REVIEW

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Curative Cell and Gene Therapy for Osteogenesis Imperfecta

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ABSTRACT

Osteogenesis imperfecta (OI) describes a series of genetic bone fragility disorders that can have a substantive impact on patient quality of life. The multidisciplinary approach to management of children and adults with OI primarily involves the administration of antiresorptive medication, allied health (physiotherapy and occupational therapy), and orthopedic surgery. However, advances in gene editing technology and gene therapy vectors bring with them the promise of gene-targeted interventions to provide an enduring or perhaps permanent cure for OI. This review describes emergent technologies for cell- and gene-targeted therapies, major hurdles to their implementation, and the prospects of their future success with a focus on bone disorders. © 2022 The Authors. *Journal of Bone and Mineral Research* published by Wiley Periodicals LLC on behalf of American Society for Bone and Mineral Research (ASBMR).

KEY WORDS: GENE THERAPY; CELL THERAPY; OSTEOGENESIS IMPERFECTA; COLLAGEN

Introduction

Osteogenesis imperfecta (OI) describes a series of genetic skeletal dysplasias that are heterogeneous and phenotypically diverse.[1] The primary clinical manifestations are recurrent low-trauma fractures and low bone mass. Individuals with OI can also have variable short stature, limb deformity, scoliosis, and extraskeletal manifestations, including blue sclera, sensory hearing loss, and dentinogenesis imperfecta.[2]

OI is clinically categorized using the Sillence scale, which originally delineated four types of OI based on their clinical presentation.[3] These "classical" OI types are associated with autosomal mutations in the genes encoding type I collagen (*COL1A1*, *COL1A2*) and still account for 85% to 90% of OI cases.[4] These mutations lead to structural or qualitative defects in the collagen protein, with reductions in collagen quantity (haploinsufficiency) typically producing milder forms of OI compared with structural mutants.[1,2] This classification system has expanded to include recessive, dominant, and X-linked mutations identified in a range of genes. Non-classical OI phenotypes produced by non-collagen mutations can have characteristic pathological features, such as the hyperplastic callus formation found in type V OI.[5]

The current management of OI is focused on reducing fracture incidence, optimizing motor function, increasing inclusion, and enhancing quality of life. This involves a multidisciplinary approach from a range of health care practitioners, including medical physicians, geneticists, orthopedic surgeons, physiotherapists, and other allied health professionals.[6] Numerous studies have also demonstrated the benefits of antiresorptive drugs to increase bone mass and decrease overall fracture risk, particularly in younger populations.[7-9] Notably, drugs such as bisphosphonates do not improve the material properties of bone (i.e., enhance bone quality), but rather induce increases in bone volume that lead to relative increases in strength.

To achieve improvements in bone quality associated with defects in the organic matrix, there is emerging potential for cell or gene therapy approaches to transform current treatment approaches. This review summarizes key recent advances in cell and gene therapy,

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how these innovations have been applied to genetic bone disease, and key hurdles to their future clinical implementation.

Identifying Gene Variants Associated With Bone Fragility

Identification and validation of gene mutations as causative for monogenic bone disorders is a key step before any targeted gene therapy can be initiated. This is particularly important in OI because although *COL1A1* and *COL1A2* mutations constitute a great proportion of the cases, there are more than 20 other genes associated with the disease. These genes can be grouped together based on their roles in collagen production, including collagen processing and cross-linking,[10-13] collagen modification,[14,15] bone mineralization,[16-18] and overall osteoblast differentiation and function[19-23] (Fig. 1). The functions of some genes associated with bone fragility, such as *TENT5A*, remain unclear.[24]

Advances in exome and genome sequencing technologies have made the process of identifying the causative mutations more accessible and affordable, whereby OI classification relies on both clinical criteria and genetic information. This trend was initiated in 2000 when Glorieux and colleagues proposed a subset of OI patients should be separately categorized based on a constellation of unique clinical and radiological features (including hyperplastic callus formations and ossification of the interosseous membrane of the forearm).[25] In 2012, whole-exome sequencing efforts revealed all these cases were caused by a single point mutation (c.-14C > T) in the 5′ untranslated region of *IFITM5*.[17,26] Although a range of additional genes not encoding for collagen are now included as causative for OI, other genes such as *LRP5* can impact on bone mass without being classified as causative for OI.[27] LRP5 is a receptor critical in the Wnt/β-catenin signaling pathway. Homozygous loss-of-function mutations are associated with osteoporosis-pseudoglioma[28,29] and heterozygous gain-of-function mutations are associated with high bone mass phenotypes.[30,31] It is possible that sequence variants in *LRP5* may underlie some cases of recurrent pediatric fracture that are mild and do not fit the criteria for an OI diagnosis.

