Use of the Cre/lox Recombination System to Develop a Non-lethal Knock-in Murine Model for Osteogenesis Imperfecta with an α1(I) G349C Substitution

VARIABILITY IN PHENOTYPE IN BrtlIV MICE*

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We utilized the Cre/lox recombination system to develop the first knock-in murine model for osteogenesis imperfecta (OI). The moderately severe OI phenotype was obtained from an α1(I) Gly349→Cys substitution in type I collagen, reproducing the mutation in a type IV OI child. We introduced four single nucleotide (nt) changes into murine col1a1 exon 23: the disease causing G→T transversion (nt 1546), an adjacent G→T change (nt 1551) to generate a GUC ribozyme cleavage site, and two transversions (nt 1567 C→A and nt 1569 C→G) to cause a Leu→Met substitution. We also introduced a 3.2-kilobase pair transcription/translation stop cassette in intron 22, flanked by directly repeating lox recombination sites. After homologous recombination in ES cells, two male chimeras were obtained. Chimeras were mated with transgenic females expressing Cre recombinase to remove the stop cassette from a portion of the progeny’s cells. To generate mice with full expression of the Gly349→Cys mutation, these offspring were then mated with wild-type females. Skeletal staining and bone histology of the F2 revealed a classical OI phenotype with deformity, fragility, osteoporosis and disorganized trabecular structure. We designate these mice BrtlIV (Brittle IV). BrtlIV mice have phenotypic variability ranging from perinatal lethality to long term survival with reproductive success. The phenotypic variability is not associated with differences in expression levels of the mutant allele in total RNA derived from tissue extracts. Expression of the mutant protein is also equivalent in different phenotypes. Thus, these mice are an excellent model for delineation of the modifying factors postulated to affect human OI phenotypes. In addition, we generated knock-in mice carrying an “intronic” inclusion by mating chimeras with wild-type females. Alternative splicing involving the stop cassette results in retention of non-collagenous sequences. These mice reproduce the lethal phenotype of similar human mutations and are designated BrtlII.

The typical clinical features of osteogenesis imperfecta (OI),¹ also known as “brittle bone disease,” are bone fragility, skeletal deformity, and growth deficiency. OI is a generalized disorder of connective tissue caused by mutations in type I collagen, the major structural protein of the extracellular matrix of bone, skin, and tendon (1). Type I collagen is a heterotrimer, composed of two copies of the α1(I) chain and one copy of the α2(I) chain. Each chain has a helical region, which is 1014 amino acid residues in length and is composed of uninterrupted repeats of the triplet Gly-X-Y, where X is often proline and Y is often hydroxyproline. The glycine present at every third residue is crucial for correct helix folding and modification (2).

Mutations in either of the genes COL1A1 or COL1A2, which code for the α1(I) and α2(I) chains of type I collagen, respectively, are responsible for the full clinical spectrum of OI. More than 150 collagen mutations have been identified in OI patients. The more clinically severe forms of the disorder (OI types II, III, and IV) are caused by structural defects in type I collagen. The great majority (85%) of these are substitutions of a glycine residue by an amino acid with a bulky, polar, or charged side chain. Exon skipping, intronic insertion, and large deletions have also been described (3). The very mild phenotype of type I OI is generally caused by the presence of a null COL1A1 allele, due to mutations which cause premature chain termination (1).

Previous murine models for OI have been generated with transgenic methodologies, resulting in variable copy number and variable integration sites. No current murine model combines dominant genetic transmission, a typical glycine substitution mutation, and physiologic levels and tissue distribution of mutation expression, as well as the skeletal outcome of non-lethal human OI. The lack of a suitable model has limited further insight into OI pathophysiology and into genetic aspects typical of dominant disease, such as variable expression. A murine model with these features is also required for the development of approaches to the gene therapy of the disorder.

