REVIEW

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Realizing the Potential of Gene-Based Molecular Therapies in Bone Repair

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ABSTRACT

A better understanding of osteogenesis at genetic and biochemical levels is yielding new molecular entities that can modulate bone regeneration and potentially act as novel therapies in a clinical setting. These new entities are motivating alternative approaches for bone repair by utilizing DNA-derived expression systems, as well as RNA-based regulatory molecules controlling the fate of cells involved in osteogenesis. These sophisticated mediators of osteogenesis, however, pose unique delivery challenges that are not obvious in deployment of conventional therapeutic agents. Viral and nonviral delivery systems are actively pursued in preclinical animal models to realize the potential of the gene-based medicines. This article will summarize promising bone-inducing molecular agents on the horizon as well as provide a critical review of delivery systems employed for their administration. Special attention was paid to synthetic (nonviral) delivery systems because they are more likely to be adopted for clinical testing because of safety considerations. We present a comparative analysis of dose-response relationships, as well as pharmacokinetic and pharmacodynamic features of various approaches, with the purpose of clearly defining the current frontier in the field. We conclude with the authors' perspective on the future of gene-based therapy of bone defects, articulating promising research avenues to advance the field of clinical bone repair. © 2013 American Society for Bone and Mineral Research.

KEY WORDS: OSTEOGENESIS; BIOENGINEERING; MOLECULAR PATHWAYS; GENE-BASED THERAPY

Clinical Need for New Bone-Regeneration Strategies

Nearly 2.2 million bone grafts are performed worldwide annually[1] and up to 20% of fractures are hampered by impaired healing.[2] The economic impact of nonunions is enormous, with the cost of spinal fusions alone reaching $20 billion annually.[3] The gold standard for repair of large segmental defects remains autologous grafts, where bone harvested from a non-weight-bearing site, usually the iliac crest, is used to repair defects. Bone grafts, however, are limited in several aspects, including potency of the grafts and the physiological detriment resulting from harvest surgery. Allografts are alternatively employed, where donor tissue is used to repair the defect, but the risk of disease transmission is always a concern. Allografts are extensively processed to reduce this risk, but osteopotency of the graft could be decreased in this way.[4] Demineralized bone matrix derived from decalcified bone can similarly act as a substitute; its potency, however, is variable and depends on the processing conditions. Synthetic scaffolds have been used to provide a hospitable environment for new bone formation. Scaffolds have been constructed from the organic (collagen) and inorganic (hydroxyapatite [HA]) components of bone, but such scaffolds are incapable of inducing osteogenesis on their own and they are more suitable for smaller defects.

Osteogenic proteins are frequently employed to render osteoconductive biomaterials osteoinductive. The clinically employed proteins for this purpose are bone morphogenetic protein (BMPs). BMPs are morphogens that can induce ectopic bone formation by causing stem cells to differentiate into osteoblasts[5] but can also act as a chemotactic agent to recruit cells at femtomolar concentrations.[6] Because of their potent bone-induction properties, BMP-7 (also known as osteogenic protein-1 [OP-1]) has been approved for Humanitarian Device Exemption for spinal fusions, and BMP-2 has been approved for clinical use in select indications, including spinal fusions, oral/maxillofacial applications, and orthopedic trauma.[7,8] In these therapies, collagenous materials are loaded with BMP-2 (INFUSE

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bone graft) or BMP-7 (OP-1 bone grafts) and implanted to induce local bone formation, where the outcomes were found to be comparable to those of autologous bone grafts. The expense of the large amount of BMPs needed in these protein therapies is impeding their widespread application. Recent studies have also raised safety concerns with protein therapies,[9–11] including osteolysis, ectopic bone growth, wound complications, and urogenital events.[10] The full extent and frequency of complications associated with rhBMPs is still under investigation. The reasons for this are currently unclear but may be associated with the exceedingly high protein concentrations required to maintain a therapeutic response. Approximately 1.5 mg/mL of BMP-2[7] or 0.9 mg/mL of OP-1[12] are required for treatment, both of which are much higher than ~ng/mL levels of endogenous proteins in bone.

Clinical deployment of BMPs is a prototypical example of regeneration strategies. These strategies are intended to restore osteogenic activity at the injury site within the framework of fracture repair.[13,14] A multitude of cells, including inflammatory cells, fibroblasts, mesenchymal stem cells, and preosteoblasts, participate in laying down the connective tissue to form a soft callus, which undergoes remodeling subsequently.[15–19] Cell-based therapies have been explored where osteogenic cell lineages are transplanted, usually on a biomaterial scaffold, to induce bone formation. The transplanted cells can deposit an extracellular matrix directly and secrete the growth factors needed to further recruit cells to the site. Mesenchymal stem cells have been pursued for this purpose because of both their wide differentiation potential and long life span, as well as their ability to home to bone marrow.[20] Even cell-based therapies, however, are not always robust enough with native cells; cells need to be genetically modified to express factors for improved potency. The patient's own cells would be the most favorable option for therapy to circumvent any potential immune response. The expansion of host cells, however, would greatly increase the cost of the therapy. The long culture period required to obtain the number of cells for success would inevitably delay the treatment. Equally undesirable is the decrease in differentiation and proliferative potential of cells during the long culture period,[21] which can lead to substantial decreases in the amount of in vivo bone formation after transplantation.[22] For these reasons, reprogramming or transdifferentiating host cells directly at the local site is appealing for functional bone regeneration.[23] It is possible to achieve cellular transformation by manipulating genetic networks, where transforming agents are coded from exogenous DNAs or regulatory pathways are altered for a desired cellular transformation. This approach bypasses the need to produce large amounts of recombinant protein in an industrial setting, as well as eliminates the issues complicating cellular harvest, expansion, and transplantation.

Possibility of Clinical Therapies Based on Genetic Elements

Both positive and negative regulation of gene expression has been explored as the basis of a therapeutic modality (Fig. 1). Positive gene expression involves introduction of DNA coding for a therapeutic gene and in situ expression of proteins by host cells. In light of safety concerns with high-dose protein therapies, gene delivery has been proposed to deliver proteins at more physiological levels.[24,25] Gene therapy is particularly amenable to bone regeneration because of the fact that a single gene, such as tissue-inducing BMPs, could induce a functional bone tissue. Genetic elements can also be employed to negatively regulate expression of proteins that inhibit osteogenesis. RNAs that prevent translation by RNA interference (RNAi) mechanism can silence specific protein expression. The specificity of protein knockdown allows treatment of diseases previously lacking any therapy, such as the inherited disorder pachyonychia congenita.[26] A recent clinical trial used short interfering RNA (siRNA) against a mutant form of keratin that causes calluses. Injection of siRNA led to a decrease in the calluses, compared with a vehicle control, providing evidence for the RNAi approach to modulate composition of tissues in a clinical setting. The dose of siRNA in each injection ranged from 0.1 to 17 mg, which is not an exuberant amount for clinical use. RNAi can be implemented with either exogenous agents, such as siRNA and oligonucleotides (ODN), or directly implemented by a plasmid for endogenous processing. Although RNAi-mediated therapies for bone regeneration are still at an early research stage, they hold untapped promise for their ability to inhibit specific pathways, as well as avoiding off-target effects that plague small molecule inhibitors.