The opportunity to achieve a genetic diagnosis is often governed by regional medical practices and resourcing, and is changing over time with increased accessibility of sequencing technology. Even recently, targeted sequencing approaches are often preferred over other sequencing efforts such as whole-genome or whole-exome sequencing because of speed and affordability.[32] Although targeted panels enable rapid screening of genes associated with OI, they are not able to detect variants in novel genes not previously associated with bone disease. Even if a variant is identified in an OI associated gene, the are not necessarily causative, whereby variant can be classified on a spectrum based on their likelihood for being benign or pathogenic.[33]

Although in silico analysis can predict some mutations as being pathogenic, functional genomics assays are emerging as methods to test variants of unknown significance (VUS). Classification of these variants will be critical to validate before any therapeutic intervention. Proof-of-principle for such functional testing has been described using CRISPR/Cas9 gene editing to engineer *COL1A1* OI patient mutations into induced pluripotent stem cells.[34,35] While these cell lines represent valuable resources to study the mechanisms of specific mutations, such approaches remain expensive and poorly amenable for diagnostic testing of individual novel VUS.

A Framework to Define Interventions for Genetic Disorders

The various gene-targeted approaches with the potential to treat or cure genetic disease can be separated based on whether they

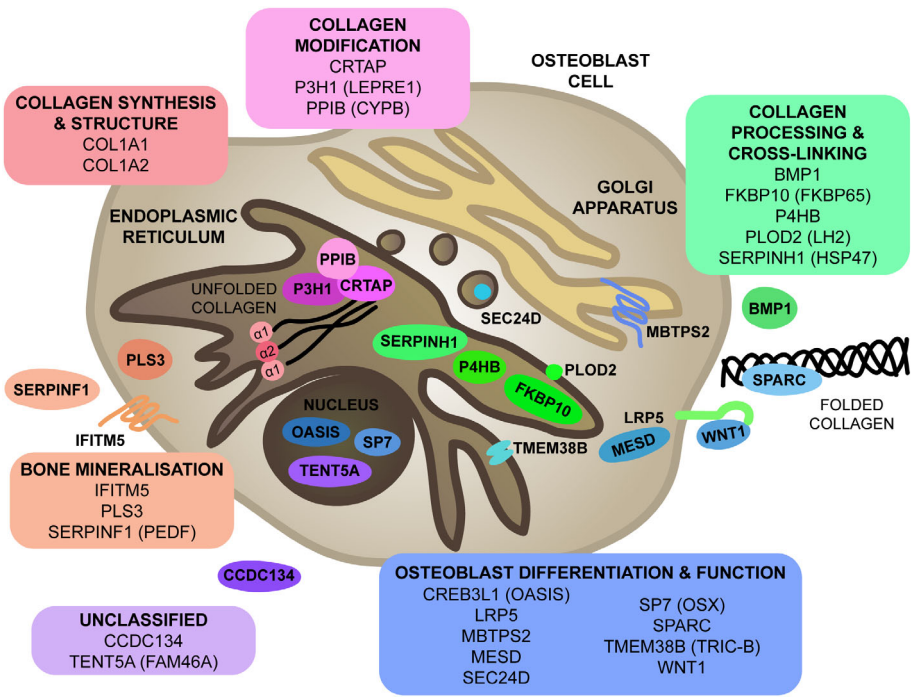

Fig. 1. Osteogenesis imperfecta–associated genes, their cellular location and mechanism of action within osteoblasts.

Table 1. Classifying Approaches for Treatment of Genetic Disorders

Approach	Methods	Utility and limitations
Suppression of harmful transcripts	• Post-transcriptional gene suppression (oligonucleotide therapy) • Promotion of alternative splicing (pharmacomodulation of splicing)	• Oligo therapy more analogous to pharmacotherapy than gene therapy; challenges with delivery to bone • Restricted to a small number of genes and mutations
Cell transplantation	• Transplant of allogenic cells to restore function to relevant tissues/organs • Transplant of gene-repaired cells, particularly stem cells	• Best use in tissues where high engraftment is possible or lower engraftment is therapeutically beneficial • Requires expansion and editing of cells before transplant; same limitations as above
Gene addition	• Drive expression of deficient or absent alleles in key cell types	• Can be impeded by large gene size as well as delivery to tissues requiring high efficiency and specificity
Gene repair	• Gene editing to • disrupt pathogenic alleles • repair or replace dominant negative or haploinsufficient alleles	• The efficiency of gene editing is still limited; concerns regarding off-target effects

modulate expression of harmful alleles, utilize cell transplantation, or employ vectors for gene addition or repair (Table 1). Although none these strategies have been optimized to reliably treat OI in a clinical setting, they have proven effective in treating other genetic disorders and/or show promise based on preclinical models of OI.

