractures usually heal spontaneously, this is not always the case. For reasons that are not completely clear, approximately 5% to 10% of fractures result in a non-union.[11] Some of this can be ascribed to underlying conditions, such as diabetes, aging, osteoporosis, alcohol abuse or smoking, among others.[12] However, in other individuals there is no obvious cause.

Even young healthy individuals, who would heal a fracture without difficulty, are unable to heal large segmental, osseous defects.[13] The reason for this is, again, unknown but it is well established that defects beyond a certain critical size or lacking sufficient soft tissue coverage will not heal spontaneously. A segmental defect becomes critical size when its length exceeds 2 to 2.5 times its diameter.[14]

Different parts of the skeleton have different propensities to heal. The distal tibia, for instance, is prone to non-union, possibly because of the poor vascular supply to this area and its minimal natural soft tissue coverage, especially in the anteromedial aspect.[11] Rib bones, in contrast, heal very efficiently, possibly because they have a thick surrounding periosteum that supplies abundant osteoprogenitor cells.[15] The calvarial bones of the adult skull, however, have no ability to heal spontaneously.[16]

Additional clinical demand for augmented osteogenesis occurs in response to iatrogenic needs, such as spine fusion and implant fixation. Moreover, large osseous defects may remain after tumor resection, the correction of congenital defects and debridement following infection.

PRESENT CLINICAL OPTIONS

Autografting is the clinical modality of choice for regenerating bone.[17] Bone is surgically harvested from a donor site, typically the iliac crest, and placed into the site of need. Although this technique is effective, amounts of autologous bone are limited and there can be considerable donor site morbidity. The fact that autografting, introduced over 100 years ago,[18] remains the treatment of choice speaks to the urgent need for improved ways to heal bone.

Allograft bone is readily available in unlimited amounts but the harsh processing procedures to which it is subjected ensure that allograft contains no living cells. It thus serves as an inert filler without intrinsic osteogenic activity; it is a mere osteoconductive construct, favored only because it can provide early structural support. However, because allografted bone does not turn over, structural allografts have a high late failure rate.[19] Nevertheless, bone is the second most frequently allografted tissue after blood,[20] reflecting the high demand for materials to aid bone healing. A number of synthetic ceramic materials that mimic, to a greater or lesser degree, the properties of the bone mineral hydroxyapatite are also FDA-approved. They are predominantly used for spine fusions. However, these materials are brittle, do not resorb well and also serve only as osteoconductive fillers.

Large segmental defects can be healed surgically by the Ilizarov technique.[21] Also known as distraction osteogenesis, this method is based upon the ability to stimulate bone growth by slowly pulling apart two cut

ends of bone. The surgery is complex and a cumbersome external fixator is used to stabilize the defect. Because new bone is formed at a rate of only about 1 mm/day, it takes considerable time to fill in large defects and many patients find the daily distraction procedure painful. Furthermore, it cannot be applied along the entire length of a long bone because the ends do not permit placing of the Ilizarov pins. Pin tract infections occur often and need immediate treatment in order to prevent progression to osteomyelitis.[22]

The Masquelet induced membrane technique offers another surgical approach for treating large segmental defects.[23,24] This is a two-step procedure in which the surgeon places into the defect a spacer of polymethylmethacrylate, already used in cranioplasty to fill cranial defects and as bone cement when securing prosthetic joints. This provokes the formation of a highly osteogenic membrane around the spacer. Approximately six weeks later a second operation removes the spacer and replaces it with morcellized bone, either autograft or allograft, a synthetic ceramic or some other material. The timing of the second procedure is still under debate. A well-vascularized membrane of certain strength is needed. When the interoperative period is too short the membrane is not well developed; when the period is too long, the membrane is mere scar tissue with a very reduced osteogenic capacity. The optimum period is probably patient dependent and no markers exist to determine the maturity of the Masquelet-induced membrane.

Besides the more material-based approach as described above, there is much interest in exploiting the osteogenic properties of mesenchymal stromal cells (MSC) to heal bone. One surgical approach, popularized by Hernigou,[25] uses autologous MSCs derived from the patient's own bone marrow for this purpose. Another takes advantage of the observation that MSCs can be allografted without provoking severe immunological reactions. Five companies sell cellular bone matrix,[26] a product that combines allograft bone with allogeneic MSCs. In line with this, the reamer irrigator aspirator (RIA) technique uses both autograft bone matrix and MSCs obtained from an intact femur.[27] This is an invasive technique with a risk of fracturing the donor femur. RIA material can be used alone or in combination with synthetic materials or allograft.

Interest in the biology of bone formation led to the identification of a family of bone morphogenetic proteins (BMPs) related to transforming growth factor-β (TGF-β), with the ability to promote osteogenesis.[10] Their osteogenic properties were first recognized in preparations of demineralized bone, which is FDA-approved for bone healing. Although stable, convenient and affordable, preparations of demineralized bone matrix have considerable batch-to-batch variability and the clinical results are equivocal.[28]

Two osteogenic components of demineralized bone matrix, BMP-2 and BMP-7, were cloned and their recombinant forms approved by the FDA for clinical use as the active components of the products INFUSE and OP-1, respectively.[10] OP-1 is no longer available, but INFUSE continues to be marketed for spinal fusion, certain dental applications and acute open tibial fractures. Nevertheless, it is widely used in an off-label fashion to treat large segmental defects and in other clinical settings where it is necessary to grow bone. However, as discussed in more detail below, the clinical application of recombinant, human (rh) BMP-2 has been problematic. Interest in using gene therapy for bone healing originated with the need for a better way to deliver BMP-2 to osseous defects.

Regardless of the technology used, the ultimate clinical requirement is for a potent, off-the-shelf bone healing product that can be applied at point-of-care in a single operative procedure, or even percutaneously, at an affordable price.

A GENE THERAPY PRIMER

Gene therapy involves the transfer of genes, usually as their cDNA equivalents, into cells in a fashion that results in the genes of interest being expressed at the appropriate levels in the correct location for the necessary duration. A variety of non-viral and viral (Table 1) vectors are available for this purpose. Of the 14 gene therapeutics approved by various jurisdictions around the world by the end of 2020, two use plasmid DNA, one uses adenovirus, one uses herpes simplex virus, four use retrovirus, three use lentivirus and three use adeno-associated virus (AAV).[3] Novel COVID-19 vaccines using mRNA and adenovirus to deliver viral antigens have recently received emergency approval in the US and elsewhere. They stand as the most broadly applied gene-based therapeutics by far and serve as a testament to overall safety and efficacy.

