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MINIREVIEW

Osteogenesis Imperfecta: Prospects for Molecular Therapeutics

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Osteogenesis Imperfecta (OI) is a dominant negative disorder of connective tissue. OI patients present with bone fragility and skeletal deformity within a broad phenotypic range. Defects in the COL1A1 or COL1A2 genes, coding, respectively, for the α1 and α2 chains of type I collagen, are the causative mutations. Over 150 mutations have been characterized. Both quantitative defects, such as null COL1A1 alleles, and qualitative defects, such as glycine substitutions, exon skipping, deletions, and insertions, have been described in type I collagen. Quantitative and structural mutations are associated with the milder and more severe forms of OI, respectively. A more detailed relationship between genotype and phenotype is still incompletely understood; several models have been proposed and are being tested. Transgenic and knock-out murine models for OI have previously been created. We have recently generated a knock-in murine model (the Brittle mouse) carrying a typical glycine substitution in type I collagen. This mouse will permit a better understanding of OI pathophysiology and phenotypic variability. It will also be used for gene therapeutic approaches to OI, especially mutation suppression by hammerhead ribozymes. The present review will provide an update of OI clinical and molecular data and outline gene therapeutic approaches being tested on OI murine models for this disorder.

Key Words: **Osteogenesis Imperfecta; collagen; Brittle mouse; ribozymes.**

OSTEOGENESIS IMPERFECTA CLINICAL AND MOLECULAR ANALYSIS

The collagens are a large family of proteins responsible for the tensile strength and structural integrity of connective tissues (1,2). Type I collagen is a member of a subclass of the collagen superfamily referred to as fibrillar collagens, which have in common a long and uninterrupted triple helical region and form a highly ordered, quarter-staggered array in the extracellular matrix (3).

Type I collagen is a heterotrimer consisting of two α1 and one α2 chains. Each α chain contains about 1000 amino acids and is composed of uninterrupted repeats of the triplet Gly-Xaa-Yaa, where Xaa is frequently proline and Yaa hydroxyproline. The amino acid glycine is necessary for the folding and stability of the triple helical structure of the protein. Its small side chain, a hydrogen molecule, is the only one able to be accommodated in the sterically constricted internal space of the triple helix (3).

Defects in type I collagen cause Osteogenesis Imperfecta (OI), also known as Brittle Bone Disease. OI is a heterogeneous group of heritable dominant disorders characterized by bone fragility and skeletal deformities. OI occurs in all ethnic and racial groups with an equal incidence of about 1 in 15,000–20,000 individuals, although the mildest forms are probably underestimated due to the difficulty of diagnosis and underreporting (4).

In 1979, Sillence proposed a classification of OI into four types (type I to IV), based on clinical, radiographic, and genetic criteria (5) (Table 1). This classification is widely accepted even though it does

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TABLE 1
Molecular Defects and Clinical Phenotypes of Different OI Forms

Quantitative defects: "null" COL1A1 allele		Qualitative defects in COL1A1 or COL1A2: Glycine substitutions, exon skipping, rearrangements, deletions, insertions		No reported defect in COL1A1 or COL1A2	
Type	Clinical outcome	Type	Clinical outcome	Type	Clinical outcome
I	Mildest OI form Close to normal stature Minimal bone deformity Blue sclerae Frequent hearing loss Variable dentinogenesis imperfecta	II	Lethal OI form Undermineralization of skeleton and calvarium Beaded ribs Compressed femurs Severe bone deformity Respiratory deficiency	V	Moderately severe OI form Mild to moderate bone deformity Variable short stature Dentinogenesis imperfecta Sporadic hearing loss High incidence of hypertrophic callus Early ossification of the interosseus membranes of the femur and leg
		III	Severest surviving OI form Progressive bone deformity High number of fractures Blue to grey sclerae Dentinogenesis imperfecta Variable hearing loss Extreme short stature		
		IV	Moderately severe OI form Mild to moderate bone deformity Moderate short stature Variable dentinogenesis imperfecta Variable hearing loss Sclerae: White or blue		

not completely describe the complexity of the OI phenotype. Type I is the mildest form of OI with occasional prepubertal fractures, usually of the long bones, minimal skeletal deformity, and stature within the normal range but slightly shorter than same gender siblings (6). Type I OI has been divided into subtypes A and B on the basis of the presence or absence of dentinogenesis imperfecta (7). OI type I patients often present with hearing loss in early adulthood due to fusion of the bones of the middle ear (8).