Oligonucleotide Therapies

Oligonucleotides are short polynucleic acid chains, which have been used in several therapeutic ways. Antisense oligos (ASOs; i.e., short reverse complementary single-stranded oligodeoxynucleotides) can lead to targeted mRNA degradation, typically via an RNase H-dependent mechanism. Alternatively, pretranscriptional gene silencing can be enabled by delivery or expression of short interfering RNA (siRNA) or microRNA (miRNA) molecules via RNA interference (RNAi).[36] Other oligonucleotide-based approaches have emerged, such as aptamers (which can bind non-gene targets, such as proteins) and CpG oligonucleotides (that target immune cells); however, these cannot be considered gene therapy interventions. Although there may be potential for aptamers as bone therapeutics,[37] it is important to note they are not acting at a genetic level.

ASOs have been used to suppress mutant *COL1A2* expression in cultured cells from a patient with dominant negative OI.[38] In this study, the mRNA of the mutant collagen allele was suppressed but at a level gauged to be insufficient for clinical translation. Systemically delivered ASOs showed more success in the gain-of-function *Notch2*$^{tm1.1Ecan}$ mice, where they ameliorated osteopenia.[39] This mouse model of Hajdu-Cheney syndrome showed significant increases in bone; however, in contrast to OI, this was associated with a suppression of osteoclast number/activity rather than osteoblast-driven bone quality.

RNA interference methods have the potential for allele-specific silencing of dominant negative OI alleles causative of severe disease.[40] In a proof-of-concept study, siRNAs were used to target small insertions and deletions (indels) in *COL1A1* and *COL1A2* in primary human bone-derived cells.[41] The group reported statistically significant but variable changes in targeted alleles, although additional proof for in vivo deliverability and efficacy would be required. Similar levels of mutant allele expression reduction were reported in a siRNA approach using fibroblasts isolated from the *Brtl* OI mouse.[42] In both instances, no in vivo targeting of bone was attempted, which remains a notable limitation.

The efficient and/or targeted delivery to bone of oligonucleotides is an area of limited study. A recent study used PEGylated lipid-PLGA nanoparticles with variable surface charge to incorporate ASOs for treatment of osteoporosis.[43] Although not focused on oligo delivery, another recent report describes the creation of bone-targeted nanomicelles for the delivery of small molecule compounds that may also be applicable for ASOs.[44] However, neither technology has been validated in vivo or in an animal model of disease; although nanoparticle delivery to bone has significant potential, it requires considerable development and validation. Thus oligonucleotide therapy in bone may have greater applications where delivery need only be localized, such as with bone healing or orthopedic injury,[45] or for intra-articular injection for osteoarthritis.[46]

Morpholinos are nonionic oligodeoxynucleotide analogs that can bind in an antisense manner to block translation, splicing, or even miRNAs and their targets. Custom morpholinos have emerged as formidable drugs for treating specific genetic disorders where exon skipping is a viable option. Duchenne muscular dystrophy patients amenable to exon 53 skipping show increased dystrophin production with golodirsen (a phosphorodiamidate morpholino oligomer) in clinical trials[47] and is approved for clinical use. Eteplirsen is another morpholino approved for exon 51 skipping in Duchenne's.[48] While exon-skipping can be highly effective, it is important to note these are treatments and not cures and only applicable for a limited number of genes and specific mutations.

Cell Transplantation for OI

Curing osteogenesis imperfecta by replacing inferior bone progenitors with healthy donor cells has been a topic of significant research and clinical interest. One of the earliest, high-profile attempts was published by Horwitz and colleagues in *Nature Medicine* in 1999, where three children with *COL1A1* or *COL1A2* mutations received donor marrow from siblings. Although high levels of hemopoietic donor cell engraftment was achieved in 2/3 patients, osteoblastic engraftment remained low (1.5% to 2%) and any functional improvements were not lasting.[49] Subsequent research in murine models of OI and in patients

reinforced the challenges of low engraftment rates and limited phenotypic improvement.[50,51]