The simplest vectors are DNA plasmids which are straightforward to engineer and then produce in bacteria. Gene transfer using a non-viral vector such as a plasmid is called transfection. The transfection efficiency of plasmids is low, but can be enhanced by various chemical and physical means.[29] Nevertheless, gene expression is usually modest and transient regardless of the transfection reagent; naked DNA is also inflammatory. However, plasmid DNA is perceived to be safe and, by gene therapy standards, inexpensive.

Because certain viruses naturally transfer genes into human cells very efficiently as part of their normal life

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
Adenovirus	Double stranded DNA genome, ~35 Kb	Straightforward production of recombinant vectors at high titers	Inflammatory and antigenic	Various generations with increasingly deleted genomes. "Gutted", high-capacity 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		
	~100 nm in size			
	Genome remains episomal in infected cells			
Herpes simplex virus	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 (But an advantage for oncolytic herpes viruses)	
	~200 nm in size	Has a natural latency in neurons		
	Genome remains episomal in infected cells	Very large carrying capacity		
Adeno-associated virus	W.t. has 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	Comparatively low immunogenicity.	Transduction efficiency sometimes low	Limitations of single stranded genome now overcome by development of double copy (self complementary) DNA viruses
	~20 nm in size			
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
	~100 nm in size			

(*continued*)

Table 1. (*Continued*)

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

cycles, they have obvious advantages as vectors for gene therapy. To develop them for this purpose, their genomes are engineered to remove sequences involved in pathology and viral replication thus creating genetic space for carrying transgenes and their regulatory elements. Gene transfer using a virus is known as transduction. Over a dozen different viruses have been investigated as possible gene therapy vectors,[30] but only the 5 listed in Table 1 have been approved for clinical use. One of these, herpes simplex virus, is cytotoxic and is used as an oncolytic agent for cancer therapy;[31] it is unlikely to be useful for bone healing.

Adenovirus vectors are non-integrating, straightforward to produce at high titers and transduce many different types of cells, both dividing and non-dividing, with high efficiency.[32] Although the first reported death of a subject in a gene therapy trial occurred with an adenovirus vector[33], they are generally perceived to be safe, especially when used in small amounts to deliver genes locally to osseous lesions. Nevertheless, adenovirus vectors are inflammatory and provoke both humoral and cell-mediated immune responses that curtail transgene expression after 2–4 weeks. Although this is an insufficient period for treating chronic diseases, it may be a favorable length of expression for initiating an irreversible osteogenic response.

AAV is increasingly popular as a gene therapy vector because it is perceived to be safe and has shown efficacy in pivotal clinical trials that led to approval by the FDA.[34] Depending on the serotype, it has a range of tropisms and although it generates humoral immune responses in the host, its ability to trigger cell-mediated immunity is muted compared to that of other viral vectors. It is able to transduce non-dividing cells within which it can persist for several years as a concatenated episome. The small packaging size of AAV can limit its utility for certain applications, but this should not affect the small growth factor genes of interest in the context of bone healing. Manufacturing, however, remains a major hurdle for the clinical use of AAV. This is very complex and expensive, which helps explain why the AAV-based drug Zolgensma used for treating spinal muscular atrophy costs over $2 million a dose.[35]

Safety, a major issue for gene therapy in general, is of prime importance when treating non-lethal conditions such as osseous defects. This consideration will make it very difficult to gain clinical acceptance for a gene therapy for bone healing that uses retroviruses. These were the first viruses developed as vectors for human gene therapy, and were used extensively in early clinical trials. While relatively straightforward to produce and manipulate, the type of retrovirus used in this early work (Moloney murine leukemia virus, a gammaretrovirus) requires target cell division for efficient transduction, which largely limits its use to *ex vivo* gene therapy. Because the retroviral genome inserts itself into the host cell genome at unpredictable sites, there is a finite possibility of insertional mutagenesis. Instances of this have occurred in clinical trials,[36] which largely restricts clinical application of retroviruses to serious conditions such as cancer and severe combined immunodeficiency disease (SCID), where the risk:benefit ratios justify their use.

Lentiviruses are also members of the retrovirus family, but unlike gammaretroviruses they transduce non-dividing cells. Nevertheless, they still risk insertional mutagenesis.[37] Indeed, a clinical trial for sickle cell anemia using a lentivirus vector was recently placed on clinical hold because of 2 cases of cancer among patients in the study. The main advantage of using retroviruses and lentiviruses is the potential for long-term gene expression but, as described later, this may not be necessary for effective bone healing.

Vectors can be deployed in an *in vivo* or *ex vivo* fashion to sites of osseous lesions. Although both are scientifically reasonable approaches, certain members of our group made an early decision to avoid *ex vivo* protocols requiring the expansion of autologous cells under good manufacturing practice conditions.[38] This was largely a result of having experienced firsthand the complications, cost and inconvenience of such a protocol in a phase I clinical trial of gene therapy for arthritis.[39]