The current challenge in bringing a gene-based therapy to a clinical trial remains the safe and effective delivery of nucleic acids. Viruses have served well in preclinical models to demonstrate the feasibility of specific gene-based therapies (Table 1). Viruses, however, are associated with risk of immune response, inflammation, and insertional mutagenesis,[27] which were evident even in some preclinical studies.[28] As of 2012, 67% of all gene therapy trials used viral carriers, with nearly 20% employing retroviruses,[29] and these numbers have not changed significantly in recent years.[30,31] Even though there are currently no approved gene delivery therapies for osteogenesis in clinical use, the promise of gene delivery has stimulated extensive explorations of various delivery approaches, in particular development of safer nonviral carriers in light of safety concerns associated with viruses.

pDNA Delivery for Bone Induction and Functional Outcomes

Although viral delivery has established the feasibility of gene delivery for bone repair (Table 1, Fig. 2, Kimelman-Bleich and colleagues[24]), a close inspection of the studies has raised questions about the actual effectiveness in a realistic setting. A particular issue is the impact of immune status on the outcome of therapy. Many viral carriers showed an attenuated response[32] or are ineffective in animals with intact immune systems.[33–41] Immunosuppression of normal animals can restore the efficacy of viral therapy,[42] but this would be undesirable (perhaps unacceptable) in a clinical setting. The impact of immune response may depend on animal model, virus type, or transgene employed because there are reports of successful bone induction in both immune-competent and immune-compromised animals.[34–37,43,44] Even if a suitable combination of carrier and transgene could be found, clinical risks of viral gene delivery have to be

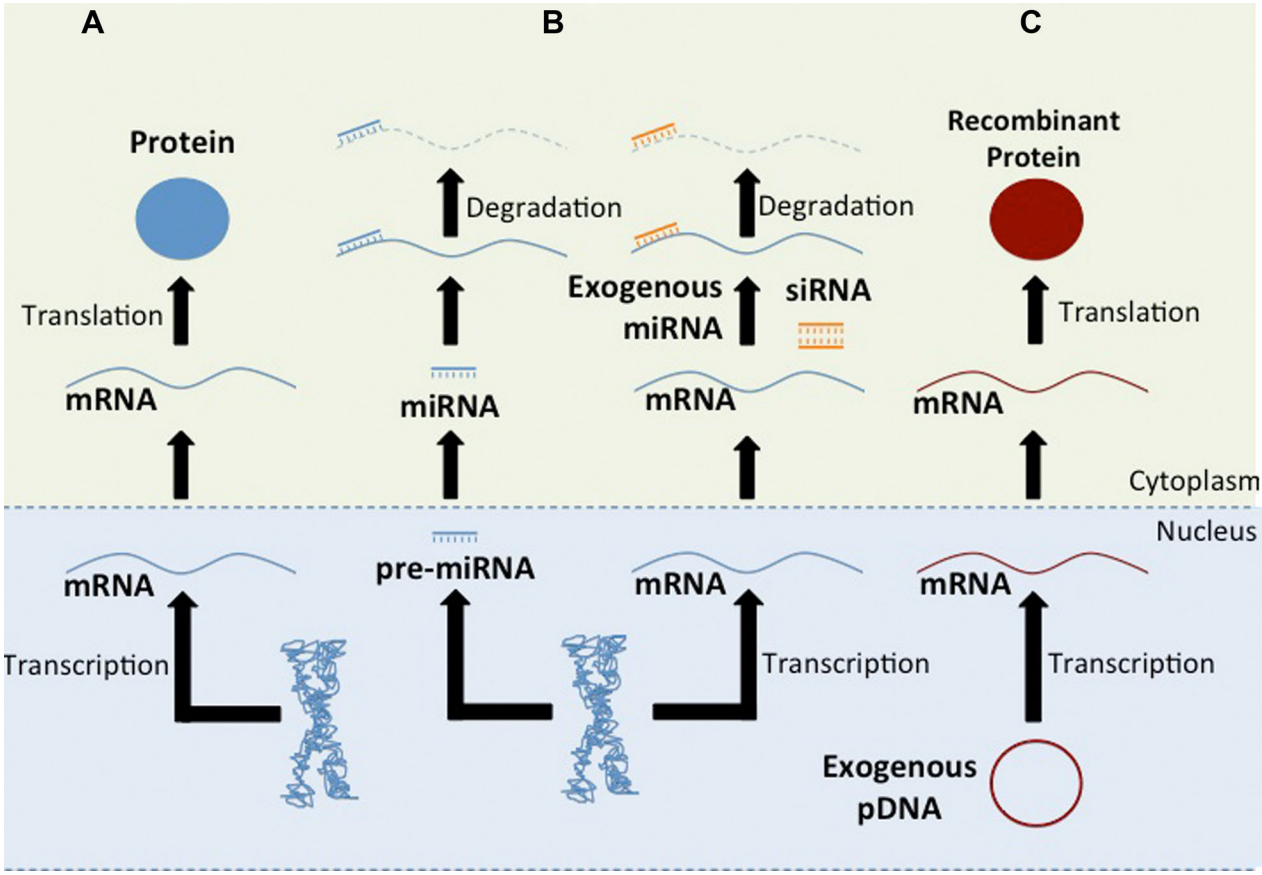


Fig. 1. Schematic of gene-based therapies. The central dogma of molecular biology is shown in pathway *A*, where DNA is transcribed into mRNA in the nucleus and proteins are translated from mRNA in the cytoplasm. Pathway *B* shows regulation of protein translation through RNAi. Endogenous miRNA, exogenous miRNA, or synthetic siRNAs can bind mRNA and induce degradation of mRNA, which can be employed to target inhibitors of osteogenesis. Pathway *C* shows production of recombinant proteins, where exogenous DNA introduced into cells is employed to produce recombinant proteins capable of stimulating osteogenesis.

mitigated; nonintegrating, short-acting viral expression systems (such as adenoviral vectors) might be preferable over integrating, long-acting vectors. Only a handful of studies employed "integrating" viruses (Table 1), which recognizes the preference of practitioners in the field. Nonviral methods, including use of naked pDNA and physical delivery methods (Fig. 3, Table 2), may alternatively be more appealing because the simpler mechanism of delivery may reduce long-term complications of the intervention.

Delivery of naked pDNA without a carrier

The simplest approach to gene therapy is the administration of pDNA alone. With injections of 500 μg BMP-2 plasmid into mouse gastrocnemius muscle,[45] bone formation was induced, but it was obtained only when pDNA dose was divided into two to eight smaller doses of 250 to 62.5 μg delivered over 2 to 8 days. A single injection of the full 500-μg dose was incapable of inducing bone formation, suggesting that pDNA without a carrier does not sufficiently sustain protein expression for a prolonged time. In a separate study,[46] pDNA delivered with or without a collagen solution led to small amounts of bone formation in a rat spinal fusion model. A high pDNA dose (500 μg) was also needed for this purpose. The specific role of the collagen in pDNA delivery was unknown, but it may retain the pDNA at the injection site longer, rather than facilitating intracellular uptake/trafficking.

In one of the earliest studies, BMP-4 plasmid (500 to 1000 mg) delivered in a collagen scaffold in a rat critical femur model[47] led to defect bridging after 9 weeks compared with fibrous tissue seen with collagen sponges alone. A combination of a PTH1-34 and BMP-4 plasmid led to accelerated healing such that the bone defect was bridged in 4 weeks. A similar collagen sponge containing 100 or 1000 μg of vascular endothelial growth factor (VEGF) plasmid increased angiogenesis, leading to bone formation in a rat critical-size effect.[48] Delivery of a PTH1-34 plasmid on a collagen sponge implanted at a beagle tibial defect model was successful without the use of a carrier.[49] Bone formation at the critical-size defect increased by 25% after 4 weeks with 40 mg, but 100 mg pDNA was required for union within 6 weeks. Although effective, it appears that excessive amounts of pDNA may be required for significant bone induction, making this approach not clinically feasible.

Physical delivery methods

Both sonoporation and electroporation have been employed to increase intracellular uptake of pDNA delivered without a carrier.

Table 1. Summary of In Vivo Studies Employing Viral Gene Delivery for Bone Induction