To overcome the limits of whole bone marrow, substantive focus has been placed on identifying mesenchymal cell (MSC) populations and subpopulations with high osteogenic potential.[52] Although this has led to several small trials, none to date have been performed on substantive patient numbers. MSC transplantation was reported in treating a type III OI case; although engraftment and bone markers were low, the authors ventured a reduction in clinical fracture rate.[53] Early in utero intervention has been suggested as a strategy for improving engraftment levels. When this was attempted using fetal MSCs, engraftment was found to reach 7.4%—higher than that found in most postnatal treatment trials—though in a single patient.[54] The Spanish TERCELOI phase I clinical trial administered multiple MSC infusions to two non-immunosuppressed OI patients. The intervention was well tolerated, and patients reported functional and quality-of-life improvements.[55] Although engraftment was not measured, the coordinators purported an engraftment-independent mechanism, suggesting that osteogenic factors including miRNAs could be induced by cell transplant. The potential for MSCs continues to be explored, with the BOOSTB4 trial using off-the-shelf MSCs for OI scheduled to complete in 2022.[56,57] Still, the concept of using MSCs as opposed to other cell types remains controversial. Horowitz's group published preclinical evidence and a case series of 5 patients that indicated non-adherent bone marrow cells were functionally superior to MSCs and showed greater engraftment.[58]

One of the ongoing challenges associated with clinical trials is the accurate measurement of donor cells, which often relies on detecting XY cells transplanted into XX patients. Not only can engraftment results vary depending on the chromosomal probe (even within a single patient[6]), but such assays are limited in their capacity to characterize the engrafted cells or their distribution within the bone. This is where preclinical studies may be particularly informative, as genetically distinct and/or fluorescent cells can be used for transplant. Such approaches have been used in two notable recent studies. The first tested whether whole body irradiation could improve engraftment rate by depleting the deficient osteoprogenitor niche. Irradiation was found to increase hematopoietic but not osteoprogenitor engraftment, and no functional rescue was found in OI mice.[59] The second study featured local injection into the femur and utilized donor osteoblast-specific reporter cells. The authors here reported a functional improvement and persistent engraftment, although such an approach may be harder to translate.[60]

Thus, while the data from preclinical and clinical studies have been mixed and inconclusive, for many, the approach still holds promise.[61] Allogenic MSC transplantation could be applied to treat other rare bone conditions, such as infants born with severe genetic hypophosphatasia.[62] Thus, breakthroughs in OI cell therapy may influence the progress of other bone diseases and vice versa. There is also the nexus to consider between gene therapy and cell therapy, as some groups are focused on ex vivo culture of autogenic cells that undergo genetic repair, expansion, and reimplantation. Repair has already been shown using OI iPSCs generated from patient monocytes.[63] Although this is an important achievement, the transplantation of gene-corrected autologous cells will face the same hurdles of delivery and engraftment as allogenic bone marrow.

Gene Therapy for OI

Conceptually, gene therapy involves the suppression of unfavorable or harmful transcripts, increased expression of healthy alleles, or repair of genetic abnormalities either transiently or permanently. The past decade has seen an exponential rise in the number and range of gene therapies reaching clinical trials or being approved for therapeutic use. While most clinically established and effective treatments involve gene addition, the advent of CRISPR/Cas9 gene editing[64] has opened myriad possibilities for repairing mutated alleles.

Currently (as of February 2022) there are no active/recruiting gene therapy trials for OI registered on clinicaltrials.gov with the majority of current trials focused on bisphosphonates or other antiresorptives. Notably, there are trials for stem cell transplantation (NCT04623606, NCT03706482), anti-sclerostin antibody (NCT04545554), as well as registries, natural history studies, or orthopedic or dental interventions. Nevertheless, the rapid advancement of the field suggests that gene therapy treatments for genetic bone fragility disorders could be practical with several key technological advancements.[65]

Gene therapy delivery

Three viral vectors have predominantly been investigated for their use in gene therapy delivery: adenovirus, lentivirus, and adeno-associated virus.[66] Each vector has its own characteristics and limitations that need to be considered when deciding the optimal approach to a specific gene therapy application. In the context of bone, these vectors have chiefly been trialed in bone regeneration or in the treatment of bone conditions that do not require specific targeting of the bone cells or bone surface. Examples will be presented illustrating the use of these vectors to target bone cells either locally or systemically in preclinical models.

Lentiviruses (LV) belong to the retrovirus family but can infect both dividing and non-dividing cells.[67] The advantages of lentiviral vectors are they do not elicit a cellular immune response at the injection site or produce a significant antibody response after administration, owing to the loss of virulence genes.[68] Lentiviral vectors integrate their transgenes into the host genome upon transduction, which was initially seen as highly desirable as it could produce stable, long-term expression of the transgene.[63] However, the risks of random integration and subsequent activation of oncogenes or conversely inactivation of tumor suppressor genes, both potentially leading to tumorigenesis, remain high.[69] It has been shown there are genomic hotspots where LV integration occurs more frequently, which can allow for screening for these events if using LV for ex vivo gene therapy approaches.[70]

A LV vector has been used systemically to treat a mouse model of hypophosphatasia, resulting in prolonged survival and improvements in bone structure after neonatal dosing.[71] However, it was noted that these improvements in the skeleton were due to the increased expression of alkaline phosphatase in the liver, and very little of the bone tissue, including bone marrow cells, was targeted by the LV vector and expressed the native enzyme.