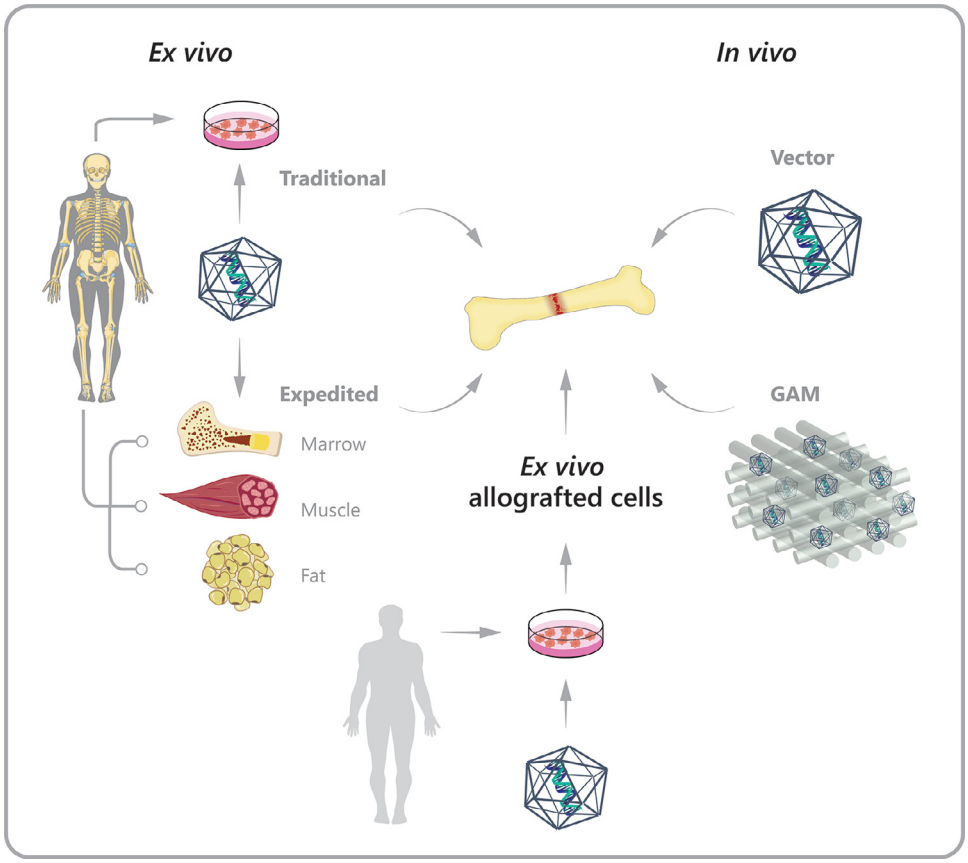

Fig 1. Strategies for gene transfer to defects in bone. There are two general strategies: *in vivo* (right hand side) and *ex vivo* (left-hand side). For *in vivo* gene delivery, the vector is introduced directly into the site of the osseous lesion, either as a free suspension (top right) or incorporated into a gene-activated matrix (GAM) (bottom right). For *ex vivo* delivery, vectors are not introduced directly into the defect. Instead, they are used for the extracorporal genetic modification of cells, which are subsequently implanted. Traditional *ex vivo* methods (top left) usually involve the establishment of autologous 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) avoid the need for cell culture and scaffolds by genetically modifying tissues such as marrow, muscle and fat, intraoperatively and inserting them into the defect during a single operative session. *Ex vivo* gene delivery could also be expedited with the use of a genetically modified, established line of allograft cells that secrete BMP-2. Redrawn and modified from reference.[44]

STRATEGIES FOR APPLYING GENE THERAPY APPROACHES TO BONE HEALING

The concept of introducing one or more osteogenic genes into an osseous lesion is attractive and has generated a large literature (reviewed in references.)[40-44] In general terms, there have been 4 main strategies for applying gene transfer to bone healing, two *in vivo* and two *ex vivo* (Fig 1). *In vivo* strategies involve the direct application of vector to the defect, either by itself or associated with a scaffold to form a gene-activated matrix (GAM).[45] One *ex vivo* strategy includes the traditional approach of removing autologous cells, expanding and genetically modifying them outside the body before returning them to an osseous lesion. Another one is an abbreviated *ex vivo* approach where suitable tissues, such as fat, muscle or marrow are harvested, genetically modified intra-operatively and re-implanted during the same surgical session.[46] Although an *ex vivo* procedure, it avoids the need to expand autologous cells under good manufacturing practice conditions. Another option, discussed later, avoids this step altogether by using a genetically modified, allograft cell line.

Table 2 provides a partial list of the large number of genes that have been tested in animal models of bone healing. As well as the BMPs, a variety of other growth factors involved in osteogenesis, such as TGF-β, insulin-like growth factors and Nell-1, have been evaluated. Investigators have also transferred cDNAs encoding transcription factors such as runx-2 and osterix, angiogenic factors, such as vascular endothelial growth factor (VEGF), and cyclooxygenase-2 whose

Table 2. Genes evaluated in animal models of bone healing

Growth Factors	
BMP-2, -4, -6, -7, -9	
IGF-1	
FGF-2	
PDGF	
VEGF	
Hormone	
PTH 1-34	
Transcription Factors	
Cbfa1 (=Runx2)	
Osterix	
Other	
LMP-1, -3	
Cyclooxygenase	
caAlk-2	
Combinations	
BMP-4 + VEGF	
BMP-2 + BMP-7	
VEGF + RANKL	
BMP:	Bone Morphogenetic Protein
IGF-1:	Insulin-like Growth Factor-1
FGF-2:	Fibroblast Growth Factor-2
PDGF:	Platelet-Derived Growth Factor
VEGF:	Vascular Endothelial Growth Factor
PTH:	Parathyroid Hormone
Cbfa1:	Core Binding Factor alpha 1
Runx2:	Runt-Related Transcription Factor-2
LMP:	Lim Mineralization Protein
caAlk-2:	Constitutively active Activin Receptor-Like Kinase-2
RANKL:	Receptor Activator of Nuclear Factor kappa B Ligand

product, prostaglandin E_2, helps bone healing. Recognizing the importance of a blood supply to bone healing, combinations of genes that pair an osteogenic growth factor with an angiogenic factor have also been evaluated. Although an attractive concept, it will be even more difficult for such combinations to obtain FDA approval.

Overall, the data from such studies are highly promising, but it is telling that nearly all of this research has used rodent models of bone healing. There have been few studies in the large animal models that are necessary before human clinical trials can start and only two, related clinical trials have emerged. These trials (NCT02293031; NCT03076138) took place in Russia and the Ukraine and used a GAM that combines a plasmid encoding VEGF with a collagen-hydroxyapatite scaffold to treat maxillofacial bone defects. The plasmid is identical to the one in the product Neovasculgen that has been approved in Russia for treating peripheral artery disease. A promising case report[47] and a summary of the pre-clinical and clinical data[48] have been published.