Gene	Site	Carrier	Outcome
BMP2	Ectopic	AV	Gene delivery to quadriceps led to bone in athymic mice[36] but not in normal immunocompetent mice[35]
			Bone formation in calf muscle of rats only with immunosuppression[42]
			AV delivered in a collagen sponge to calf muscle led to bone in immunocompetent rats but not when AV particles were injected without the sponge[41]
			Bone formation in the calf muscle of rats was observed only with immunosuppression[38–40]
			Bone induction in quadriceps was stronger in athymic nude rats compared with immunocompetent rats[32]
			AV delivery to soleus muscle of rats resulted in bone only when ischemic degeneration was induced via muscle grafting. Bone was not observed without grafting[152]
		AAV	Bone induction via endochondral ossification was observed in hindlimb muscle of immunocompetent rats[101,102]
			Viral particles delivered on hydroxyapatite scaffold to the back muscles of immunocompetent rats led to bone formation[103]
			Bone formation in thigh muscle after delivery of a tetracycline-sensitive expression system was observed in mice only when a tetracycline analogue was administered[153]
	Orthotopic	AV	Increased regeneration in a mandibular distraction osteogenesis model in rats[154]
			Increased bone regeneration in an osteoporotic fracture model in tibia of sheep[155]
			Bone formation or increased regeneration was observed in several defect models, including a critical-size mandibular defect,[33] critical-size nasal defect in athymic nude mice,[156] rib defect in horses,[157] metatarsal defect in horses,[158] and femoral critical-size defect in rats[159]
			Injected AVs led to partial regeneration of critical-size calvarial defects in rats, with a more vigorous response when particles were delivered in a gelatin scaffold[117]
			Healing of iliac crest critical-size defects in sheep was delayed compared with no treatment when viral particles were injected to injury site[160]
			Bone formation was observed in a dental model in immunocompetent dogs[107]
			Enhanced cartilage and subchondral bone was observed in femur condyle defect in immunocompetent ponies[161]
			Delaying administration of viral particles improved healing of femur critical-size defects in rats[162]
		AAV	Bone formation in femur defects in immunocompromised rats, but addition of human mesenchymal stem cells did not improve the outcome[163]
BMP4	Ectopic	AV	Bone induction in hindlimb muscle,[164] calf muscle,[34] and quadriceps[35] of athymic nude rats
			Bone formation in the thigh muscle was observed in athymic nude rats but not in immunocompetent rats[36]
	Ectopic	AAV	Bone formation in hindlimb muscle of immunocompetent rats[104]
	Orthotopic	AV	Enhanced bone formation around implants in femur defects in ovariectomized rabbits[165]
		RV	Increased callus size and enhanced healing in a femur fracture model in immunocompetent rats[166]
BMP6	Ectopic	AV	Bone formation in quadriceps in athymic nude mice,[35] in thigh muscle of various immunocompetent rat strains,[36,37] and in calf muscle of nude athymic rats[34]
	Orthotopic	AV	Enhanced cartilage and subchondral bone formation in a femur condyle defect model in immunocompetent ponies[161]
BMP7	Ectopic	AV	Bone formation in quadriceps of athymic nude mice[35] and thigh muscle of athymic nude rats[36] but not in thigh muscle of immunocompetent rats[36]
	Orthotopic	AV	Better osseointegration of dental implants by enhanced aveolar bone formation in immunocompetent rats[167]
			Bone regeneration in calvarial defect in mice when viral particles were delivered in a silk fibroin scaffold[124]
BMP9	Ectopic	AV	Bone formation in quadriceps in athymic nude mice[35] and rats[43,44] and in thigh muscle of immunocompetent mice[36] and rat strains[37,43,44]

Table 1. (*Continued*)

Gene	Site	Carrier	Outcome
	Orthotopic	AV	Regeneration in critical-size mandibular defect[33] and healing of spinal arthrodesis model in athymic nude mice[168]
TGFβ	Orthotopic	AV	Increased epiphyseal thickness was observed after injection of viral particles into the humerus of rats[169]
VEGF	Orthotopic	AV	Viral particles injected into muscle surrounding a femur defect in immunocompetent rats led to faster healing and repair[170]
			No bone formation in a dental implant model in immunocompetent dogs[107]
PDGF	Orthotopic	AV	Enhanced aveolar bone repair and regeneration in periodontal lesions[171] and alveolar ridge defects[172] in immunocompetent rats
LMP1	Orthotopic	RV	Improved regeneration and healing in femur fractures in immunocompetent rats[150]
Cbfa1	Orthotopic	AV	Robust bone regeneration in an idiopathic osteonecrosis model in rats[173]
Nell1	Orthotopic	AV	Viral particles in demineralized bone matrix enhanced spinal fusion in immunocompetent rats[149]

AV = adenovirus; AAV = adeno-associated virus; RV = retrovirus; BMP = bone morphogenetic protein; TGFβ = transforming growth factor-β; VEGF = vascular endothelial growth factor; PDGF = platelet-derived growth factor; LMP1 = latent membrane protein 1; Cbfa1 = core binding factor alpha1 subunit protein (Runx2); Nell1 = NEL-like 1.

Sonoporation uses collapsing microbubbles, compromising plasma membrane integrity to allow pDNA passage into the cell.[50] Sonoporation with 75 µg BMP-2 plasmid led to radiographic bone formation in a mouse intramuscular model, although pDNA without sonoporation also led to some ectopic bone in this study.[51] Multiple cycles of injections and sonoporations were required to induce bone.[51] Electroporation similarly allows cellular internalization of pDNA after plasma membrane integrity is compromised by electrical pulses.[52] Electroporation of 25 to 50 µg BMP-2/7 plasmid yielded radiopaque bone in a rat intramuscular model.[53] A similar system was also effective for intraosseous application, where electroporation with 50 µg of BMP-9 plasmid induced bone formation in a critical-size defect in a mouse radius.[54] No bone formation was evident without electroporation. The use of only 50 µg pDNA to heal a defect with electroporation represents ~10-fold decrease in the pDNA dose needed for naked pDNA delivery (from above studies). This is a significant improvement. A separate study also demonstrated osteogenesis in an ectopic model after electroporation of 100 µg BMP-4 plasmid, but this study also found dystrophic calcification in electroporation groups.[55] This excessive tissue damage is worrisome, particularly if such a physical intervention is applied at a site that is already injured. Finally, a comparison of electroporation and sonoporation with BMP-9 plasmid in a mouse intramuscular model found that the volume of bone formed was ~30-fold higher with sonoporation compared with electroporation.[56] It must be noted that in preclinical models, the surrounding tissue is relatively thin and the bone tissue is easily accessible. The thick tissues surrounding human bones may greatly limit the ability of percutaneously applied electroporation or sonoporation to perturb cellular membranes in situ and internalize pDNA.

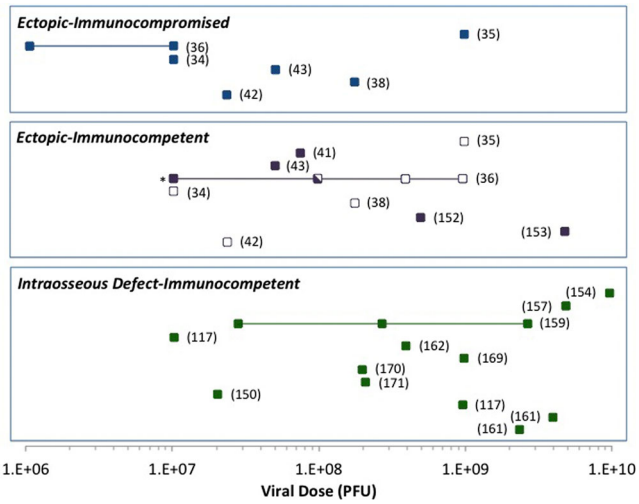


Fig. 2. Summary of viral doses employed in bone induction studies. The summary shows viral doses for ectopic implantation in immunocompromised animals, ectopic implantation in immunocompetent animals, and intraosseous defect in immunocompetent animals. The viral dose is given as plaque-forming units (PFU) per implant. Open squares (□) indicate studies with no bone formation, whereas closed squares (■) indicate successful bone formation. Connected squares indicate the range of doses employed in the study. In the case of Li 2003a (*), the studies employed only one dose for each of five different BMPs and are not indicative of any dose response, only the differences in BMP potency.

Delivery with synthetic carriers

Synthetic carriers are intended to facilitate intracellular uptake of pDNA without nonspecific membrane disruptions. Because unmethylated CpG motifs on naked pDNA can be recognized by Toll-like Receptor-9[57] and stimulate an immune response, synthetic carriers can also mask the immunogenic CpG motifs.[58] The broadly effective carrier 25 kDa polyethylenimine (PEI25) has been employed to deliver BMP-4 plasmid (200 µg) in a poly (lactic-*co*-glycolic) acid implant for regeneration of a rat critical-size skull defect.[59] Bone formation was observed around defect

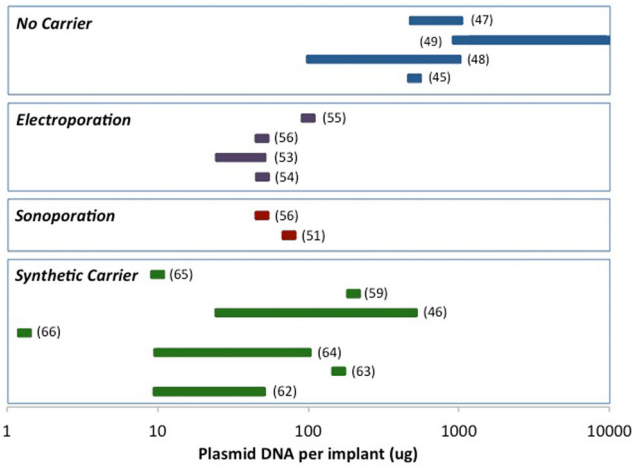


Fig. 3. Amount of pDNA used in bone induction studies involving nonviral gene delivery. The graph summarizes the range of pDNA doses used in osteogenesis studies, which were classified based on the type of nonviral gene delivery: no carrier (naked pDNA), electroporation, sonoporation, or synthetic carriers. Experimental details of each study are described in Table 2.

edges after 8 weeks, whereas naked pDNA and scaffolds alone gave no bone formation. Limited bone formation may in part be owing to the toxicity of PEI25; although the amount of implanted polymer was not provided, this is likely to be in excess of 32 µg (polymer:pDNA ratios are typically >1.0). We previously found that relatively small amounts of PEI25 (16 to 32 µg) was sufficient to inhibit BMP-2 (protein)–induced bone formation at ectopic sites,[60] so that the excess PEI25 in the above study might have limited a robust bone tissue formation.