Type II is the most severe form of OI; it is lethal in the perinatal period. Patients with OI type II show striking deformity of the long bones and thorax and *in utero* fractures. Pulmonary insufficiency is the leading cause of death. About 60% of the affected individuals die in the first day of life and 80% within the first month from pulmonary infections or insufficiency or congestive heart failure. OI type II patients have a characteristic facial appearance with dark blue sclerae, a beaked nose, and a soft calvarium (9).

In type III OI, frequent fractures and the tension of muscles on soft bones cause progressive bone deformity. Extreme short stature, triangular facies, blue sclerae, and dentinogenesis imperfecta are common features. The growth plates are often disorganized microscopically and "popcorn" formation is noted on radiography (10). Scoliosis and kyphosis are common and contribute to cardiopulmonary insufficiency, which is the major cause of death in OI patients (11).

In type IV OI, affected individuals have mild to moderate deformity, long bone fractures that decrease in frequency after puberty, moderate short stature, dentinogenesis imperfecta (present in subtype A, absent in subtype B), and normal to gray or blue sclerae. Stature is frequently normal at birth but by 2 years is generally below the 5th percentile (12). The OI type IV group covers a wide range between the severe OI type III and the mild OI type I. There is often substantial intrafamilial variability. Recently, a new group of OI patients has been proposed, called OI type V (13). Its unique features are a high incidence of hypertrophic callus and early ossification of the interosseous membrane of the forearms and legs. It is transmitted as a dominant trait. Intriguingly, these patients do not have detectable alteration in the sequence of either the COL1A1 or the COL1A2 gene.

At a biochemical level, OI is characterized by either quantitative or qualitative defects in the synthesis of type I collagen. The mildest form of the disease (OI type I) is associated with mutations that result in loss of mRNA from one COL1A1 allele, leading to the synthesis of half of the normal amount of type I collagen. The reduced synthesis of collagen can alter the architecture and strength of bone. It can also compromise the relative amount of other macromolecules in bone matrix and fibril formation (14).

Structural defects of type I collagen are responsible for the clinically significant forms of the disease (OI types II, III, IV). About 85% of the mutations that have been described are single nucleotide changes responsible for a glycine substitution in the triple helical domain of the protein (15). During helix formation, these mutations affect propagation and folding. In the extracellular matrix, they may affect procollagen processing or interfere with collagen–collagen and collagen–extracellular matrix protein interactions (16). Exon skipping defects account for about 12% of the structural mutations. Insertions, deletions, and rearrangements in the triple helical domain have also been reported as the cause of OI. An additional group of mutations in the C-propeptide of type I collagen can cause OI by interfering with chain assembly (15).

The relationship between genotype and phenotype in OI is still incompletely understood. In general, qualitative defects are more severe than quantitative defects. Among the collagen structural mutations, a wide range in clinical outcome has been detected. Outcome is related to a variable extent to the location and nature of the mutation and the biochemical role of the chain in which the mutation is located (17).

Mutations in COL1A1 affect three-fourths of the type I collagen helices, with half of the molecules containing one abnormal proα1(I) chain and a quarter containing two abnormal proα1(I) chains. In contrast, mutations in COL1A2 affect only half of the synthesized molecules. As collagen mutations first began to be delineated, theoretical considerations led investigators to postulate that mutations in COL1A1 would be more severe than those in COL1A2 (18). Numerous exceptions to this model in both α chains showed that it was not the functional basis of phenotype. A subsequent model took into account the linear direction of helix formation and the resultant variation in helix over modification caused by mutations (19). This "gradient model" proposed that mutations cause lethal to severe to moderate phenotype, as their location moves from the C- to the N-terminal position along the α chains. This model accounts for some glycine substitutions in α1(I), but does not account for the phenotype of α2(I) mutations.