As noted in the introduction to this review, research in this area was stimulated by clinicians who were enthusiastic about the recent availability of rhBMPs as potent osteogenic agents but concerned about delivery problems that limited clinical usefulness. The clinically approved product INFUSE uses a collagen sponge as the delivery scaffold. The rhBMP-2, supplied as a powder, is dissolved in water in the operating room and applied to the sponge 10 to 15 minutes prior to implantation. However, rhBMP-2 diffuses away from the sponge very rapidly after implantation. Thus, to achieve healing it is necessary to apply very large amounts of rhBMP-2, typically in milligram quantities, which are approximately 10^6 -fold greater than the levels of BMP-2 found naturally in bone. These high amounts are very expensive and produce off-target side effects, some of them serious.[49] Despite the large amounts of rhBMP-2 applied to the sponge, the clinical efficacy is modest and its cost-effectiveness has been questioned.[50] There was thus a feeling that BMP-2 could become a more useful clinical product if it were delivered locally at high doses in a sustained fashion. One approach to achieving this has been the use of smart scaffolds to deliver precise amounts of rhBMP-2 for an extended period. Gene transfer offers an alternative technology. Although the latter has not yet delivered a clinical product for bone healing, research towards this goal has provided insight into the biology of segmental defect healing and has suggested new biologically-based approaches to achieving this end.

DELIVERY OF BMP-2 CDNA IN ANIMAL MODELS OF SEGMENTAL BONE DEFECTS

Initial experiments by Lieberman's group[8] and our own[9,51] used adenovirus as a vector to deliver BMP-2 (Ad.BMP-2). As noted earlier in this review, adenovirus holds many advantages in this respect. As a vector it is straightforward to construct and, once generated, it can be produced easily in the laboratory at high titers. It is relatively stable and highly infectious towards many cell types. When used *in vivo* with a strong constitutive promoter it typically provides robust transgene expression peaking at around 2 weeks and then declining during weeks 3 to 6; this could be a favorable expression profile for bone healing.

In Lieberman's pioneering studies, Ad.BMP-2 was used to transduce mesenchymal stromal cells (MSCs) which were then seeded onto a collagen sponge, incubated and implanted into a rat, diaphyseal, critical sized, segmental defect.[52] Because MSCs can differentiate into osteoblasts, this approach has the advantage of supplying primed osteoprogenitor cells, as well as

transgenic BMP-2, to the defect. This method produced efficient bone healing.

In an alternative, *in vivo* strategy we injected Ad. BMP-2 directly into a similar defect.[53] This produced healing in about 50% to 75% of the rats. In the other animals we saw the formation of cartilage, a normal evolution in the process of endochondral ossification, but this failed to undergo transformation into bone. Nevertheless, this approach was taken into a sheep segmental defect model, but the data were very disappointing because none of the sheep healed.[54] However, a subsequent ovine study using osteoporotic sheep provided interesting findings. Ovariectomy in sheep proved insufficient to generate osteoporosis, so the ewes were also subjected to a high dose of steroid. In these animals, bone healing was improved by Ad. BMP-2.[55] Although we did not realize it at the time, this opened up the possibility that the immunogenicity of adenovirus might be an impediment to healing in sheep. This should have been evident from our own earlier studies in rodents showing that intra-muscular injection of Ad.BMP-2 only produced large amounts of ectopic bone in immunosuppressed mice.[56]

One interesting observation from the studies using rats concerns the quality of the bone healed using rhBMP-2. Although the bones appear solidly healed on X-ray, the latter is a 2-dimensional projection of a 3-dimensional reality. When observed in histological sections and by 3-dimensional micro-computed tomography (Fig 2) it is clear that the regenerate formed in response to rhBMP-2 looks solid in X-ray because of overlay. The histology, in contrast, reveals new bone with more of an egg shell appearance, where thin cortices enclose a tissue containing thin, wispy trabeculae (Fig 2). This has also been noted by Lieberman. The callus formed by rhBMP-2 is very large. The regenerate formed by BMP-2 gene transfer, in contrast, has a much smaller callus and lacks the egg shell appearance of defects bridged by rhBMP-2 (Fig 2).

Lieberman's group also examined the potential for using lentivirus to deliver BMP-2 for healing large segmental defects. The idea was to improve the quality of the healed bone by expressing BMP-2 for an extended period of time. This successfully extended the period of transgene expression, with adenovirus expressing a luciferase reporter gene within the defect for 3 weeks, whereas lentivirus expressed the same gene for at least 3 months.[57] However, the quality of healed bone formed when expressing BMP-2 via a lentivirus was only marginally better than that obtained with adenovirus.[58] Safety concerns when using lentivirus prompted examination of including a suicide gene that could render cells susceptible to ganciclovir[59] or inducible caspase-9,[60] in case it would become necessary to eliminate them. Subsequent research confirmed the