Adenoviruses (AdV) are DNA viruses capable of infecting both dividing and non-dividing cells but replicate episomally rather than integrating into the host genome. High titers of AdV are easily obtainable and high-efficiency gene transfer both in vitro and in vivo is readily achieved in a range of cells and tissues.[72] Adenoviruses are strongly immunogenic, with types 2 and 5 causing respiratory infection in humans. Serious adverse events have occurred from clinical trials using adenovirus for gene therapy purposes, which has led to a reduction in their

use for this purpose globally.[73] There are also no drugs currently in use for treatment of natural adenoviral infections, and hence the risk of disseminated infections occurring through use of AdV vectors in gene therapy remains a concern.

AdV has long been used for ex vivo gene therapy of bone cell precursors before transplantation. Most experiments have used AdV to express bone morphogenetic proteins (BMPs) ex vivo to enhance the osteogenic potential of cells and aid in regenerating bone defects when transplanted in vivo[74-76] or directly injected in vivo to induce ectopic bone formation.[77,78] However, to our knowledge no AdV vectors have been used to specifically target bone surfaces and bone cells for gene therapy purposes.

Adeno-associated virus (AAV) is a member of the Parvoviridae family, a group of small, non-enveloped animal viruses with icosahedral symmetry that infect humans, some other species of primates, other mammals, and birds.[79] AAVs were first isolated as a contaminant in preparations of simian AdV, and it was discovered the AAV particles could only be obtained in the presence of AdV.[80] Therefore, AAVs cannot replicate effectively within a host cell without the presence of a "helper virus," which is usually an adenovirus[81] but can also be a herpesvirus or vaccinia virus. Current AAV vectors used preclinically and clinically do not have the capacity for site-specific integration and latency that wild-type AAVs possess. This is because engineered vectors do not contain the viral genes required for replication. AAVs are the smallest viral vectors, with each capsid approximately 20 to 25 nm in diameter, giving them a limited package capacity.[82] Further, as the AAV capsid is non-enveloped, it is directly involved in virus-host cell interactions, and consequently confers tissue- and cell-specific entry to these vectors.

AAV vectors have since been used to transduce a range of cell types and organs in both animal models and human trials, with the most common targets being the liver, eye, heart, lungs, and skeletal muscle. Many serotypes of AAV have been isolated and used for gene therapy purposes; however, now there are methods of engineering variants and modifying capsid proteins to alter transduction efficiency for a given target tissue or cell type. There are two processes by which the targeting of an AAV can be altered: pseudotyping and capsid engineering. Pseudotyping involves producing a virus that has an AAV2 genome packaged in a viral capsid that is not native AAV2. For AAVs, it was first used to generate recombinant AAV2 (rAAV2) vectors, which were not targeted by neutralizing antibodies in vivo, and vectors that had altered affinities for heparin.[83] Capsid engineering is a different approach that involves generating hybrid capsids with novel targeting capabilities. The process can involve using a library of capsid sequences to produce hybrid capsids that are placed under selective pressure to select for capsids able to overcome the desired stimulus, e.g., evade the preexisting immune response.[84] Alternatively, capsid sequences can be intentionally manipulated as the regions within the AAV capsid that are involved in cell attachment are being increasingly identified.[85] Together, these two processes have been used to develop a range of AAV capsids with specificity for target tissues.

AAVs have also been used widely to deliver bone formation genes in ex vivo gene therapy applications for bone regeneration. However, AAVs can also be applied directly in vivo to target bone cells, both locally and systemically.[86] The serotype AAV-DJ was used locally to deliver the COX2 transgene to aid fracture healing in a femoral fracture mouse model.[87] Two different studies have also used AAV9 to target osteoclasts[88] and osteoblasts[89] in vivo, both showing proof-of-concept for the treatment of osteoporosis.

Nonviral methods of delivery

To avoid the issues associated with viral vector gene therapy, nonviral methods have also been investigated as a potential alternative. Nanoparticles are a popular choice because they are small enough that they are nonimmunogenic, do not show acute toxicity, and can accommodate larger DNA payloads.[90] Again, in the context of bone, nanoparticles have mainly been investigated for their ability to deliver DNA, particularly BMP-2 transgenes, to assist in bone regeneration.[91-93]

However, direct delivery and targeting the bone surfaces and cells have also been investigated. In a rat model of rheumatoid arthritis, folate-chitosan-DNA nanoparticles were used to deliver IL-1Ra systemically and were able to induce protection from inflammation and abnormal bone resorption.[85] Another study used encapsulation to produce polyurethane nanomicelles capable of selectively delivering miRNAs to osteoclasts on the bone surface in osteoporotic rats.[94] Exosomes are also being investigated for their ability to deliver gene therapy to bone. A recent example used expression of CXCR4 on exosome-liposome hybrid nanoparticles to specifically target bone cells within the bone marrow and promote osteogenesis.[95] Therefore, nanoparticle technology could prove to be a suitable carrier for gene therapy for targeting bone and overcoming the issues associated with viral approaches.