A "regional model" was proposed several years ago to give a rationale for the mutations found in the COL1A2 gene (20). This model accounts for 86% of known glycine substitutions and single exon skipping defects (21). According to this model, the mutations in the COL1A2 gene are clustered in alternating lethal and nonlethal domains whose function is undetermined, but which may be involved in interactions of collagen and noncollagenous proteins of extracellular matrix. A better knowledge of collagen–collagen and collagen–extracellular matrix protein interactions will increase understanding of the relationship between collagen mutations and clinical outcome, thus opening the possibility of investigating not only the collagen molecules but also their behavior in the extracellular matrix. In particular, the study of bone extracellular matrix organization will enhance insight into why OI is predominantly a bone disease although type I collagen is also highly expressed in skin and tendons.

ANIMAL MODELS FOR OSTEOGENESIS IMPERFECTA

The development of transgenic technology in recent decades made it possible to generate murine models for many human disorders. Transgenic mice were particularly useful in verifying that mutations in type I collagen cause OI and in better understanding OI pathophysiology (Table 2).

Two transgenic mice were created with collagen mutations. Stacey *et al.* generated a transgenic mouse expressing a mutant col1a1 gene in which there was a 2623G > C substitution (G859C). The mutant gene produced a dominant lethal phenotype similar to the lethal OI type II in human patients. The mice presented with short wavy ribs, short and broad long bones, and generalized poor mineralization. A lethal outcome was obtained when as little as 10% of total collagen synthesis was from transgene.

Khillan *et al.* produced another transgenic mouse expressing a human COL1A1 minigene under the control of the 2.5-kb collagen promoter (23). This minigene is deleted for most of the collagen helical region (442-3336del). The mice displayed a variable

TABLE 2
Murine Models for Osteogenesis Imperfecta

Murine models	Molecular defect	OI type	Reference
Transgenic	col1a1 mutated construct G- > T 2623 nt Gly 859- > Cys α1(I)	II	Stacey *et al.* (22)
Transgenic	Human COL1A1 construct Δ ex6-46	Variable	Khillan *et al.* (23)
Naturally occurring mutation (oim mice)	Δ G nt 3983 col1a2	III	Chipman *et al.* (24)
Knock-out heterozygous Mov13	Insertion of MMLV in col1a1	I	Bonadio *et al.* (26)
Knock-out homozygous Mov13	Insertion of MMLV in col1a1	II	Schnieke *et al.* (25)
Knock-in BrtIII	Insertion in col1a1 1482-1483 ins 147 nt SV40 T antigen sequence	II	Forlino *et al.* (27)
Knock-in BrtIIV	G- > T 1456 nt col1a1 Gly349- > Cys α1(I)	III/IV	Forlino *et al.* (27)

phenotype including extensive fractures of ribs and long bone similar to the lethal OI type II in humans.

A spontaneous mutation in the col1a2 gene has been characterized in a murine strain that develops a severe OI phenotype resembling human OI type III (24). The mice homozygous for the mutation (named oim/oim) have a 3983delG of both col1a2 genes. This frameshift mutation results in an alteration of the sequence of the last 48 amino acids of the C-terminal propeptide and prevents the incorporation of the mutant proα2(I) chains into type I collagen trimer. Instead the mouse generates a proα1(I)$_3$ homotrimer. The phenotype has a recessive transmission. The oim/oim mice have osteopenia, fractures, skeletal deformity and thin cortical bone.