A more biocompatible carrier is smaller (2 kDa) PEI modified with lipids.[61] Although this carrier yielded the same recombinant protein expression rate as viral vectors (based on in vitro assessment), no bone formation was observed with 10 to 50 µg of BMP-2 plasmid at subcutaneous sites in rats.[62] The same outcome was observed with the PEI25 carrier as well. This may be in part owing to insufficient transgene expression (dose or duration). Another study employed a rat critical-size skull defect and delivered 160 µg of BMP-2 plasmid[63] by using a triacrylate/amine polycationic polymer (TAPP) for delivery. The resulting particles were incorporated into gelatin microspheres and implanted in a poly(propylene fumarate) scaffold. Despite the large amount of implanted pDNA, gene delivery had no effect on bone formation. The reasons for this are unclear, but given that the TAPP only led to a small increase in transfection compared with free pDNA in vitro,[63] the low efficiency of the carrier may be responsible for this outcome.

When 50 to 100 µg of BMP-2 plasmid condensed with calcium phosphates were implanted in HA scaffolds, radiopaque masses were seen at the subcutaneous implant site after 4 weeks.[64] A lower (10 µg) plasmid dose was also effective but required 12 weeks to show radiopaque tissue. HA may be an optimal scaffold as the scaffold itself induced formation of calcified tissue in this study. HA scaffolds containing 10 µg of BMP-2 plasmid in cationic liposomes (SuperFect) were also employed in an intraosseous model.[65] The amount of pDNA to be delivered was limited by the toxicity of the carrier. The defect containing BMP-2 liposomes was bridged after 6 weeks; however, most of the new bone was formed on the periphery of the scaffold with little bone tissue penetrating the scaffold. Longer-term studies might prove otherwise, but low potency of the delivery systems and/or suboptimal scaffolds might have hindered a robust response. Finally, a unique study employed only 1.3 µg pDNA expressing both runt-related transcription factor 2 (Runx2) and constitutively active form of activin receptor-like kinase 6 (caALK6),[66] two intracellular mediators of osteogenesis. pDNA was condensed by a block co-polymer of polyethyleneglycol-aspartate-diethylenetriamine and mixed into a calcium phosphate cement to fill the cranial defect. Unlike polymeric PEI25 or liposomal Fugene that led to no regeneration, this novel carrier led to histological bone covering approximately half of the original defect after 4 weeks. This is a noteworthy result because the pDNA dose was significantly lower than any other studies reported. The aspartic acid residues in the polymer are expected to enhance the affinity of pDNA complexes to calcium/phosphate scaffold, better localizing the pDNA to the defect site. The calcium/phosphate cement might have directly contributed to this result because it is noted to stimulate endogenous mineralization,[67] but whether it also supports enhanced transgene expression per se remains to be investigated.

Bone Induction by RNA Interference

Small druglike inhibitors are being investigated as a means to overcome negative regulators of osteogenesis. Examples of such inhibitors include: the small GTPase Rho and Rho-associated protein kinase (ROCK) inhibitor Y27632,[68] histone deacetylase inhibitor Trichostatin A,[69] CXCR4 inhibitor AMD3100,[70] and the cGMP-dependent phospodiesterase-5 inhibitor Sildenafil.[71] Unlike the synthetic entities, however, inhibitory RNAs in the form of microRNAs (miRNA) and small interfering RNAs (siRNA) are more appropriate (ie, physiological) means to alter gene expression. Single-stranded miRNAs bind to RNA-Induced Silencing Complex (RISC), which then binds target mRNA at the 3′ untranslated region (URT) to reduce or inhibit the translation. miRNAs with exact complementary sequences may result in cleavage of the bound mRNA, whereas base mismatches likely lead to translational repression.[72] The miRNA have recently been shown to regulate osteogenic commitment of mesenchymal stem cells.[73] The miRNAs can act as negative regulators of osteogenesis,[74–79] or as promoters of osteoblast differentiation (Fig. 4).[80–83] Pro-osteogenic miRNAs can upregulate Wnt[81] and BMP/Runx2[76] signaling. miRNA alterations are implicated in musculoskeletal diseases; mutations in miR-2861 are associated with Type 1 osteoporosis in adolescents resulting from functional loss of miR-2861.[80] In contrast, mice lacking miR-155 do not develop collagen-induced arthritis and had reduced bone destruction because of diminished osteoclastic activity.[84] There are no published studies that investigated direct delivery of miRNA for bone induction. Human mesenchymal stem cells, however, transfected with miR-138 or antimiR-138 expression systems, were explored for enhanced

Table 2. Details of Studies Described in Fig. 3

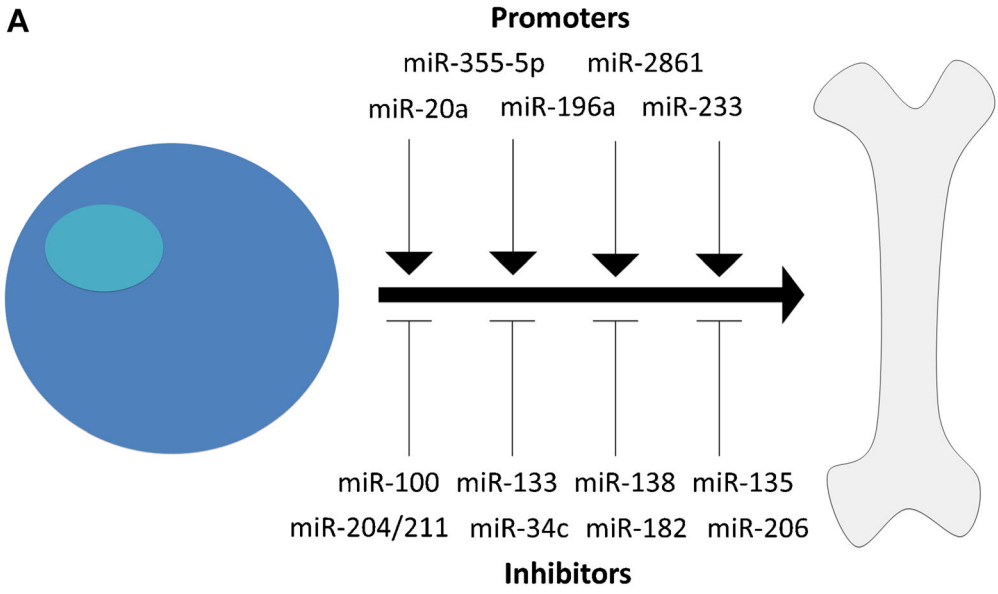
Reference	Gene	Scaffold	Model	Outcome
No./carrier				
47	BMP-4, hPTH1-34	Collagen	Rat femur-critical defect	Bone formation and gap healing after 9 weeks with BMP-4; the combination of BMP-4 and hPTH1-43 was more potent than individual factors, with bony bridging after 4 weeks
49	hPTH1-34	Collagen	Beagle tibia-critical defect	Although some regeneration was seen after 4 weeks with 40 mg of pDNA, 100 mg was required for major regeneration after 6 weeks
48	VEGF	Collagen	Rat cranium-critical defect	The defect was bridged after 6 weeks
45	BMP-2	No scaffold	Mouse radius-critical defect	Bone formation was detected after 3 weeks but only when multiple injections were given
Electroporation				
55	BMP-4	No scaffold	Mouse SC implantation	Bone formation after 2 to 4 weeks of BMP-4 electroporation but dystrophic calcification in all groups receiving electroporation
53	BMP-2/7	No scaffold	Mouse IM injection	Bone formation was observed after 10 days
56	BMP-9	No scaffold	Mouse IM injection	Bone formation after 5 weeks of electroporation
54	BMP-9	Collagen	Mouse radius-critical defect	Critical-size defect was bridged with electroporation of BMP-9 after 5 weeks
Sonoporation				
56	BMP-9	No scaffold	Mouse IM injection	Sonoporation led to bone formation to a lesser extent than electroporation
51	BMP-2	No scaffold	Mouse IM injection	Bone was observed with 7 cycles of injection/sonoporation after 3 weeks
Synthetic carrier				
65	BMP-2	HA	Rabbit cranium-critical defect	SuperFect-mediated gene delivery led to bridging of the critical defect, but the new bone was outside the scaffold
59	BMP-2	PLGA	Rat cranium-critical defect	Limited bone formation was observed with 25 kDa PEI delivery after 15 weeks
46	BMP-7	No scaffold, collagen	Rat spinal fusion	Limited bone formation was observed histologically when pDNA was delivered with cross-linked collagen
66	Runx2 and caALK6	Ca/P cement	Mouse cranium-critical defect	A novel polymeric carrier, composed of block co-polymers of polyethyleneglycol-aspartate-diethylenetriamine, led to bone formation at 4 weeks
64	BMP-2	HA fiber	Rat SC implantation	Bone formation was observed after 4 weeks with calcium phosphate
63	BMP-2	PFF	Rat cranium-critical defect	No bone formation was observed with triacrylate/amine polycationic polymer (TAPP) as a gene carrier
62	BMP-2	Gelatin	Rat SC implantation	2 kDa polyethylenimine modified with linoleic acid (PEI-LA) gene delivery led to extensive tissue induction but no bone formation