Gene addition therapy

Gene addition therapy involves driving the corrective expression of deficient genes in affected cells. For some conditions, such as spinal muscular atrophy, gene addition therapy has proven transformative.[96] However, it has a range of caveats—conditions that must be met for it to be most effective. First, gene addition cannot correct a dominant negative mutation—which many heterozygous mutations causing severe OI are. In instances where a truncated collagen chain affects triple helix assembly and formation of collagen fibrils,[97] production of additional normal collagen will not rescue the phenotype. Second, different conditions require different proportions of cells to be affected and/or additional gene expression to be effective. For example, near or complete correction has been achieved for hemophilia using hepatic in vivo gene transfer to express factor VIII (hemophilia A) or factor IX (hemophilia B).[98] In contrast, cell targeting and reaching enough collagen production throughout the skeleton to improve haploinsufficient OI may be extremely challenging. Third, in some cases there may be risks associated with gene overexpression causing deleterious results; although this is unlikely for collagen mutations, it may be problematic for other genetic causes of OI. Genes affecting WNT signaling (e.g., *WNT1, LRP5*) are examples where uncontrolled expression could be ill advised. Lastly, gene size can be a constraint, particularly for AAV vectors with a limited packaging capacity. For collagen, this is likely to be limiting, unless minigene constructs could be generated as they have been in the case of dystrophin.[99] However, for non-collagen mutations causative for OI, gene addition may be less constrained.

Nevertheless, several groups have performed preclinical studies to test the concept of gene addition. Niyibizi and colleagues described the transfer of *Proα2(I)* cDNA by AdV vector into cultured cells from collagen-deficient *oim* mice.[100] While the data supported the idea of procollagen expression for collagen null mutations, subsequent planned studies for targeted retroviral vector delivery never eventuated. In a more recent study, lysyl

Table 2. Requirements for In Vivo AAV-Based CRISPR/Cas9 Gene Editing for Osteogenesis Imperfecta (OI)

Requirement	Achievements	Hurdles/limitations
Streamlined methods for functional assessment of OI VUS	• Proof-of-principle for in vitro assessment of OI mutations[34,35]	• Affordable and broadly accessible mutation testing for OI patients
Targeting of AAV vectors to the skeleton	• Efficient and specific targeting of the skeleton achieved in mice with an AAV8-Sp7 vector[81]	• AAV skeletal targeting efficacy not yet shown in a human • AAV tropisms can differ between humans and mice
Capacity to package Cas enzymes into AAV vectors (*Sp*Cas9 too large)	• *Sa*Cas9 enzyme variants[102,103] • Split intein assembling *Sp*Cas9[104]	• *Sa*Cas9 has a less common PAM site (more limited utility) • Dual vector systems are fundamentally less efficient
Efficient disruption of dominant negative OI alleles using CRISPR/Cas9 editing	• NHEJ[a] is the most efficient form of CRISPR gene editing and has strong emerging clinical trial data outside of OI[105] • Cas9n approaches[102] may allow for more specific allele targeted disruption	• Small indels created by NHEJ may be insufficient to overcome a DN phenotype • Approach highly limited in terms of which OI mutations NHEJ can treat
Efficient repair of single-base or small insertion/deletion mutations using CRISPR/Cas9 editing	• Base editing[106] and prime editing[107] Cas9 variants show higher efficiency than HDR	• Efficiency levels may still be insufficient, particularly for prime editing • Base editing currently limited to C→T and A→G single base substitutions
Efficient gene repair of large indels causing OI by HDR	• Homology-independent targeted integration (HITI) emerging as a new method for exon replacement[108] • Potential for Cas-protein engineering and small molecule additives to improve efficiency rates	• HDR remains a poorly efficient process and has a requirement for cell cycle
Risk of off-target effects in other genes and/or in other cell types	• Development of tools to minimize off-target sequences during design[109] • Cas9n approaches can reduce off-target effects[101]	• Even low-efficiency off-target effects could produce significant challenges if oncogenic.
Persistence of gene editing enabling long-term improvements to bone health	• Well-characterized human bone stem cell markers will facilitate analysis of progenitor targeting[110]	• Concerns that "curative" gene therapies could be transient if progenitors are not targeted
Immune resistance to first or subsequent gene therapy attempts (Cas9 or AAV)	• Broad efforts being undertaken to overcome this via de-targeting immune cells, capsid modification, and immunosuppression	• This remains a fundamental limitation to all AAV-CRISPR gene therapy