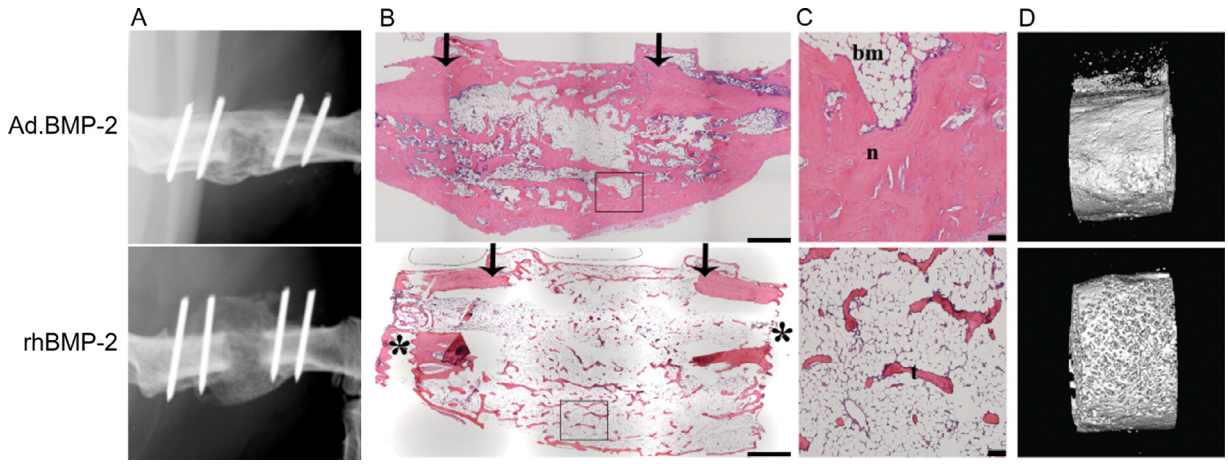

Fig 2. Histological and radiological appearance of rat segmental defects healed with BMP-2 delivered as a recombinant protein or by gene transfer. A rat, 5 mm, segmental, femoral defect was created and filled with either 11 μg rhBMP-2 (bottom row) or BMP-2 gene transfer using adenovirus transduced, rat, adipose-derived MSCs (top row). Column A shows radiographs of both treatments 8 wk after surgical implantation. Column B depicts the histological appearance of these defects after 8 wk using H&E at low magnification (scale bar = 1 mm). Column C shows higher magnification images of the healed defects from the area enclosed within the black boxes present in column B, emphasizing the defect cortex (scale bar = 100 μm). Column D shows the outer cortex of the regenerated bone, in 3-dimensional micro-computed tomography reconstructions, evidencing the difference in cortical porosity between treatments. Asterisks indicate the pin holes for the fixation plate. Arrows indicate the original defect location. "Ad.BMP-2" denotes adenovirus transduced BMP-2 treatment; "rhBMP-2" denotes recombinant human BMP-2 treatment; "bm" denotes bone marrow; "n" denotes new cortical bone formation; "t" denotes trabecular bone. From reference.[68]

utility of this approach, although it proved difficult to eliminate every last transduced cell.

Because lentivirus is so efficient in transducing non-dividing cells, it allowed the prospect of "same-day"[61] or "next day"[62] gene therapy where the patient would give bone marrow, have the buffy coats transduced and then returned, all on the same day or the next day. Pre-clinical data in rats were highly encouraging.

Studies in rabbits[63] and rats demonstrated that when adenovirus vectors were injected into segmental defects, much of the transgene expression occurred in the muscle that surrounds the defect. Because muscle is known to contain osteo-progenitor cells[64] and forms heterotopic bone in response to injury[65] and conditions such as *Fibrodysplasia ossificans progressiva*,[66] it seemed likely that the surrounding muscle supplied the cells responsible for forming new bone in the defects. This conclusion fits with our earlier demonstration that the intra-muscular injection of Ad.BMP-2 into SCID mice provoked bone formation.[56]

To take advantage of this insight, we investigated the merit of a strategy where muscle biopsies were harvested, transduced and implanted into the osseous defect intra-operatively.[46,67] This approach worked well in rats, but came with certain caveats. Initial experiments were conducted with Sprague-Dawley rats which, although inbred, are not syngeneic. Muscle was harvested from donor rats, transduced with Ad.BMP-2 and then implanted into defects in recipient animals. Only 1/3rd of rats healed well using this method. An additional 1/3rd formed bone but did not bridge, and the final 1/3rd exhibited no osteogenic response. This issue was only resolved following discussions with a transplant immunologist who recommended using Fischer 344 rats, which are syngeneic. With Fischer rats, all defects healed using the muscle graft approach, which was also shown effective using fat grafts.[46] These studies pointed towards the relevance of the immune response to bone healing.

Because of the plan to take this technology into human clinical trials via a sheep model, we explored the ability of sheep muscle transduced with Ad.BMP-2 to heal critical size segmental defects in rats. It was proposed to use athymic rats to eliminate the xenograft response. However, athymic rats were found to mount strong xenograft responses as well as being often on back order; the rats were also very variable in size, which created problems for the fixation hardware. In response, we used regular Fischer rats and immunosuppressed them with a combination of FK506 (tacrolimus), a drug used to prevent rejection of organ transplants, and SEW2871, a compound that reduces the number of circulating T-lymphocytes. This series of experiments proved to be very informative.[70]

Healing in the presence of immunosuppressants was much greater than expected from previous experience. Subsequent research focused on a possible osteoinductive role for FK506, but instead we found that the stimulatory effects of FK506 were probably due to suppression of the immune response to the adenovirus vector.[68] This susceptibility of bone healing to immune activation reflected the earlier finding that allograft muscle grafts were inferior to syngeneic muscle grafts in the rat model,[46] as well as the murine[56] and ovine[54,55] data mentioned earlier in this review. Immunohistochemical examination of the fate of muscle grafts from GFP + rats, transduced with Ad.BMP-2 and implanted into the segmental defects of wild-type animals, confirmed that donor muscle cells differentiated into chondrocytes, osteoblasts and endothelial cells in the recipient animals' osseous regenerate.[69]

The sheep muscle-in-rat studies were also informative in that we measured the amount of BMP-2 protein present in defects that went on to heal.[70] Although the standard dose of rhBMP-2 recommended for healing critical sized femoral defects in rats is 11 μg, the total amount of BMP-2 produced in the defects that healed via gene therapy was of the order of 50 ng or less (Fig 3). This is over two log orders lower than the level needed when using recombinant protein. The other insight afforded by these studies was to reveal that long-term transgenic BMP-2 expression is not necessary for efficient healing; BMP-2 was undetectable after 1 week (Fig 3).

The approach of using autologous, genetically modified muscle was further tested in a sheep, tibial, critical sized defect model. There was no evidence of an osteogenic response in any of the animals, although histology suggested a marked angiogenic response to transgenic BMP-2 (our unpublished data).

LESSONS LEARNED

The main insights from the data described so far are that immune responses to materials within the defect impair healing of large segmental defects and that healing requires only low, transient levels of transgenic BMP-2 expression. The latter conclusion is interesting in light of data from Gazit's group who achieved remarkable healing in a porcine tibial, segmental defect using a BMP-6 plasmid delivered by sonication.[71] Expression of BMP-6 persisted for less than 10 days and expression peaked at less than 150 pg.