Recently, the Cre/lox recombination system has become a major technology for modification of gene expression (4, 5). Cre recombinase, isolated from bacteriophage P1, is a 38-kDa protein, which is able to catalyze conservative reciprocal recombination in mammalian cells (6). Cre recognizes a 34-bp loxP sequence and, in the presence of two directly repeating loxP sites, excises the intervening DNA sequence (7, 8). The in vivo use of the Cre/lox system involves both mice expressing Cre enzyme and mice with loxP sites inserted at a selected transgenic or endogenous locus of interest. The mating of the two strains generates progeny in which Cre is expressed and exerts its effects on loxP sequence.

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‡ The abbreviations used are: OI, osteogenesis imperfecta; bp, base pair(s); kb, kilobase pair(s); nt, nucleotide(s); PAGE, polyacrylamide gel electrophoresis; RT, reverse transcription; PCR, polymerase chain reaction; tk, thymidine kinase.

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cises the sequences between the loxP sites. Selection of the appropriate promoter controlling Cre expression allows the production of mice with temporal and tissue-specific modification (5).

Here we report the generation of a knock-in murine model for OI, obtained using the Cre/lox system. This mouse is heterozygous for a glycine substitution, Gly349→Cys, in one col1a1 allele (9). The heterozygous mutant mice accurately reproduced the molecular findings in human OI, with balanced and tissue-specific expression of normal and mutant alleles, dominant genetic transmission, and moderately severe skeletal phenotype. Furthermore, the phenotypic variability, ranging from moderately severe to lethal, that we found in these mice reproduces the variability described in some unrelated patients who carry identical mutations but have variation in clinical severity (10, 11). We designate these mice BritIV (Brittle IV).

A murine model for lethal type II OI was also obtained from this system. Non-collagenous sequences, derived from the floxed stop cassette with an intronic placement, were inserted into collagen helical sequence by alternative splicing. The lethal phenotype of these mice reproduced the skeletal outcome described in patients with similar molecular rearrangements (12). We designated these mice BritII.

**Experimental Procedures**

**Construction of the pAFES Targeting Vector and Generation of Chimeric Mice**—The targeting vector contained four point mutations in exon 23, a floxed stop cassette in intron 22, and two selectable markers. The relevant segment of the murine col1a1 gene was contained in the pcol1a1–22 plasmid kindly provided by R. Jaenisch. A 4.2-kb KpnI/XbaI fragment from this vector was subcloned into pBSII-SK+ (Stratagene) and used as the template for site-directed mutagenesis with the Chamaeleon double-stranded site-directed mutagenesis kit (Stratagene). The primer for mutagenesis was a 45-mer (MF4) complementary to col1a1 nt 1537–1581, located in exon 23. Use of this primer resulted in the four nucleotides changes shown in bold letters: 5′-GGTTAGACCTTGCTGCCCAGTGGAAGCTGTGCTGATTGCAAAG-3′, including: 1) a G→T transversion at nt 1456, creating a Gly349→Cys substitution in the α1(I) collagen chain, 2) a C→T transition at nt 1551 generating a GUC ribosome-coding site and 3) C→A and C→G transversions at nt 1567 and 1569, respectively, changing the amino acid Leu350 to Met. Subsequently, a lox-stop-lox cassette was inserted into intron 22 by blunt-ended ligation at a unique BsaI site. For positive selection, a neomycin-resistant gene (neo) was inserted inside the floxed stop cassette. The neo gene was under the control of the phosphoglycerokinase promoter (PGK-neo) and was inserted in inverse orientation with respect to the endogenous col1a1 gene. The resulting vector, pAF10, contained 9.1 kb of inserted sequences.

Finally, we added a thymidine kinase gene, under the control of the PGK promoter (PGK-th), for positive selection. This was done by first inserting PGK-th at an SspI site in pBSII-SK+ by blunt-ended ligation. Then, an 8.1-kb EcoRV/NotI-modified col1a1 fragment from pAF10 was subcloned into the pBSII-SK+ vector downstream of the th gene.