A knock-out mouse for the col1a1 gene has also been generated. Schniecke *et al.* inactivated the col1a1 gene by inserting the moloney murine leukemia virus into intron 1 (25). These mice (named Mov13) have a recessive embryonic lethal outcome; embryos died *in utero* around Day 12 p.c. because of failure in the development of the vascular system. Heterozygous Mov13 mice produce half of the normal amount of type I collagen and have a phenotype similar to the mild OI type I. On biomechanical testing, the femurs have diminished strength not associated with spontaneous fractures. Moreover, Mov13 heterozygous mice also have reduced hearing measured by evoked auditory brainstem responses (26).

GENERATION OF TWO KNOCK-IN MURINE MODELS FOR OSTEOGENESIS IMPERFECTA: BrtlII AND BrtlIV

Although useful for investigating different aspects of human OI, none of the murine models described above reproduce both the molecular defect and the clinical outcome of severe human OI. None introduces a typical molecular defect of human patients, a glycine substitution in the triple helical domain of type I collagen which is present in single copy in the genome, under the control of the endogenous promoter and with dominant negative transmission. Furthermore, the transgenic mice contain an altered number of collagen genes in their genome, depending on the copy number of the transgene, in addition to the presence of the two endogenous wild-type collagen alleles. This results in nonphysiologic ratios of mutant and normal collagen chains.

The lack of an appropriate model for severe OI has limited the development of gene therapeutic approaches for this disorder. Recently, we generated a knock-in murine model for moderately severe OI in our lab, inserting in its genome a G349C substitution in the endogenous col1a1 gene (27) (Table 2). This mutation had been already described in one of our human OI type IV patients (28). In our model, the mutant allele is under the control of the endogenous col1a1 promoter, is present in single copy in the murine genome, and generates a dominant transmission of the OI phenotype. We named these mice Brittle IV (BrtlIV). BrtlIV was generated using standard techniques of homologous recombination in ES cells (29). The electrophorated construct was a subcloned fragment of murine col1a1 gene that had been subjected to *in vitro* mutagenesis. We introduced four mutations into exon 23: 1456G > T responsible for the causative mutation G349C, a 1461C > T to create a GUC ribozyme cleavage site, and two transitions 1567C > A and 1569C > G, respectively, to change the L356M. In addition, the mutated allele was made conditionally null by the insertion of a transcription/translation stop cassette, flanked by two loxP sites, into intron 22 (30). The presence of the lox–stop–lox cassette itself gen-

erated alternative splicing of the mutant collagen mRNA. F1 mice obtained from matings of the chimeras with wild-type mice and carrying the floxed allele had a lethal outcome resembling a lethal OI type II (BrtlII). Four alternatively spliced mRNAs were identified, three of which contained a stop codon generated from insertions of different length coming from the stop cassette itself and one of which did not contain a stop codon and was in-frame with the collagen coding sequence. This transcript was translated and the mutated protein identified in cultured cells.

Mating of the chimeras with transgenic mice expressing cre recombinase resulted in removal of the stop cassette and expression of the allele carrying the single glycine substitution. BrtlIV mice show a wide range of clinical outcome: 70% of the mutant mice have a lethal outcome and about 30% have a moderately severe phenotype. The lethal BrtlIV mice die a few hours after birth, apparently from respiratory distress. They show generalized undermineralization of the skeleton, especially of the skull, and multiple rib fractures resembling human lethal OI. The surviving BrtlIV mice live to maturity and are reproductively successful. They have an undermineralized skeleton, thin ribs and long bones, and flaring of the ribcage. Their long bones and tail vertebrae have disorganized trabeculae. Histologically, they also have dentinogenesis imperfecta, which is a common finding in human OI. Their growth is delayed with respect to wild-type littermates. BrtlIV surviving mice display a phenotype similar to the human OI patient who carries the same molecular defect.