In recent work we have studied the mechanism through which low concentrations of transgenic BMP-2 trigger an osteogenic response. To facilitate this, we have used a genetically modified, murine, mesenchymal cell line that expresses luciferase when SMAD signaling occurs in

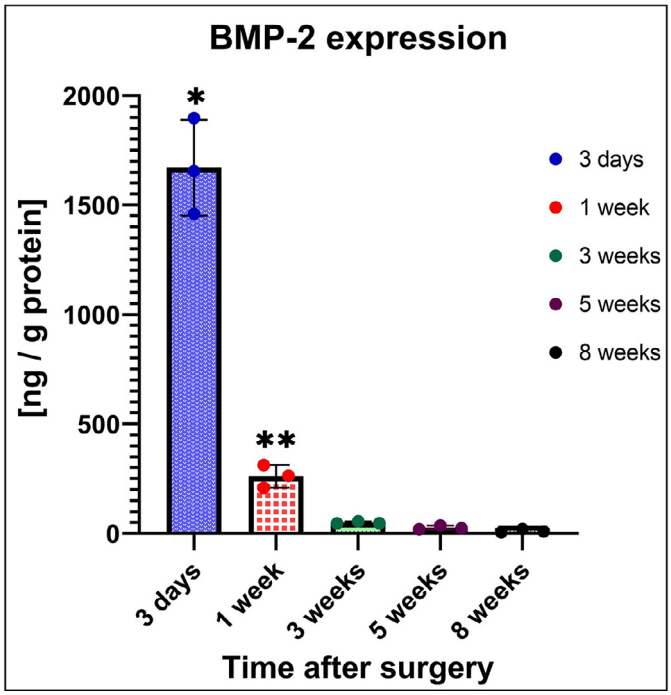

Fig 3. Expression of transgenic BMP-2 in segmental defects in the rat under conditions of healing. BMP-2 content of defects treated with sheep muscle transduced with Ad.BMP-2. BMP-2 content within extracts of rat femur defects was measured by ELISA and standardized to total protein. * indicates a significant increase ($P < 0.05$) of the 3 d group compared to all other groups; ** indicates a significant increase ($P < 0.05$) of 1 wk group compared to all later time points. Redrawn from reference.[70]

response to BMP-2.[72] In certain experiments the cells were transduced with adenovirus or lentivirus carrying BMP-2 cDNA. Remarkably, endogenously synthesized BMP-2 produced by the transduced cells was approximately 100-fold more effective than rhBMP-2 when inducing luciferase expression (Fig 4). Moreover, much of the BMP-2 produced by the transduced cells remained cell-associated. Indeed, when medium conditioned by the transduced cells was added to cultures of untransduced cells there was no induction of luciferase. Further investigation revealed that close proximity, possibly cell-to-cell contact, was required for cells expressing BMP-2 to induce luciferase in untransduced cells. We thus conclude that endogenously synthesized BMP-2 was stimulating luciferase expression via autocrine and close paracrine mechanisms.[73] This explains how the low concentrations of BMP-2 found in nature can stimulate osteogenic responses during bone healing and provides optimism about the prospects of stimulating this process by gene transfer.

NEW APPROACHES

Use of genetically modified allograft cells. Use of an established line of well-characterized cells expressing BMP-2 to heal osseous defects would take advantage of *ex vivo* gene delivery and cell-cell induction of SMAD signaling, while avoiding the complications of having to expand autologous cells (Fig 1). An additional advantage would be the creation of a standardized, uniform product synthesizing a known amount of BMP-2. *In vivo* gene transfer, in contrast, does not allow such control over the delivered dose of BMP-2 because this is determined by transduction efficiency within the defect; the latter is intrinsically variable, especially in clinical settings.

As proof of concept, we have used lentivirus to engineer HEK293 cells to express BMP-2 constitutively. This produced efficient bone healing (Fig 5) when incorporated into a fibrin gel and implanted into critical size femoral defects in rats whose immune response was suppressed by FK506.[74] HEK293 cells do not differentiate into chondrocytes or osteoblasts, indicating that their sole function was to deliver BMP-2 to the defect. In the absence of FK506 there was no healing.

While these are encouraging results, this cell line is not a good candidate for clinical translation because of concerns about malignancy and its questionable performance as an allograft. For this reason, we are now exploring the possibility of using MSCs. These are already used clinically, in an unmodified form, as allografts for bone healing as a component of cellular