The resulting construct, pAFES, was used for gene targeting in 129Sv/J ES cells. 40 μg of NotI-linearized pAFES was electroporated into ES cells using a Bio-Rad Gene Pulser (0.4 kV, 25 microfarads, 0.4 s). The cells were plated in 60-mm plates. G418 (400 μg/ml, Life Technologies, Inc.) and FIAU (1-[2-deoxy-2-fluoro-β-D-arabinofuranosyl] (5 mM, Syntex) were added to the media 24 h after the electroporation. Resistant colonies were picked from day 8–10 following electroporation, transferred to 24-well plates, and expanded in the presence of G418. At
The expression of the mutant allele and the presence of alternatively spliced sequences was analyzed using total RNA. Total RNA was extracted from 18.5-day embryos from CXWT and CXEIIa-cre matings, as well as from cultured dermal FB from both wild-type and mutant offspring of those matings, using TRI reagent (Biotra), according to the manufacturer’s protocol. For the offspring of CXWT matings, RT-PCR was performed using total RNA and primers located in exon 21 and 25, respectively: AF1 (5’-TGCCCGAAGAAAGGAGTGACGCCG-3’, nt 1354–1375) and AF6 (5’-GCCAGGTGGTTGGATGAGGATTCC-3’, complementary to nt 1691–1713). We detected the expected 360-bp product and an additional less abundant product about 100 nt larger. Both products were subcloned into TA cloning vector pCR® 2.1 (Invitrogen) and sequenced. Further delineation of the variety of inserted sequences from intron 22 was obtained by additional RT-PCR amplifications, pairing primers specific for each end of the insert with AF1 or AF6, respectively. Insertion-specific primers were Ins-sense (5’-ATTCACACCTATGGAACACTGATGAAATGGGAG-3’) and Ins-antisense (5’-CTTCTCTCTTGGTTGGAGAGTAGAGG-3’). The products of these amplification reactions were also subcloned into TA cloning vector and sequenced. Radiolabeled antisense riboprobes for the four mutant alternatively spliced transcripts were synthesized as described (14), using a plasmid carrying each form of mutant cDNA or 245 nt of β-actin cDNA, respectively, T3 or T7 RNA polymerase (Promega), and [α-32P]CTP. The assay was performed using the RPA III kit (Ambion) according to the manufacturer’s protocol. Denaturation was performed with ScanMaker 600ZS MICROTECK, Analysis was performed using the public domain NIH Image Program 5.55.

**Fig. 2.** Genotyping of the offspring resulting from the matings: CXWT, CXEIIa-cre, and BrtlIV. The expression of the mutant allele and the presence of alternatively spliced sequences was analyzed using total RNA. Total RNA was extracted from 18.5-day embryos from CXWT and CXEIIa-cre matings, as well as from cultured dermal FB from both wild-type and mutant offspring of those matings, using TRI reagent (Biotra), according to the manufacturer’s protocol. For the offspring of CXWT matings, RT-PCR was performed using total RNA and primers located in exon 21 and 25, respectively: AF1 (5’-TGCCCGAAGAAAGGAGTGACGCCG-3’, nt 1354–1375) and AF6 (5’-GCCAGGTGGTTGGATGAGGATTCC-3’, complementary to nt 1691–1713). We detected the expected 360-bp product and an additional less abundant product about 100 nt larger. Both products were subcloned into TA cloning vector pCR® 2.1 (Invitrogen) and sequenced. Further delineation of the variety of inserted sequences from intron 22 was obtained by additional RT-PCR amplifications, pairing primers specific for each end of the insert with AF1 or AF6, respectively. Insertion-specific primers were Ins-sense (5’-ATTCACACCTATGGAACACTGATGAAATGGGAG-3’) and Ins-antisense (5’-CTTCTCTCTTGGTTGGAGAGTAGAGG-3’). The products of these amplification reactions were also subcloned into TA cloning vector and sequenced. Radiolabeled antisense riboprobes for the four mutant alternatively spliced transcripts were synthesized as described (14), using a plasmid carrying each form of mutant cDNA or 245 nt of β-actin cDNA, respectively, T3 or T7 RNA polymerase (Promega), and [α-32P]CTP. The assay was performed using the RPA III kit (Ambion) according to the manufacturer’s protocol. Denaturation was performed with ScanMaker 600ZS MICROTECK, Analysis was performed using the public domain NIH Image Program 5.55.