SC = subcutaneous; IM = intramuscular; HA = hydroxyapatite; PLGA = poly(lactic-co-glycolic acid); PFF = poly(propylene fumarate).

osteogenesis; when seeded on a HA/calcium-phosphate scaffold and implanted subcutaneously in SCID mice, miR-138–modified cells decreased ectopic bone formation by ~80%, whereas anti-miR-138 more than doubled the ectopic bone formation.[74] miR-138 inhibited osteogenesis through extracellular signal-receptor kinase (ERK) via focal adhesion protein (FAK), although the precise signaling pathway is still unclear. Presumably the obtained effect was because of reprogramming of transplanted pluripotent cells. Direct delivery of miRNA to reprogram host cells, instead of delivery via cell transplantation, is going to be more clinically relevant.

The siRNA have been alternatively employed to selectively silence protein expression in support of osteogenesis. siRNAs are synthetic nucleic acids (19 to 23 base pairs) but can be introduced into the cell to silence gene expression through endogenous the RNAi pathway.[85] siRNA-mediated downregulation of STAT3,[86]

A



B

miRNA	Role in or Link to Human Disease	Pathway or Mechanism	Reference
miR-20a	Expression of miR-20a promotes osteogenic differentiation	Inhibits PPARϒ, Bambi, and Crim1, antagonists of BMP/Runx2 signalling	76
miR-34c	Overexpression of miR-34c into osteoblasts leads to defective mineralization and osteoporosis	miR-34c decreases Notch signalling	78
miR-100	Overexpression of miR-100 inhibits osteogenic differentiation of hADSC, whereas knockdown enhanced osteogenesis	miR inhibits BMPR2 expression	77
miR-133	Overexpression of miR-133 inhibits expression of osteogenic genes	miR-133 targets Runx2	172
miR-135	Overexpression of miR-135 inhibits expression of osteogenic genes	miR-135 targets Smad5	172
miR-138	Inhibition of miR-138 induces bone formation	miR-138 inhibits ERK pathway by targeting FAK	74
miR-155	Mice without miR-155 did not develop collagen-induced arthritis.	miR-155 is involved in innate and adaptive immunity	84
miR-182	Osteogenesis and bone formation inhibited by overexpression of miR-182	miR-182 inhibits FoxO1	173
miR-196a	hADSC osteogenesis was inhibited by knockdown and enhanced by overexpression of miR-196a	miR-196a down regulates the HOXC8	83
miR-204/211	miR-204 expression stimulates adipocyte differentiation from bone marrow stromal cells	miR-204 decreases expression of osteogenic Runx2 expression	79
miR-206	Osteogenesis is inhibited by over-expression and induced by knockdown of miR-206	miR-206 decreases Cx43 expression	75
miR-233	Overexpression prevents osteoclastogenesis	Unknown	82
miR-355-5p	Delivery of miR-355-5p increases expression of osteogenic genes	Inhibits DKK, an antagonist of Wnt signalling	81
miR-2861	Mutation in miRNA linked to osteoporosis. Knockdown inhibits bone formation, whereas overexpression increases osteogenesis.	The miRNA represses HDAC5, which enhances Runx2 degradation	80

Fig. 4. (*A*) miRNA identified to promote or inhibit bone regeneration. An miRNA whose expression leads to osteogenesis (or depletion hinders osteogenesis) was categorized as a promoter of bone regeneration. In contrast, an miRNA whose expression impedes bone formation was categorized as an inhibitor of bone regeneration. (*B*) List of miRNAs with potential for bone regeneration and their reported mechanisms of action. PPARγ = peroxisome proliferator-activated receptor gamma; BMPR2 = bone morphogenetic proteins receptor II; ERK = extracellular signal-regulated kinases; FAK = focal adhesion kinase; FoxO1 = forkhead box protein O1; HOXC8 = homeobox-containing protein C8; Cx43 = connexion 43; DKK1 = Dickkopf-related protein 1; HDAC5 =histonedeacetylase 5.

Table 3. In Vivo Studies Using siRNA-Mediated RNAi for Osteogenesis

siRNA target	Carrier	Amount	Model	Reference no.
Plekho1	Novel carrier of DOTAP with six repeats of aspartate, serine, and serine	1 mg (est.)	Mouse, systemic delivery targeting to bone surfaces	97
GNAS1, PDH2	Naked siRNA with silk fibroin-chitosan scaffold	Unknown	Sheep, intramuscular implantation	94
Noggin	Electroporation	10–16 μg	Mouse, intramuscular implantation of collagen sponge containing 5 μg of BMP-2 after injection and electroporation of siRNA	90
Noggin	No carrier	10–16 μg	Mouse, intramuscular of novel scaffold (poly-D,L lactic acid-co-dioxanone-co-polyethyleneglycol hydrogel) containing 2.5 μg of BMP-2 and siRNA	91

Abundant in Neuroepithelial Area (ANA),[87] Hoxc8,[88] Protein related to DAN and cerberus (PRDC),[89] Noggin,[90,91] Notch,[92] zinc finger Zfp467,[93] guanine nucleotide-binding protein alpha (GNAS1),[94] and prolyl hydroxylase domain-containing protein 2 (PHD2)[94] have been all shown to enhance osteogenic activity of various cell types in vitro. siRNA has been additionally investigated as a supplement to protein delivery, where siRNA against Noggin have been deployed in support of BMP-induced osteogenesis.[90,91] In one study, the muscle surrounding the implantation site was primed through electroporation of Noggin siRNA. After BMP-2 implantation, a small increase in BMD was observed with Noggin siRNA.[90] A subsequent study found similar results when Noggin siRNA was delivered alongside BMP-2 in a synthetic scaffold.[91] For these studies, we estimate that 10 to 16 μg of siRNA was delivered in each implant, which is practical for clinical scale up. These in vivo studies were in line with reported effects of Noggin on osteogenesis from some in vitro studies, but there are also conflicting reports on Noggin effect on BMP-2–induced osteogenesis. siRNA-mediated Noggin suppression in human bone marrow–derived stem cells reduced expression of osteogenic markers and in vitro calcification in our hands,[95] unlike its well-known effect on rodent cells and in animal models. Others also noted a stimulatory role of Noggin protein on osteogenesis of human mesenchymal stem cells under a variety of inducing conditions.[96] Such a contradictory Noggin effect calls for better understanding of reasons behind this observation. In addition to species effects, pharmacokinetic differences, culture conditions, nonspecific effects of delivery, and inherent differences in cell populations (ie, activity of osteogenesis-related intracellular pathways or receptor repertories) could all be likely reasons for the differential response to Noggin suppression.