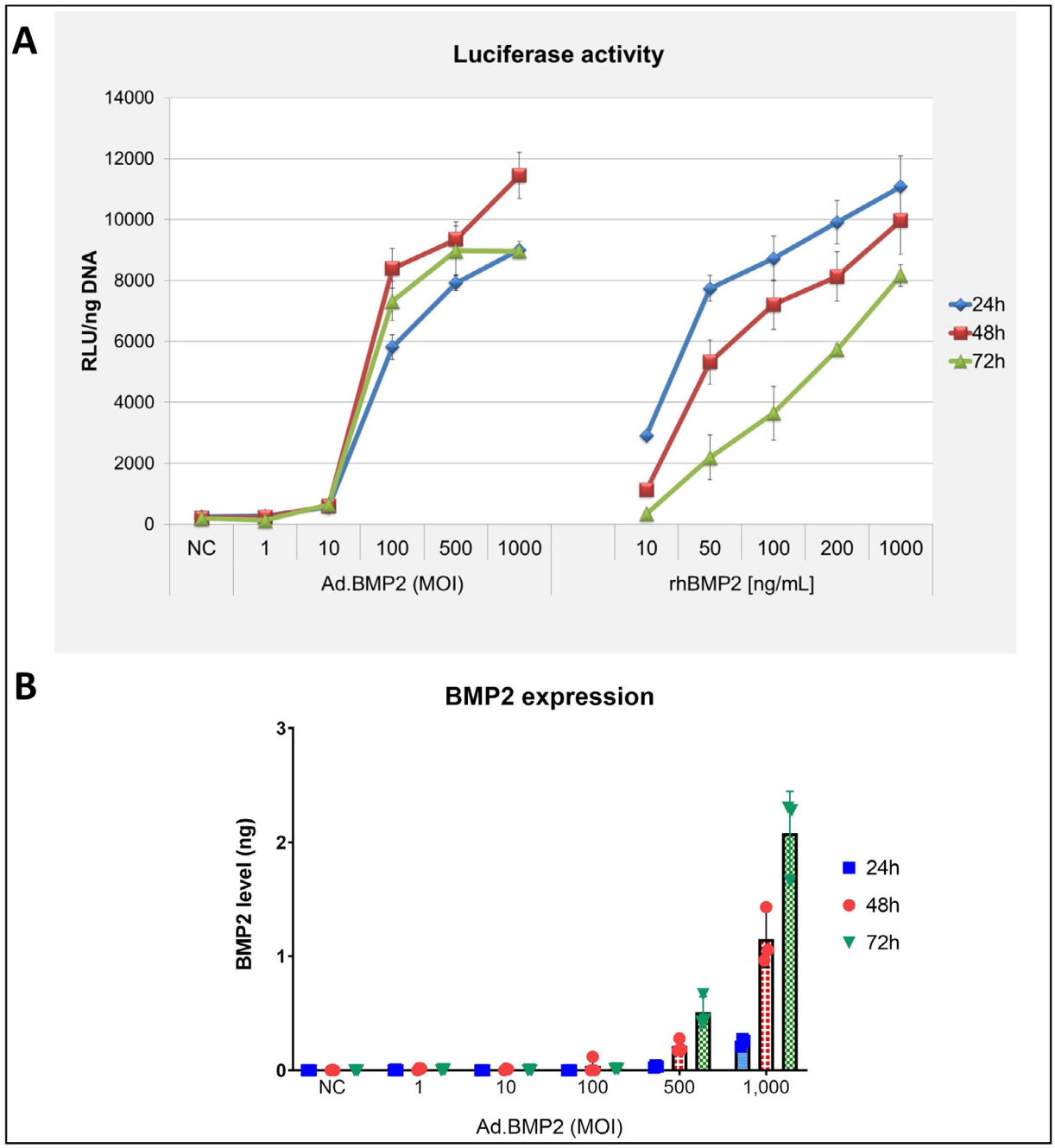

Fig 4. SMAD signaling in response to rhBMP-2 and transgenic BMP-2. A reporter cell line expressing luciferase in response to BMP signaling[72] was transduced with Ad.BMP-2 at the multiplicities of infection (MOI) shown in panels A and B. These cells produced the amounts of BMP-2 shown in panel B, with approximately equal amounts secreted and cell associated. The sub-nanogram amounts of BMP-2 produced by cells transduced with Ad.BMP-2 at MOIs of 100 and 500 were equally as effective as 200 ng/ml rhBMP-2 in inducing luciferase. From reference.[73]

bone matrix.[26] Unlike the case with 293 cells, there are no immortalized human MSC cell lines, but even if there were such cells, it would be difficult to translate into clinical use because of concerns over possible malignancy. Although MSCs possess a Hayflick limit, they have sufficient replicative potential for present purposes. Human, adipose-derived MSCs seem able to reach particularly high passage numbers and Lieberman's data suggest they are more effective than human bone marrow MSCs for healing osseous lesions

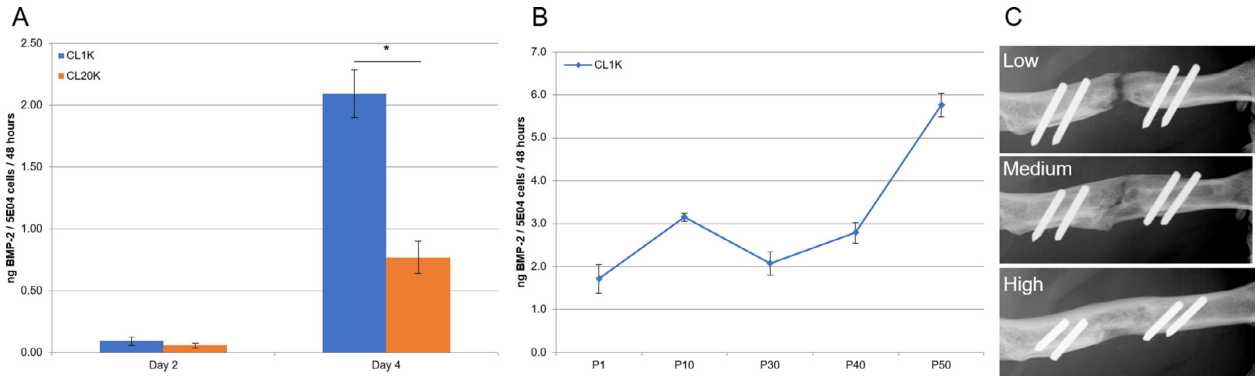

Fig 5. Rat, critical size femoral defects healed using a genetically modified, allograft cell line. HEK293 cells were transduced using a lentivirus to express BMP-2 constitutively at low doses. (A) Two clones were selected, expanded and their BMP-2 production characterized. (B) Clone CL1K was passaged up to 50 times and its BMP-2 production capacity characterized prior to *in vivo* use. (C) Clone CL1K was encapsulated in fibrin at ascending cell numbers and implanted into rat critical size bone defects in the presence of immune suppression with FK506. Asterisk in panel A denotes statistically significant difference in BMP-2 production between the two clones ($P < 0.05$). From reference.[74]

in rats by *ex vivo* gene therapy.[75] One advantage of using MSCs is the potential to compensate for the loss of soft tissue support that often occurs with large segmental defects. One possible disadvantage is signaled by the report of Bougioukli et al[76] that AAV serotypes 2 and 6 have limited ability to transduce human MSCs. In this case, alternative serotypes or transduction conditions may need to be explored.

Use of chemically modified messenger RNA. The finding that BMP-2 need be expressed only transiently at low levels to heal large segmental defects in the rat brings into focus alternative genetic strategies for bone healing. The use of mRNA is particularly intriguing in this regard and holds several advantages over classical gene therapy using DNA.[77-79]

Unlike DNA, mRNA does not need to be translocated to the nucleus of the cell before it becomes active; once it is in the cytoplasm translation can start immediately. Unlike plasmid DNA and viral vectors, the production of mRNA does not require bacterial or eukaryotic cell culture. Instead, it is produced by *in vitro* transcription, a biochemical process that is easier to control and to scale-up. The disadvantages of mRNA include its short half-life and the inflammation it triggers through interaction with toll-like receptors (TLRs). Both of these disadvantages have been recently addressed by chemical modification.