**X-ray, Skeletal Staining, and Histological Analysis—**Radiographs of the F1 and F2 wild-type and mutant mice were performed by Faxitron (30 kV for 1 min). For skeletal staining, skin was removed from dead newborn mice within a few hours after birth. The pups were fixed in 95% ethanol for 7 days and then stained with 0.3% Alcian Blue 8GS and 0.1% Alizarin Red S (15).

For light microscopy, necropsy of mutant and wild-type mice was performed at 2 months of age. The tissues were fixed in neutral buffered 10% formalin for 24 h, embedded in paraffin, sectioned, and stained with hematoxylin and eosin and with Masson’s Trichrome (SAIC, Frederick, MD).

**Immunohistochemistry**—Collagen was extracted from the skin of newborn mice (16). The denatured samples were electrophoresed on 6% SDS-urea-PAGE (MiniPROTEAN II, Bio-Rad) and transferred by electroblotting (90 V, 2 h) onto 0.45 μm nitrocellulose membrane (Bio-Rad). The membrane was incubated with antibody (kindly provided by Dr. L. Fisher) specifically reacting with the COOH end of α(I) chain. The secondary antibody was anti-rabbit IgG-horseradish peroxidase conjugate (Promega). ECL (Amersham Pharmacia Biotech) was used to visualize antibody binding on x-ray film.

**F2 Mutant Mice (BrtlIV)—**Mice expressing the mutant allele carrying only the Gly349 → Cys substitution were obtained by mating the...
m又好 with wild-type CD-1 and C3H/HeJ females or by crossing the F2 with the same strains of wild-type mice. Genotyping was performed on DNA from tail clippings by Southern blot as described previously, using probes A and B and probe Cre (196-bp BamHI/EcoRV fragment) from cre gene.

Analysis of the Type I Collagen from Tissues and Cultured Fibroblasts of F1 and F2 Mice—Fibroblasts from F1 and F2 wild-type and heterozygous mutant mice were plated at equal density (2.5 × 10^5 cells/well) in six-well plates (30 mm) and grown for 24 h in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. Collagen labeling and analysis were performed as described previously (17). Cyanogen bromide peptides were prepared by CNBr digestion of gel slices containing [35S]- and [3H]-labeled α1(I) and α1(I) dimer followed by electrophoresis onto 10% SDS-urea-PAGE (18). Collagen was extracted from skin of newborn F2 mice. Skin was digested for 48 h with pepsin (1:10 pepsin:skin, dry weight) and the collagen was precipitated with 2 M NaCl, 0.5 M acetic acid. Samples were electrophoresed on 6% SDS-urea-PAGE and stained with Silver Stain Plus kit (Bio-Rad).

### Table II

| Allele with stop cassette/total col1a1 alleles | Skin | Bone | Lung |
|----------------------------------------------|------|------|------|
| Mouse 19                                     | 40   | 41   | 30   |
| Mouse 21                                     | 44   | 29   | 18   |
| Mouse 22                                     | 18   | 27   | 13   |
| Mouse 23                                     | 38   | 50   | 23   |
| Mouse 24                                     | 38   | ND   | 5    |
| Mouse 26                                     | ND   | 52   | 41   |
| Mouse 28                                     | ND   | 16   | ND   |
| Mouse 29                                     | ND   | 24   | 21   |
| Mouse 30                                     | ND   | 50   | 29   |

*Efficiency of recombination at the lox sites in col1a1*

Southern blot on XbaI digested genomic DNA extracted from tail clippings of chimera X EIIa-cre pups was performed. The efficiency of recombination at the lox sites was calculated using densitometry of the 9- and 10-kb bands detected by probe B. These bands correspond, respectively, to the allele with stop cassette and to the co-migrating wild-type allele and the mutant allele after cre recombination. Values shown were calculated as 9-kb band/9-kb band with [3H]proline. Collagen was harvested from medium as described. Samples were analyzed on 6% SDS-urea-PAGE and stained with Silver Stain Plus kit (Bio-Rad).