AAV = adeno-associated virus; VUS = variant of unknown significance; DN = dominant negative; HDR = homology directed recombination.
[a]This study involving treatment of transthyretin amyloidosis utilizes lipid nanoparticle delivery of Cas9/sgRNA guides rather than an AAV vector.

hydroxylase activity was supplied in a cell model of type IV Ehlers-Danlos Syndrome, a disease caused by deficient lysine hydroxylation of collagen.[100] Restoration of ~20% of enzyme activity with a Ad5RSV-LH vector yielded some positive effects in cell assays, but it was noted that curative gene therapy for the condition would ideally need to be delivered during fetal development.

Gene editing with AAV-based CRISPR/Cas9 vectors

CRISPR/Cas9 gene editing has been one of the most transformative genetic technologies discovered in the past several decades, enabling the straightforward and versatile editing of sequences in mammalian cells.[101] This fundamental approach involves supplying the RNA-guided Cas nuclease (usually *Sp*Cas9 or *Sa*Cas9) along with a sgRNA guide that allows for specific targeting. In the absence of a template, gene disruption occurs at the target site by non-homologous end joining (NHEJ). Provision of a single-stranded oligonucleotide (ssODN) template can drive homology directed recombination (HDR), and although this can enable perfect repair at a given locus, the poor efficiency of this process remains a major barrier to in vivo gene therapy.[97]

Although there is substantive scope for gene therapy to provide a curative solution for osteogenesis imperfecta and other genetic disorders affecting the skeleton, there remains numerous technical hurdles that need to be overcome. These are summarized in Table 2, which features not only the exigent challenges but also notable achievements that may lead to the current hurdles being overcome.

The field of Cas enzyme bioengineering is likely to have the greatest impact on the feasibility and practicality of gene therapy generally (not just in bone). Base-editing using modified Cas9 enzymes[102] has significant potential for making single A→G or C→T base changes. Although this technology has not been applied to osteoblasts, it has been successfully used to target hematopoietic stem cells in the bone marrow to rescue sickle cell disease in mice.[111] Base-editing still requires delivery with dual-AAV vectors; however, a recent study highlighted a new base-editing enzyme featuring a cleavable deoxycytidine deaminase inhibitor domain that reduces off-target mutations.[112]

Table 3. Murine Models of Osteogenesis Imperfecta (OI)

Mouse strain	Models OI type	Affected gene	Lethal phenotype	Reference no.
Oim/oim	OI III	*Col1a2*	—	(113)
Oim$^{/+}$	OI I	*Col1a2*	—	(113)
G610C/G610C^{Neo+}	OI IV	*Col1a2*	—	(115)
+/G610C^{Neo+}	OI I/IV	*Col1a2*	—	(115)
+/G610C^{Neo-} (Amish)	OI IV	*Col1a2*	—	(115)
Mov-13$^{-/-}$	OI II	*Col1a1*	Embryonic lethality	(116)
Mov-13$^{/+}$	OI I	*Col1a1*	—	(117)
G859C	OI II	*Col1a1*	Perinatal lethality	(118)
Col1a1$^{+/-365}$	OI I	*Col1a1*	—	(119)
Col1a1^{m1Btlr} or Col1a1^{seal}	OI III	*Col1a1*	—	(120)
Aga2$^{/+}$	OI III	*Col1a1*	Variable—42% to 69% postnatal lethality	(114)
Aga2$^{-/-}$	OI II	*Col1a1*	Embryonic lethality	(114)
BrtlII	OI II	*Col1a1*	Perinatal lethality	(121)
BrtlIV$^{/+}$	OI IV	*Col1a1*	Variable—30% perinatal lethality	(121)
BrtlIV$^{-/-}$	OI IV (mild)	*Col1a1*	—	(121)
Col1a1(Jrt)$^{-/-}$	Uncharacterized	*Col1a1*	Lethal	(122)
Col1a1(Jrt)$^{/+}$	OI IV	*Col1a1*	—	(122)
Human *COL1A1* minigene mouse	OI II, IV	*Col1a1*	Variable—high levels of transgene expression leads to perinatal lethality	(123)
Ifitm5 c.−14C > T	OI V	*Ifitm5*	Perinatal lethality	(124)
Ifitm5 S42L	OI VI	*Ifitm5*	—	(125)
Pedf$^{-/-}$	OI VI	*Serpinf1*	—	(126)
Crtap$^{-/-}$	OI VII	*Crtap*	—	([15])
P3h1$^{-/-}$	OI VIII	*Lepre1*	—	(127)
Ppib$^{-/-}$	OI IX	*Ppib*	—	(128)
Hsp47$^{-/-}$	OI X	*Serpinh1*	Embryonic lethality	(129)
Fkbp10$^{-/-}$	OI XI	*Fkbp10*	Perinatal lethality	(130)
Osx$^{Flox/-}$;Col1a-Cre	Possible OI XII	*Sp7*	—	(131)
Wnt1$^{sw/sw}$	OI XV	*Wnt1*	Variable—29% postnatal lethality	(132)
Wnt1$^{G177C/G177C}$	OI XV	*Wnt1*	—	(133)
Tric-B$^{-/-}$	OI XIV	*Tric-B (TMEM38B)*	Perinatal lethality	(134)