The delivery of siRNA has been attempted in animal models (Table 3). In a ground-breaking study, an siRNA against Pleckho1 (casein kinase-2 interacting protein-1) was delivered systemically using a novel carrier consisting of cationic lipid DOTAP with six repeat of a tripeptide aspartate-serine-serine.[97] Peptide-modified liposomes containing Pleckho1 siRNA led to an increase in bone mineral density compared with unmodified liposomes or free siRNA over the course of 9 weeks. Bone mineral density returned to normal levels in ovariectomized rats upon delivery of Pleckho1 siRNA in peptide-modified liposomes over a 13-week period. Based on an average mass of 300 g, each rat probably received ~1 mg of siRNA. siRNA has been employed locally to enhance bone formation, where delivery of siRNA alone was investigated to induce osteogenesis. Silk fibroin-chitosan scaffolds were loaded with siRNA against GNAS1, PDH2, or a combination of siRNAs against both targets and were implanted intramuscularly.[94] No specific carrier for siRNA was employed, and it was unclear how much siRNA was delivered. An in vitro model used mesenchymal stem cells seeded onto the scaffolds and the commercial reagent siPort Amine siRNA, and showed minimal changes in osteogenic gene expression. In agreement with in vitro data, minimal increases in bone formation (compared with scaffold alone) were observed in vivo after delivery of GNAS1 or PDH2 siRNA.

These studies, however, require better controls to confirm the efficacy of RNAi-mediated bone induction. Of foremost importance is the inclusion of scrambled (control) siRNA in studies. With systemic delivery of ~1 mg of Pleckho1 siRNA, off-target effects could be potentially exacerbated,[98] leading to immune responses, knockdown of nonspecific mRNA, and unpredictable cellular effects. The lack of other controls, such as siRNA delivery with unmodified (ie, non-bone-seeking) carriers, make it difficult to evaluate the specificity of both molecular action and tissue targeting. Scrambled siRNA was also omitted from local (electroporation) delivery studies,[90,94] which is known to cause dystrophic calcification.[55] It is important to note that local siRNA delivery still required the presence of a stimulant to induce bone formation, and delivery of Noggin siRNA alone would have led to minimal, if any, calcification at intramuscular sites.[90] Given that Noggin siRNA led to slight enhancement of BMP-2–induced bone formation in employed models (we estimate a "savings" of ~20% of implanted BMP dose), selection of targets more potent than Noggin will be needed to significantly reduce and/or obviate the recombinant protein.

Pharmacokinetics and Pharmacodynamics of Gene Delivery

Nonviral delivery for induction of osteogenesis remains inefficient based on amount of pDNA required to achieve functional bone

formation (ie, significant defect bridging achieved only with large (1 to 100 mg) quantities of pDNA).[47,49] Based on estimated defect volumes, the concentration of pDNA required for efficacy borders that of protein in clinical therapies (0.9 to 1.5 mg/mL). The need for more effective synthetic carriers calls for a better understanding of pharmacokinetic and pharmacodynamic issues affecting gene medicines. The pharmacodynamics of pDNA and siRNA differ from small molecule drugs because of the number of processing steps required for bioactivity, and the specificity of the intracellular compartment to which the nucleic acid must be delivered. In the context of pDNA, the production of a therapeutic protein is the desired end result, so that pDNA must be internalized by the cells, dissociated from carriers, and trafficked to nucleus for transcription. On the other hand, microRNA and siRNA must be internalized and dissociate from its carrier in cytoplasm to achieve the desired silencing. These considerations are different from systemic attempts at gene delivery[99] because transfection, protein secretion, and tissue induction are all occurring locally in the case of bone repair.

In situ pharmacokinetics of pDNA

As in protein therapeutics, a critical issue with gene medicines is the local residence time of delivered pDNA (we will restrict this discussion to pDNA because no information exists on miRNA and siRNA). One would expect in situ retention of pDNA to allow a high level of cellular uptake at the local site but release from scaffold to be equally important as well for free (unsequestered) availability of pDNA to invading and surrounding cells. In studies where the pDNA was administered freely in a collagen scaffold,[49] pDNA was shown to remain at bony defect sites for 6 weeks, with significant loss from 2 to 6 weeks (kinetics not quantitated). Although significant reduction in transgene expression was evident during the healing period,[54] whether this decrease was because of physical loss of pDNA, pDNA degradation, or transcriptional silencing is not known. Carrier-to-carrier differences in functional bone induction were evident, for example with the use of (PEG)-b-(Asp-[DET]) versus linear-PEI versus Fugene6,[66] but whether pDNA pharmacokinetics or cell uptake was the underlying reason for this difference is not known. The carriers, by converting long, stringlike pDNA into charged nanoparticles, can obviously affect local pDNA pharmacokinetics, and along with the appropriate scaffold features,[63] it may be possible to release the pDNA over a period of 3 to 4 weeks. However, not all such attempts resulted in robust bone formation. In one study, PEI25 was beneficial when pDNA was delivered in scaffolds in a cranial defect site.[59] Other studies with sustained release formulations[63] or a carrier functional in vitro (*N,N,N*-trimethyl chitosan[100]) did not result in improved bone formation in a similar intracranial site. It remains to be seen whether the scaffold effects (chemical composition, physical architecture controlling cell invasion, degradation products, etc.) contributed to these conflicting results.[59,63] Fundamental studies on pDNA pharmacokinetics are still needed to better reveal the underlying basis of these observations. Particular areas of desired investigations include 1) long-term pharmacokinetics of pDNA (ie, duration exceeding 1 month even if improved analytical techniques might be needed for accurate assessment of low levels of pDNA in situ); 2) relating pDNA pharmacokinetics to gene expression (ie, whether the presence of pDNA corresponds to transcriptionally active therapeutic genes); 3) pharmacokinetics of various carriers with special emphasis on comparing free versus bound (either to a synthetic carrier or scaffold itself) pDNA among carriers; and 4) effect of release rate of pDNA (or its complexes) on bone formation.

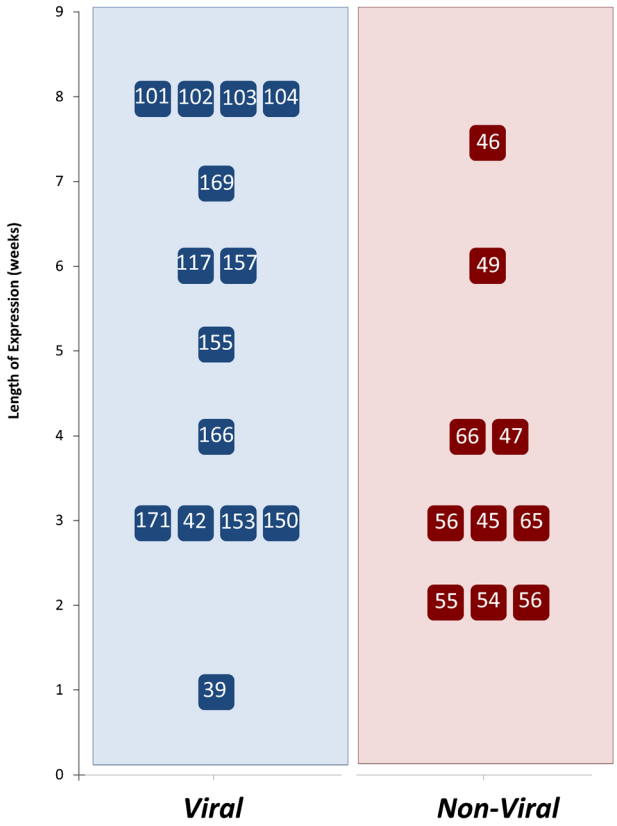


Fig. 5. Comparison of duration of gene expression in viral and nonviral studies. The numbers indicate the specific study. The length of expression was determined by how long the recombinant protein was detected. In some cases, however, the studies did not include sufficient time points to inspect the loss of expression and may therefore underestimate the expression length.