**Expression of Mutant Allele—**Expression of the mutant allele was confirmed by RT-PCR. Total RNA was extracted from tail clippings, skin, calvarium, and lungs of surviving and lethal mutant mice using TRI reagent (BMC) according to the suggested protocol, after homogenization of the tissues. RT-PCR using AF1/AF6 primers and a hemi-nested PCR using AF3 (5′-GTGGACCTGGTACCGTGAGTTTCTC-3′, nt 1430–1452)/AF6 were performed. Allele-specific oligonucleotide hybridization was performed using 5 μg of total RNA slot-blotted onto nylon membrane (Hybond+., Amersham Pharmacia Biotech). An antisense oligonucleotide specific for the mutant allele (5′-ACCGGGACCA-CAAGCTTC-3′, complementary to nt 1540–1557) was synthesized and 3′-labeled using terminal transferase (Roche Molecular Biochemicals) with [α-32P]dGTP. Hybridization was performed at 45 °C using 1.5 × 10^6 cpm/ml probe in 10 ml Rapid Hyb Buffer (Amersham). Washes were: 2 × 30 min with 6XSSC, 0.5% Na4O7P2 at room temperature and 2 × 10 min with 3M TMAC, 50 mm Tris pH 8.0, 2 mm EDTA, 0.1% SDS at 45 °C. Under these conditions, the oligonucleotide probe was >99% specific for synthetic mutant transcript as compared with synthetic transcript from the normal allele. Membranes were hybridized with β-actin after stripping. Densitometry was performed with a ScanMaker 6002S MICROTECK and autoradiographs were analyzed with the public domain NIH Image Program 5.55.
RESULTS

Generation of Chimeras Carrying a Mutated col1a1 Allele and Production of Heterozygous Mutant Mice—A genomic clone, pcol1a1-22, spanning exons 8–40 of the murine col1a1 gene, was used to generate the mutant targeting construct. Site-directed mutagenesis (Fig. 1A) was used to introduce four point mutations into exon 23. A G→T transversion was inserted at nt 1546, changing the amino acid Gly349→Cys in the \( \alpha(1) \) chain. This mutation has been previously characterized in a moderately severe OI proband (9). A C→T transition was introduced at nt 1551 to create a standard GUC ribosome cleavage site close to the disorder-causing mutation. Additionally, C→A and C→G mutations, changing Leu356→Met, were introduced to be able to follow the mutant chains by CNBr digestion. The presence of these point mutations was confirmed by sequencing. In addition, a \( \text{lox-Stop-lox} \) cassette was inserted into intron 22 (Fig. 1C).

Mutant ES cells were generated by homologous recombination (Fig. 1, B–D). Two recombinant clones (76 and 90) were microinjected into C57Bl/6J blastocysts. Two male chimeras were obtained. The chimeras were mated with wild-type females (Table I). Germ line transmission of the mutated allele was confirmed by Southern blotting analysis (Fig. 2, A and B) of DNA extracted from tail clippings of the F1. We expected the F1 heterozygous mutant mice to have a null mutant allele and a mild phenotype. Instead, the allele with the stop cassette was expressed and resulted in a lethal phenotype (see “Characterization of BrtlIV Mice: The Floxed Stop Cassette Is Responsible for Alternative Splicing, Generating Abnormal col1a1 Transcripts”).

Mating of Chimeras with EIIa-cre Females Generates Heterozygous F1 OI Mice with Incomplete Removal of Floxed Cassette—Transgenic mice expressing Cre recombinase under the control of the adeno virus EIIa early promoter (13) were mated with the chimeras (Table I). Expression of Cre in embryos permits reciprocal recombination at the loxP sites and removal of the \( \text{lox-Stop-lox} \) cassette to yield heterozygous mutant mice carrying only the Gly→Cys substitution. Southern blotting analysis with Probe A and probe B to confirm cassette removal from the recombinant allele. The absence of Cre from their genome was confirmed by Southern blot using probe Cre. RT-PCR using total RNA extracted from tail clipping confirmed the expression of the mutated allele. The phenotype ranged from moderately severe to lethal (Table I).

We have now propagated over 100 non-lethal mutant mice. Their size is about 50% that of normal littermates until 6 weeks of age, after which their size increases to about 80% of normal. Deformity of the rib cage is apparent. In addition, both fore and hind legs are bowed and thinner than control littermates. X-rays show a general undermineralization of the skeleton, especially of the skull, a thorax with a narrow apex and flared base, and gracile ribs. The ramus of ischium appears to be laterally flared (Fig. 4).