Zhang et al[80] created an improved, chemically modified, mRNA (cmRNA) encoding BMP-2 by engineering several key molecular alterations. Interaction with TLRs was reduced by the inclusion of 5-iodo-pyrimidine residues. The physiological stability of the molecule was improved by including certain sequences in the 5'-untranslated region (UTR) and extending the polyA tail, and the efficiency of translation improved by including a translation initiator of short UTRs sequence. Fig 6 shows the cmRNA construct developed by Zhang et al.[80] The figure also highlights key mRNA structural elements that were modified to enhance translation and stability of mRNA.

This cmRNA construct was combined with a liposome and absorbed into a collagen sponge to form a "Transcript Activated Matrix".[81] When placed into a rat, femoral critical sized defect this material induced bone formation in a dose-dependent manner, with full bridging of the defects at the appropriate dose (our unpublished data). The same cmRNA construct also added osteogenic properties to titanium. When coated onto titanium to form a "Transcript Activated Coating", seeded myoblast cells showed high alkaline phosphatase activity and robust mineralization.[82] This may be an innovative approach to enhance the osteointegration of metallic implant surfaces in orthopedics.

To increase vascularization during bone healing, Geng et al[82] used a combination of VEGF and BMP-2 cmRNAs. In this study, the authors used a rat calvarial defect model to demonstrate the synergy between osteogenesis and angiogenesis stimulated by the cmRNA combination. Overall, the study indicated that the precise ratio of BMP-2 and VEGF cmRNAs is of ultimate importance for osteogenesis.

A BMP-9 cmRNA has also been developed with 100% of pyrimidines substituted with pseudouridine and 5-methylcytosine. Using this construct, Khorsand et al[83] demonstrated osteogenesis in cultures of bone marrow MSCs and in a rat cranial defect model. Neither the cmRNA combination of Geng et al[82] nor the

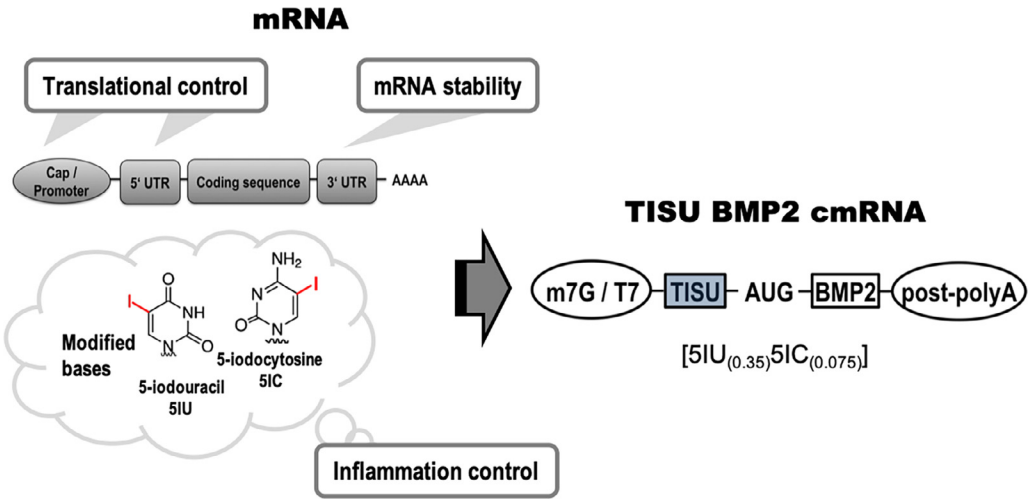

Fig 6. Salient chemical modifications to mRNA encoding BMP-2. The basic elements of mRNA shown in the top left of the figure have been modified in several ways. Iodinated pyrimidines reduce interaction with toll-like receptors to lower inflammation (bottom left-hand side); the efficiency of translation is improved by including a translation initiator of short UTRs (TISU) sequence (right hand side); mRNA stability is enhanced by extending the polyA tail and including sequences in the 5'-untranslated regions (UTR). Modified from reference.[80]

BMP-9 cmRNA of Khorsand et al[83] has been evaluated in a long bone, critical sized defect model.

CONCLUSIONS AND PERSPECTIVE

The original motive for the genetic delivery of BMP-2 for bone healing was to deliver into the defect as much BMP-2 as possible for as long as possible. For the reasons described in this review article, such a strategy no longer seems appropriate. Data obtained with a rat segmental defect model suggest a more subtle approach in which only small amounts of transgenic BMP-2 are produced locally within the defect for a short period of time. This promises to be much more effective, safer and probably less expensive than the present clinical practice of delivering very large amounts of rhBMP-2. The use of an engineered cell line and cmRNA are two genetic strategies for achieving this in a clinically acceptable fashion. Nevertheless, it will be necessary to show that transient, low expression of BMP-2 can heal recalcitrant segmental defects in animals larger, older and less healthy than the young rats studied so far.

Our gene therapy focus has remained with BMP-2, partly because the recombinant protein is the most potent osteoinductive cytokine that is FDA-approved, which should facilitate clinical translation. Moreover, there is a large amount of valuable data concerning the biology of rhBMP-2 in human patients with broken bones. Nevertheless, much remains unknown. Progress would be helped by greater insight into the role of inflammation and specific inflammatory mediators in bone regeneration, as well as the contributions of the immune and nervous systems. Often overlooked by biologists is the role of the mechanical environment in bone healing.[84] It is likely that important synergies exist between biological and mechanical approaches to regeneration which could be exploited clinically for bone healing.[85]

Moving novel bone healing molecular therapies from the rat model to the clinic is not easy.[86] Models in larger animals, such as sheep, goats and pigs, are more challenging than rodent models and much more expensive, but necessary for clinical translation. As mentioned in this article, two of our gene therapy approaches that worked well in rats were ineffective in sheep. Apart from demonstrating efficacy, preclinical biodistribution and toxicity testing will need to be performed under Good Laboratory Practice conditions before contemplating human trials.

Gene therapy has entered a period of rapid growth for a number of different indications, and the regulatory authorities have granted marketing approval to a growing number of gene-based products. Moreover, the field of RNA therapeutics is undergoing massive and rapid expansion. Such circumstances provide optimism for the future of genetic approaches to healing bone.

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