Duration of transgene expression

The durations of gene expression from viral and nonviral delivery studies are summarized in Fig. 5, which compares duration of detectable exogenous proteins in situ. Certain AAVs led to 8-week-long detectable proteins,[101–104] although other viruses showed a much shorter duration of protein expression. Nonviral carriers showed similar lengths of detectable protein levels on average, but fewer studies reported extended (>6 weeks) protein delivery. For small animal models, 3 to 4 weeks of gene expression observed with most studies are likely sufficient for bone formation, but a more sustained protein expression will be needed for clinical studies. Although the naked pDNA can also lead to protein expression for 2 to 4 weeks,[47,49] the mRNA and pDNA can be detected for much longer,[49] suggesting active repression of protein synthesis. Electroporation similarly led to detectable protein levels for 2 to 3 weeks,[45,54–56,] whereas sonoporation

gave detectable proteins for ~3 weeks.[56] It was interesting to note that physical methods of delivery and synthetic carriers have been traditionally pursued on their own, and it is likely that combining synthetic carriers with physical delivery will lead to enhanced (additive or synergistic) protein expression.

A more direct measure of delivery efficiency is the amount of recombinant protein produced because increased protein secretion is generally expected to correlate with the repair response (note that a nonlinear dose-effect relationship was noted for some nonviral systems).[105] Exogenous proteins can be detected in preclinical models through immunohistochemistry, but this provides at best a semiquantitative assessment of production. Quantitative assays such as ELISA might be more useful provided full recovery of proteins from the physiological milieu is attained. Using this approach, we observed a maximal BMP-2 secretion rate of ~0.3 ng/implant/day (based on ex vivo measurement of BMP-2 secretion from recovered implants) with lipophilic PEI.[62] An independent study reported a BMP-2 secretion rate of ~0.1 ng BMP-2/implant/day with ex vivo AV transduction of fat pads, which was sufficient for healing in a critical-size femur defect. Similarly, 0.25 ng BMP-2/clot/day was produced by chondrocyte clots transduced ex vivo with a retrovirus for repair of an osteochondral defect.[106] Higher secretion rates (1 to 5 ng/day for BMP-2[107] and BMP-7[108]) were reported with another AV system used for transfecting human bone marrow stromal cells for intraosseous implantation. It appears that BMP production rates range from 0.1 to 5 ng/day with current delivery systems, but the absolute level of protein secretions (~ng/day) are significantly lower than amounts used in equivalent preclinical studies (>1 mg/implant). Sustained protein production at repair sites could be more potent in inducing new tissues, and further studies to correlate protein secretion rates to obtained responses will better reveal the efficiency of a delivery system.

Stability of gene delivery systems

Unlike viral delivery systems with molecularly defined structure, nonviral systems are composed of free molecules (pDNA) or nucleic acid/carrier nanoparticles that are heterogeneous and range in size from ~100 nm (typical of polyplexes) to >1 mm (typical of liposomes). The stability of gene delivery systems is frequently reported in terms of their physicochemical features (eg, size), with several studies investigating methods to stabilize the formulations for pharmaceutical use. Complexes stored at body[109] and room temperature[110–112] both exhibit a time-dependent decline in transfection. Lowering the storage temperature[110–112] and lyophilisation[110,113] can minimize the loss of activity, but these measures are not useful to maintain transfection efficiency once implanted. In the case of systemic delivery, size stability is an important factor, as complexes must travel unobstructed through capillary beds, but such stringent size requirements are not necessary for local delivery, provided that the size does not impact cell uptake and transgene expression. Strategies employed to ensure a size-stable complex (eg, PEG addition) compromised in vivo transfection efficiency[114] or increased susceptibility to nucleases.[115] The authors recently investigated this issue (Fig. 6*A*, Rose and colleagues[116]), and found that the loss in transfection efficiency is immediate and large, with up to a 90% loss compared with freshly made complexes after a 24-hour exposure to body temperature. Some carriers, however, can better retain the activity for short duration

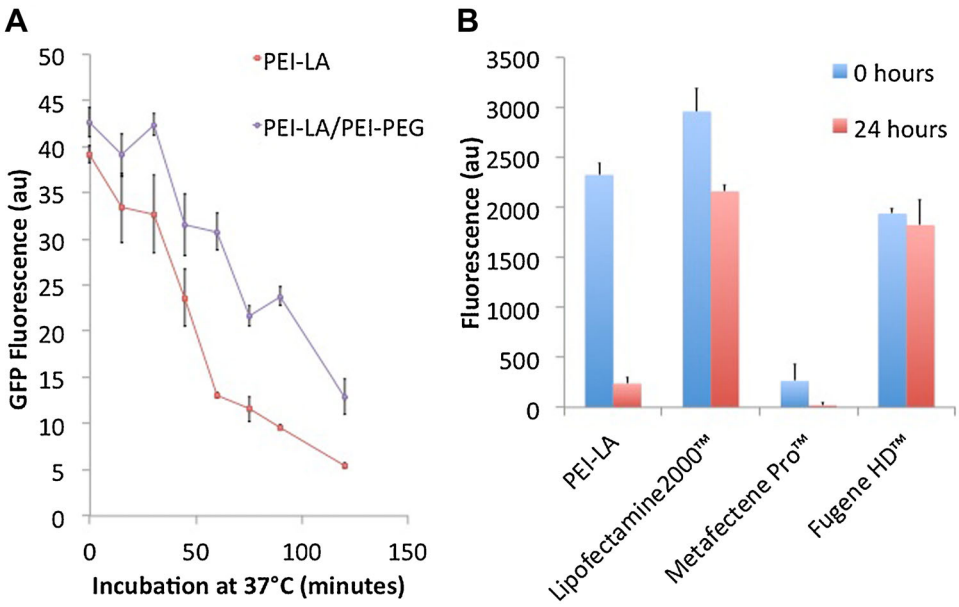


Fig. 6. Changes in transfection efficiency during incubations at body temperature. Complexes made of plasmid DNA and either 2-kDa polyethylenemine modified with linoleic acid (PEI-LA) or a mixture of PEI-LA and PEI modified with polyethyleneglycol (PEI-PEG) show a steady decrease in transfection efficiency when exposed to 37°C (*A*) before addition to 293T cell culture. Note that even PEI-LA/PEI-PEG, which maintain a constant size during this period, also show a decline similar to PEI-LA and PEI-LA/PEI complexes, both of which show a steady increase in size during this time (data not shown). Incubation of commercially available reagents led to minimal changes for Fugene HD, whereas PEI-LA, Lipofectamine2000, and Metafectene Pro all led to decreases in transfection efficiency (*B*).

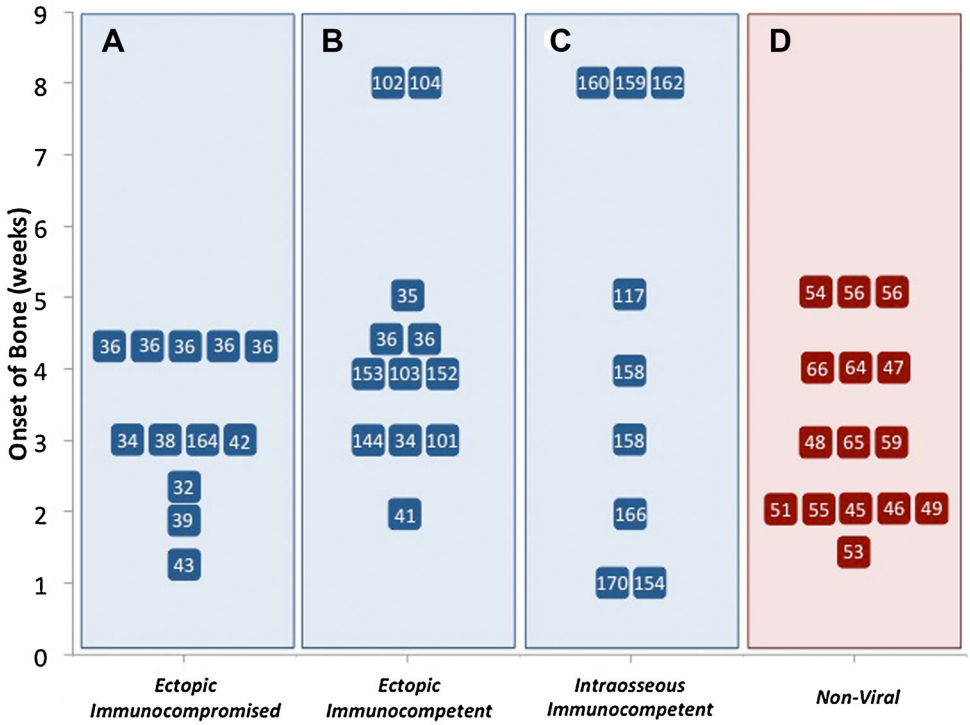


Fig. 7. Onset of bone formation with gene-based therapeutics. For studies involving viral delivery, the onset of bone formation is categorized into ectopic implantations in immunocompromised animals (*A*), ectopic implantations in immunocompetent animals (*B*), and intraosseous defects in immunocompetent animals (*C*). Studies involving nonviral delivery (*D*) are in immunocompetent animals. In some studies, a limited number of time points were investigated such that some points may overestimate the time for onset of bone formation.