Light microscopy analysis was performed on tissues from 2-month-old BrtlIV mice (Fig. 5). The calvarium was thinner and poorly mineralized. The vertebral bodies were more disorganized and osteoporotic in OI mice than in control littermates. Analysis of sections through the nasal turbinates of mutant mice showed the presence of osteoid proliferation and tissue composed of fibroblasts rather than mature bone (Fig. 5). There were disorganized islands of bone formation in the maxilla in the area around the molars, as compared with the organized collar of cells surrounding the wild-type molars. The pulp cavity of molars appeared necrotic and infected with bacteria. The presence of necrosis and fibrotic tissue in the pulp cavity is well described as a common feature associated with dentinogenesis imperfecta in OI patients.

At the other end of the spectrum, 40–60% of the mutant F2 died within a few hours after birth from respiratory distress. Histological analysis of the mutant embryos showed pulmonary hemorrhages (data not shown). The size and weight of the...
pups were similar to the normal littermates. X-rays and skeletal staining revealed a flared ribcage with multiple rib fractures, long bone fractures, shorter and flattened vertebral bodies, and narrow pelvis. Additionally, decreased calvarial mineralization was noted (Fig. 6, BrtlIV).

Variable Phenotype of BrtlIV Mice Not Dependent on Variation in Levels of Mutant Collagen mRNA or Protein Expression—Expression of the mutant col1a1 allele in BrtlIV mice was demonstrated by RT-PCR and digestion with HinIII (data not shown). The level of expression of the mutant allele in lethal and moderately severe BrtlIV mice was analyzed by hybridization of total RNA from lung, skin, and bone with allele-specific oligonucleotide. There was no significant difference in expression of mutant collagen transcripts between the two phenotypes (Table III). As expected, collagen expression in these tissues correlated with relative levels of endogenous type I expression, being most abundant in bone, abundant in skin, and less abundant in lung.

Expression of the mutant collagen protein in BrtlIV mice was demonstrated first in cultured dermal fibroblasts (Fig. 3B) by the presence of an a1(I) dimer band on SDS-urea-PAGE. Since normal a1(I) chains contain no cysteine residues, the a1(I) dimer can form only with the expression of the Gly<sup>490</sup> → Cys mutation. The identity of the dimer band was verified by cyanogen bromide (CNBr) digestion (Fig. 3B). The mutant collagen was well secreted into the media, as was found in the patient with this mutation (9). We attempted to quantitate the levels of mutant chain using the methionine residue introduced patient with this mutation (9). We attempted to quantitate the levels of mutant chain using the methionine residue introduced to determine the molecular cause of the unexpected lethal outcome. RNA was extracted directly from 18.5-day embryos from chimera X wild-type matings as well as from fibroblast cultures established from those embryos. RT-PCR were performed with primer pairs AF1/AF6, AF1/Ins antisense, and Ins sense/AF6 to amplify the region between exons 21 and 25 and delineate any sequences from intron 22 remaining in cDNA. PCR products were subcloned and sequenced. We identified four alternatively spliced transcripts, which resulted in the insertion of non-collagenous sequences between exons 22 and 23. All transcripts resulted from the use of the same splice donor, contained in the sequences for SV40 large T antigen present in the stop cassette, but each used a different splice acceptor (Fig. 7). The smallest insertion was 147 bp, maintained the collagen reading frame, and did not contain a stop codon. The other insertions were 581, 633, and 691 bp, respectively, and each contained a stop codon in the collagen reading frame. Quantitation of the alternative transcripts from the allele with the stop cassette was performed by RNA protection analysis (data not shown). The values for the collagen transcripts were normalized using a co-protection assay of RNA for β-actin, then corrected for transcript length. We estimate that the proportions of the alternatively spliced products were: 25% for the in-frame transcript, 12% for the 580-bp, 37% for the 529-bp, and 26% for the 638-bp out-of-frame transcripts, respectively. The representation of alternatively spliced transcripts in poly(A)<sup>+</sup> mRNA was not determined.