Bioengineering of Cas enzymes will also need to focus on designing smaller variants (such as those based on *Sa*Cas9[98,99] to better enable efficient packaging within single-AAV vectors). This is particularly the case if bone-targeted promoter sequences and guide RNA expression are also to be included.

Although there is an understandable urgency from clinicians and patient families to create and trial new therapeutic interventions, proof-of-concept in animal models is the standard for most gene therapy development. Numerous brittle bone mouse models are in common use (Table 3), with the most common being the *oim* mouse. This mouse features a mutation leading to a disruption of α2(I) collagen.[113] The *Aga2* mouse is an example of an ER-stress-induced dominant negative model and features a T→A mutation that dramatically affects gene splicing.[114] Lastly, the *Col1a2* G610C mutant mouse (also known as the Amish mutation) is an engineered knock-in mouse featuring a point mutation that leads to a mild-moderate bone phenotype.[115] None of these models are amenable to current Cas9 base-editing enzymes; however, the *oim*/+ and *Aga2*/+ mice may be amenable to targeted disruption of their dominant negative alleles. Despite the general characterization of these models, there may be arguments to generate new mouse strains featuring patient mutations selected based on being highly amenable to specific gene editing approaches.

Patient selection

Gene therapy remains a relatively new therapeutic avenue and there are certain ethical considerations that need to be taken into consideration. First, there are some risks associated with delivery, such as those associated with AAV vectors. Higher vector titers are generally more effective but also at greater risk of adverse events (e.g., liver toxicity). Second, both AAV vectors and Cas enzymes are capable of generating an immune response that will antagonize subsequent attempts at therapy. AAV neutralizing antibodies have been detected up 15 years post-AAV gene therapy administration in the first long-term AAV gene therapy follow-up,[135] demonstrating how persistent these immunological responses are. There is a prospect for some patients that they may only have "a single shot" at a gene therapy approach. Thus, the desire for early intervention must be balanced against the prospect of future, more effective treatments. This may be a challenging conversation to have between clinicians and patients and their families, but it is critical to explain the potential for involvement in a trial to limit future therapeutic attempts. Moreover, as for many diseases where early gene therapy is available, the risks of therapy must be weighed against the impact of untreated disease. Hence, it is likely that the initial candidates for therapy in OI will be those with severe, deforming OI (e.g., type III). Although it is likely that gene therapy for OI will

have the greatest impact in the young, phase 1 and initial phase 2 clinical trials are likely to start with adults and older children, before being undertaken in younger children and babies.

As gene therapy becomes a more likely option, there will be an increasing need to establish causative mutations. Gene diagnostics is often limited and not necessarily a priority when it is not a necessary diagnostic criterion. Because 90% of individuals with OI have collagen mutations, it is likely that therapies for treating these mutations are to be prioritized.

It will also be important to select outcome measures of gene therapy trials that are scientifically valid and meaningful for the affected cohort. In OI, this will likely include assessment of bone quality and strength, e.g., fracture rate, histomorphometry, and bone density assessment, as well as functional outcome measures and quality of life.

Conclusions

Although there are substantive obstacles that need to be overcome to treat genetic bone disorders using emerging gene technology, breakthroughs in AAV vectors and CRISPR/Cas9 gene editing are paving the way toward effective and accessible treatments. Although there are gene therapy trials for a range of other genetic conditions, this review has highlighted several areas where technical innovation is required—focused on efficient bone delivery and efficient gene repair. Unlike currently successful genetic therapies, the rate of gene correction for OI will likely need to be higher—possibly more than 50%. Nevertheless, the rapid progress in Cas enzyme bioengineering and AAV vector design make it not unreasonable to anticipate human trials for OI gene therapy within 5 to 10 years.

Disclosures

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Conflict of Interest

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Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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