(Fig. 6*B*). This reduction in transfection ability is ignored in development of gene delivery systems and must be tackled because delivered agents do not come in contact with target cells immediately and must remain "active" for weeks. Even the viral systems display a loss of activity at 4°C; for example, ~85% loss of activity was seen after 1 month with an AV system lyophilized into scaffolds, and no infectivity after 2 weeks in solution.[117] This loss of activity is expected to be accelerated at the physiological temperature.[118] Various methods to stabilize viral particles during freezing and lyophilisation were reported,[118,119] but these methods offer no solution to in vivo changes in infectivity.

Scaffolds in gene delivery

As in BMP therapies, a scaffold is going to be indispensable in gene-based bone repair and sustaining pDNA release. Bone induction with gene delivery displayed relatively fast (1 to 2 weeks) bone formation with both viral and nonviral delivery irrespective of the anatomical site (Fig. 7), so that a prolonged release system might not be necessary in these cases. Some studies indicated bone induction after 8 weeks, so that a more prolonged release might be needed in these cases. Clinical studies with humans are expected to mimic the latter scenario. Controlling the structural features of scaffolds[120] or their degradation rate[121] are obvious means to control the pDNA release. However, such systems remain to be tested for bone repair, and relationship between pDNA release rates and osteogenic potency remains to be established. Scaffolds have been used to deliver BMP-2–expressing AVs to regenerate bone around dental implants.[107] Scaffolds were capable of improving viral-mediated transfection in vitro compared with "free" virus in solution.[122] The release of AVs from the scaffold is expected to be relatively rapid, with a typical half-life of 24 hours,[122] but it was possible to control the release rate by controlling the surface features of scaffolds. Fibronectin, for example, was shown to slow the release of an AV from a PLGA scaffold, while not influencing the release of a lentivirus. A diverse range of scaffolds was compatible with bone regeneration induced with AVs, for example, collagen/chitosan composites[108,123] and silk fibroin scaffolds.[124] Scaffolds made from HA, however, did not show efficacious delivery of a BMP-7 AV in a subcutaneous implant model,[125] unlike the experience with such scaffolds in nonviral delivery. It is likely that the subcutaneous site was suboptimal for evaluating such a delivery system because others had shown the HA scaffolds to be compatible with AV delivery for BMP-2.[117] Scaffolds might have to be employed for viral delivery systems, and it is likely that scaffolds will require tailoring for particular delivery systems because a "universal" scaffold suitable for all delivery systems is unlikely.

Perspective

Delivery of nucleic acid–based therapeutic agents for bone regeneration is an attractive option, but many obstacles remain for a successful clinical therapy. Although bone induction has been observed with small amounts of pDNA,[66] clinically significant results such as bridging of critical-size defects remain challenging without employing large amounts of pDNA. Developing more effective gene delivery strategies will aid in

this goal, but this requires a better understanding of not only the pharmacokinetics of delivery formulations but also the pharmacodynamics of transgene expression both in the form of protein production and resultant tissue response. Because the success of a delivery system ultimately depends on the amount of protein produced at the local site, further examination and quantitation of recombinant protein expression is needed. Many studies use indirect measures to gauge transfection efficiencies, such as mRNA expression or biological changes known to be associated with the transgene. Biological changes, such as extent of angiogenesis or newly induced bone, are highly dependent on the surrounding environment and can significantly misjudge protein expression depending on the preclinical model.

The microenvironment at the repair site is obviously different from a prototypical cell culture environment; there is no guarantee that the effective carriers optimized in vitro will be most effective for in vivo use. Comparison of in vivo gene delivery efficiencies of different carriers is crucial for continued advancement of gene-based therapies. Unlike transfections in monolayer culture, transfection in three-dimensional scaffolds may better mimic the events at repair sites. However, clearance of delivery systems in vivo is difficult to reproduce in any culture system and may be the primary reason for failure of gene delivery in vivo. The risks associated with virus-based gene delivery systems have been recognized and resulted in many exclusion criteria in clinics for patients' own safety. For example, because AV can be associated with liver toxicity, patients with impaired liver function are not well suited for viral therapy. Immunodeficient patients or patients with viral infections such as hepatitis possess similar grounds for exclusion. The nonviral pDNA-based delivery is not as limiting, but transfection efficiency of naked pDNA is likely going to be very low and the amount of administered pDNA accordingly high. Development of efficient and safe synthetic carriers will obviate the shortcomings of viral systems and naked pDNA delivery.

The miRNA- and siRNA-based approaches have untapped potential at this stage but require a more thorough understanding of effective molecular pathways involved in healing to identify the most appropriate and effective targets. They face similar delivery challenges with respect to development of carriers that are effective in vivo. With siRNA, effective carriers have been intensely explored in the cancer therapeutics field.[126] It is likely that some of the effective carriers used for anticancer therapy will be effective in delivering siRNA to stimulate bone repair. Delivering miRNAs or oligonucleotides capable of modulating intracellular microRNAs is at infancy, with no animal studies reported to date. Carriers employed in pDNA delivery may be employed for RNAi molecules as a starting point because our experience indicates that carriers effective for pDNA are also effective in siRNA delivery. Whether that holds true for miRNA or oligonucleotides is unknown at this stage; differences in the way oligonucleotides interact with synthetic carriers are known,[127,128] which suggest that optimal delivery formulations might need to be tailored for each class of nucleic acids. Nonspecific physical methods (electroporation and sonoporation) might provide more effective delivery initially in the absence of experience with synthetic carriers.

Finally, identifying the most potent gene or gene combinations is paramount for clinical translation. Although BMP-2 and BMP-7 may represent the initial choice for gene delivery, more potent proteins were recently identified that may allow for a greater response with lower amounts.[129] Because of the crucial role of vascularization in new bone formation, angiogenic proteins can enhance bone formation.[130] In particular, VEGF[131] and bFGF[132,133] have been shown to be beneficial on their own for osteogenesis and in combinations with BMPs.[134,135] Angiogenic genes have been delivered in clinical studies but not in the context of bone regeneration. VEGF-expressing AVs have been delivered for treatment of coronary heart diseases[136] and end-stage renal disease,[137] whereas pDNA-expressing VEGF have been injected for treatment of diabetic neuropathy[138] and critical limb ischemia.[139] pDNA-expressingVEGF and bFGF has similarly been used for no-option coronary disease.[140] The effects of osteogenic-angiogenic combinations, however, might be closely linked to the delivery mode; whereas BMP-2 gene delivery benefited from VEGF protein co-delivery, delivering VEGF gene along with BMP-2 gene did not offer an obvious benefit in a canine dental implant model.[107] Slower expression of VEGF (compared with readily available protein) presumably did not provide a robust angiogenic activity in that study. A combination of AV-based BMP-7 and PDGF-B (as a mitogen) expression systems, on the other hand, provided a synergistic activity in the same animal model, enhancing bone deposition around dental implants,[108] clearly indicating the importance of gene combinations to be chosen. Other proteins associated with canonical osteogenic pathways, such as BMP-6,[141] BMP-9,[142] and Wnt,[143] have been investigated for regenerative therapy, but their gene delivery remains to be explored in detail. Four and half lim protein-2 (FHL2) and Lim mineralization proteins 1 and 3 (LMP 1 and LMP3) are recently investigated proteins that were shown to induce bone regeneration.[144,145] LMP-1 and LMP-3 can also act synergistically with BMPs.[146,147] NEL-like molecule-1 (Nell-1) is another molecule under investigation for its osteogenic properties in repair of large segmental defects.[148–150] Nell-1 was shown to be as effective as BMP-2 in repair of critical-size defect[151] and also works synergistically with BMPs[129] that are endogenously expressed at the defect site. These proteins and, their combinations, are expected to provide further leads in aiding or even replacing BMPs in functional bone regeneration.

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Authors' roles: Data acquisition and analysis: LR. Data interpretation: LR and HU. Drafting manuscript: LR. Revising manuscript: HU. Approving final version of manuscript: LR and HU.

Disclosures

Both authors state that they have no conflicts of interest.

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