Because procollagen protein uses an alignment region in the carboxy-terminal extension to incorporate into the procollagen heterotrimer, abnormal pro-a1 chains that were able to incorporate into helix could only result from transcripts with the small in-frame insertion. Procollagen synthesized by cultured fibroblasts from mutant offspring of chimera X wild-type matings was digested with pepsin, and the resulting collagen was electrophoresed on SDS-urea-PAGE. In the cell-layer fraction, we detected a band migrating faster than both type I a chains (Fig. 3A). Western blotting using anti-a1 antibody confirmed the identity of the faster band as a partial a1 chain (Fig. 3A).

The insertion of 49 non-collagenous amino acids presumably creates a pepsin-sensitive site in the normally pepsin-resistant trimer, and a partial a1 fragment is released. As would be

![WT](image1.png) ![BrtlIV](image2.png)

**Fig. 5. Light microscopy analysis of BrtlIV tissues.** Light microscopy analyses of vertebral bodies (top), calvarium (center), and nasal section (bottom) were performed on mice at 2 months of age. Sections were stained with Masson's Trichrome. The vertebral bodies appeared disorganized and less mineralized in BrtlIV than wild-type (WT). The calvarium is thinner and less mineralized in BrtlIV. In the massilla sections, disorganized islands of bone formation are evident in the OI mice compared with the well organized bone tissue present in the wild-type littermates. The pulp cavity of the BrtlIV molars is necrotic with acute inflammation and bacterial infections. The identity of the dimer band was verified by cyanogen bromide (CNBr) digestion (Fig. 3B).
predicted, this fragment is also detected in collagen synthesized by fibroblasts from mosaic offspring of CxElIa-cre matings.

**DISCUSSION**

We describe here the first knock-in murine model for osteogenesis imperfecta. These mice are an excellent model for OI biochemistry and molecular biology, in that their genome contains a single copy per cell of a mutation causing a typical glycine substitution, under the control of the endogenous promoter and with dominant genetic transmission.

Several mice with type I collagen defects have been described previously. Although the generation of these mice confirmed that collagen defects cause brittle bone disease, none of these mice is suitable for studies of OI pathophysiology or development of gene therapy approaches. They lack the necessary combination of a characteristic molecular defect, physiologic levels of mutant mRNA expression, and dominant genetic transmission. There are two transgenic OI mice. Stacey et al. (19) generated a mouse with a Gly\(^{859}\)Cys substitution in \(\alpha_1(I)\), reproducing the most common type of OI defect. Khillan et al. (20) created a mouse with a collagen minigene that contains the 5' and 3' ends of the human COL1A1 gene but lacks the 41 central exons coding for collagen helix. Because both models are transgenic, the mutant gene is present in the genome in varying copy number. Even in a murine line with only one copy of the transgene, the two normal alleles are still present and alter the ratio of mutant and normal collagen (21). Furthermore, in the case of the transgenic carrying the human minigene, a mutated human collagen was synthesized in the murine background, which generated a hybrid mutant type I collagen.

There are two mice with brittle bones due to recessive transmission of a type I collagen defect. First, the oim mouse originated spontaneously in the Jackson Laboratory. These mice
have a defect in the α2(I) carboxyl-terminal propeptide that prevents the incorporation of their α2 chains into the procollagen trimer (22). Production of α1(I) trimers has been described in only one OI family. Second, Schnieke et al. (23) generated a lethal OI model, which was homozygous for a null Col1a1 gene, obtained by inserting a Moloney murine leukemia virus in the first intron of Col1a1.

In the mice described in this report, an α1(I) 1456G→T point mutation was knocked-into the triple helical domain of the murine Col1a1 locus by gene targeting in ES cells. This mutation generates a Gly349→Cys substitution in exon 23 of the α1(I) chains and reproduces a mutation previously characterized in an NIH OI type IV proband (9). The heterozygous mutant mice described here express the undermineralization of the skeleton and the bone fragility and deformity characteristic of the human patient. Their growth pattern, with normal size at birth followed by growth deficiency until 4–5 weeks of age, resembles the early childhood growth pattern reported for moderately severe OI patients (24). No significant deformities in long bones were evident in mutant mice after puberty. Long bone fractures also appeared to be infrequent in adult mice. Similarly, moderately severe OI patients experience a dramatic decrease of fracture frequency following puberty.