APPROACHING GENE THERAPY BY SUPPRESSION OF MUTANT ALLELE EXPRESSION

OI is a dominant negative disorder: a mutation in a single α-chain affects not only the individual collagen helix that incorporates the mutant chain but, due to the higher order associations of type I collagen, it affects also fibril formation and eventually extracellular matrix structure (1). For OI, as well as for other dominant negative disorders, it is not sufficient to develop molecular therapies by expressing an exogenous normal gene, as has been the approach taken for recessive enzymatic diseases (31). In dominant disorders, it is not the lack of functional protein that is the cause of the disease, but the production of an altered form. This requires alternative approaches such as suppression of mutant message or correction of the defect at the DNA level to affect the tissue level outcome (32).

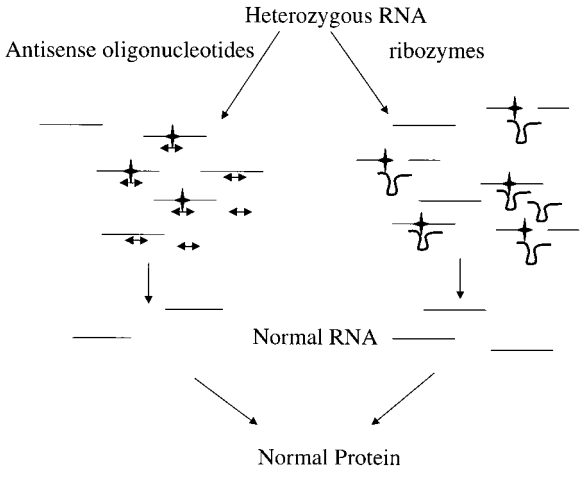

FIG. 1. Antisense strategies to suppress mutant collagen transcripts. Antisense oligodeoxynucleotides (double arrows) and hammerhead ribozymes, specific for mutant mRNA, are synthesized and incubated in the presence of mutant and normal transcripts. Abnormal RNA is cleaved with both approaches and wild-type RNA remains intact, generating normal protein. However, antisense oligodeoxynucleotides also bind with high efficiency to the normal transcripts, which limits their use in gene therapeutic trials.

Two potentially useful antisense strategies were developed in the past decade: antisense linear oligonucleotides and hammerhead ribozymes (Fig. 1). Their potential usefulness at a practical as well as theoretical level was demonstrated by the more classical antisense experiments of Khillan *et al.* using the transgenic mice expressing the human collagen minigene (33). These mice were mated with other transgenic animals expressing the minigene in antisense orientation. In the F1 mice that inherited both genes, the incidence of the lethal fragile bone phenotype was reduced from 92 to 27% and the effects of the antisense gene were directly demonstrated by an increase in the ratio of normal mouse proα1(I) chains to human mini proα1(I) chains in the tissues. But this antisense gene was targeted to human sequences in a murine background, so that allele specificity was not a limiting factor.

A first attempt to test the sequence specificity of oligonucleotides was done by Laptev *et al.* (34). They tested a series of antisense oligonucleotides (ASOs) specific for the human collagen transcript expressed by NIH3T3 murine fibroblasts after transfection with a human minigene for procollagen type I. The

ASOs were designed to target sites in which there were at least two base differences within a 20-nucleotide sequence between human and mouse transcripts. In human patients, the presence of two alleles differing only by a single nucleotide necessitates the use of short antisense oligonucleotides specifically targeting the abnormal allele. Our group reported the use of antisense oligonucleotides in fibroblasts cultured from OI patients (35). Although significant suppression of the mutant transcripts was demonstrated, the antisense effect on the transcripts from the normal allele was unacceptably high (35).

Ribozymes represent an alternative agent for suppression of mutant transcripts in dominant negative disorders (32). They are theoretically able to selectively reduce the intracellular level of a specific RNA. Cleavage by trans-active ribozymes requires three elements. Two elements are part of the ribozyme itself: first, the central hairpin sequence, which is the catalytic core for cleavage, and second, the binding arms flanking the catalytic core and designed to be complementary to any target sequence. The third component is on the target RNA molecule and is a three nucleotide cleavage site NUX, where N is often a G and X can be any nucleotide except G. The most common cleavage site in nature is GUC. This combination of requirements for a binding site and a cleavage site enhances the specificity of ribozymes in comparison to linear antisense oligonucleotides. Furthermore, the ability of ribozymes to cleave a target and release and cleave again on a second target molecule, and so on, provides the potential for increasing efficiency and stability. Our group has already demonstrated *in vitro* the potential of hammerhead ribozymes in gene therapeutic approaches for human OI. We used collagen point mutations that generated a novel ribozyme cleavage site in the mutant transcript (36). Synthetic target transcripts were generated from subclones of both mutant and normal cDNA. Active ribozymes were able to bind both transcripts because they contain identical binding regions. However, only the mutant transcript was cleaved. Ribozymes with an inactive catalytic core cleave neither substrate. Cleavage by the active ribozyme generates products of the expected size. Furthermore, active ribozyme retains its ability to cleave mutant transcript in the presence of a mixture of normal and mutant target. Competitive binding effects can be overcome by increasing the ratio of ribozyme to target or by the introduction of mismatches into the ribozyme's binding arms to facilitate release from target in the absence of cleavage. The specificity and efficiency of ribozyme cleavage demonstrate the potential role of ribozymes in gene therapy for OI.

BrtlIV mice represent a suitable target for testing such an approach. BrtlIV carry a GUC ribozyme cleavage site near the causative G349C mutation. The proof of principle for the efficiency of this gene therapy trial will be obtained by the study of F1 pups from matings of BrtlIV with transgenic mice expressing the specific ribozyme. Additional studies will be subsequently required to develop more appropriate ways to deliver ribozyme to the mutant animal and, eventually, to the OI patients.

The limitations of this approach in practice include (a) its applicability only to the approximately 25% of known collagen mutations that generate a ribozyme cleavage site, and (b) the time and expense required to test a ribozyme targeted to each individual mutation. The application of ribozymes to OI cases can be expanded by targeting a common polymorphism rather than the disease-causing mutation per se. Millington-Ward *et al.* demonstrated efficient *in vitro* cleavage of one form of a COL1A1 polymorphic site with a heterozygosity frequency of 0.42 (37). In principle, this site could be targeted in all OI patients with a collagen mutation on the same COL1A1 allele as the cleavable form of the polymorphism, or about 20% of patients with α1(I) mutations.

APPROACH TO GENE THERAPY BY CELL REPLACEMENT

Since the mid-1980s, we have known that a small proportion of the clinically unaffected parents of children with OI are mosaics for the mutations that affect their children (38–40). The mutations present in the parents presumably arise during their fetal development and result in a situation in which the parents have a mixture of normal and heterozygous cells. Some of these individuals have more than 50% heterozygous cells in their skin, yet have minimal to no symptoms. This suggests that some level of mutation burden can be tolerated before the consequences become symptomatic. In gene therapy, we might hope to mimic this situation by replacing a portion of an affected individual's cells with a normal cell population.

In the past decade, cell replacement strategies for gene therapy have progressed to the stage of active investigation. While not yet practical, they do con-

tinue to hold theoretical promise. The multipotent ability of mesenchymal cells to generate bone, cartilage, and muscle has been demonstrated (41). In principle, a successful transplantation could create a "mosaic" recipient and cure their skeletal dysplasia.

In animal and human experiments it has proven difficult to transplant more than a minimally detectable level of cells. Pereira *et al.* infused marrow stromal cells from normal mice into mice expressing a collagen minigene construct and displaying a fragile skeletal phenotype (42). The recipients had been prepared with near-lethal irradiation. Small improvements in OI outcome were reported at modest levels of transplanted cells. In children with severe OI pretreated with chemical ablation, Horowitz *et al.* demonstrated that transplantation with whole marrow resulted in the presence in the recipient of 1–2% donor bone cells at 101 days post-transplant (43). It is possible that use of more vigorous pretreatment and transplantation with expanded stromal cells could achieve sufficient levels of donor cells to affect phenotype. Additional animal studies on mesenchymal cell replacement should enhance the potential of bone cell transplants.

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