Of particular interest is the phenotypic variability found in the mice heterozygous for the Gly349→Cys substitution, ranging from moderately severe (as described above) to lethal. The mice with lethal outcome died at birth from apparent respiratory insufficiency, a common cause of morbidity and mortality in human OI. Both x-ray and skeletal staining of the lethal mice showed undermineralization of the skull as well as the multiple rib beads and long bone fractures typical in lethal human OI.

In patients with OI, phenotypic variability has been reported in several instances of both related and unrelated probands with the same collagen mutation. There are currently 22 glycine residues in Col1a1 and 8 in Col1a2 for which substitution of the glycine by the same amino acid has been reported in more than one proband. At 9 of those residues in Col1a1 and 4 in Col1a2, there has been variability in OI outcome sufficient to diagnosis different OI types. This variability of phenotype has included both lethal and non-lethal cases at α1(I): Gly1552, Gly1415, or Gly862→Ser (10, 25–32), and at α2(I): Gly811 and Gly859→Ser (33, 34). Only one OI patient with Gly349→Cys substitution has been described (9), so the range of human phenotype for this mutation is currently unknown. The occurrence of phenotypic variability in OI has been ascribed to discrete non-collagenous modifying factors. Since these mice are knock-in models, rather than transgenics, they can be used for investigating the factors modulating OI outcome.

We demonstrated that mice with variable phenotype have equivalent expression of mutant α1(I) mRNA in several tissues, including bone and skin. We also demonstrated that the incorporation of mutant α1 chain into mouse skin was equivalent in mice with variable phenotype. These data support the proposal that discrete non-collagenous modifying factors are responsible for variable severity in OI.

We also plan to use BrtIV mice as a target for a new gene therapy approach. The mice described in this report contain an additional mutation in the allele that carries the Gly349→Cys substitution. This is a silent C→T transition that generates a novel ribozyme cleavage site in the same exon as the disease-causing mutation. We have previously published our data showing the in vitro allele specificity and efficiency of hammerhead ribozyme directed against this murine cleavage site (35). In vivo, intracellular synthesis of ribozyme targeted to the ribozyme site in the mutant allele transcript will be used to suppress expression and ameliorate the severe OI phenotype. Since the mutated allele is an endogenous Col1a1 gene, we will be able to accurately analyze the effects and limitations of our gene therapeutic strategy.

Finally, we utilized the Cre/lox recombination system to generate our Brtl mice. This is the first report of an intronic placement of a floxed transcription/translation stop cassette to suppress gene expression. We planned to use the Cre/lox system to delay the expression of the collagen mutation by blocking expression of the mutant gene in the F1, generating a null allele. In both human and murine examples, a null COL1A1 allele is associated with a very mild, barely detectable, phenotype (36).

We demonstrated limitations in the use of intronic stop cassettes, which should be taken into account in planning the generation of knock-in models. In this particular placement, the stop cassette was responsible for alternative splicing of mutant transcript. Thus, instead of the expected mild phenotype from a null allele, the F1 mice presented with a perinatal...
lethal outcome due to partial expression of an α1 chain with a non-collagenous insertion. Fortuitously, this outcome resembled the lethal phenotype of the human OI cases with in-frame intronic retention (12) and generated a second murine model. We designate these mice BrtlII, in parallel with lethal human type II OI.

Furthermore, mating the chimeras with transgenic mice expressing Cre recombinase under the control of the EIIa promoter, active at the one-cell stage of embryo development (13), was expected to bypass the problem of alternative splicing by eliminating the stop cassette. Our data revealed an incomplete efficiency of the recombinase activity for our construct. The F1 offspring were mosaic for the two forms of the modified allele, i.e. the stop cassette was still detectable in a portion of multiple tissues. Our results establish the relevance of the location and size of the floxed DNA sequence for Cre efficiency.

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