ER Stress-Mediated Apoptosis in a New Mouse Model of Osteogenesis imperfecta

Thomas S. Lisse1, Frank Thiele1, Helmut Fuchs1, Wolfgang Hans1, Gerhard K. H. Przemeck1, Koichiro Abe1b, Birgit Rathkolb1,2, Leticia Quintanilla-Martinez2, Gabriele Hoelzlwinter3, Miep Helfrich4, Eckhard Wolf2, Stuart H. Ralston5, Martin Hrabe de Angelis1*

1 Institute of Experimental Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, Neuherberg, Germany, 2 Institute of Molecular Animal Breeding and Biotechnology, Ludwig-Maximilians University, Munich, Germany, 3 Institute of Pathology, Helmholtz Zentrum München, German Research Center for Environmental Health, Neuherberg, Germany, 4 Department of Medicine and Therapeutics, University of Aberdeen, Institute of Medical Sciences, Foresterhill, Aberdeen, United Kingdom, 5 Molecular Medicine Centre, University of Edinburgh, Western General Hospital, Edinburgh, United Kingdom

Osteogenesis imperfecta is an inherited disorder characterized by increased bone fragility, fractures, and osteoporosis, and most cases are caused by mutations affecting the type I collagen genes. Here, we describe a new mouse model for Osteogenesis imperfecta termed Aga2 (abnormal gait 2) that was isolated from the Munich N-ethyl-N-nitrosourea mutagenesis program and exhibited phenotypic variability, including reduced bone mass, multiple fractures, and early lethality. The causal gene was mapped to Chromosome 11 by linkage analysis, and a C-terminal frameshift mutation was identified in the Col1a1 (procollagen type I, alpha 1) gene as the cause of the disorder. Aga2 heterozygous animals had markedly increased bone turnover and a disrupted native collagen network. Further studies showed that abnormal proc1(I) chains accumulated intracellularly in Aga2/+ dermal fibroblasts and were poorly secreted extracellularly. This was associated with the induction of an endoplasmic reticulum stress-specific unfolded protein response involving upregulation of BiP, Hsp47, and Gadd153 with caspases-12 and –3 activation and apoptosis of osteoblasts both in vitro and in vivo. These studies resulted in the identification of a new model for Osteogenesis imperfecta, and identified a role for intracellular modulation of the endoplasmic reticulum stress-associated unfolded protein response machinery toward osteoblast apoptosis during the pathogenesis of disease.

Introduction

Mutations in type I collagen genes (COL1A1/2) typically lead to Osteogenesis imperfecta (OI), the most common heritable cause of skeletal fractures and bone deformation in humans [1]. OI is classified into eight human subtypes, and to date greater than 500 human COL1A1 mutations have been reported representing a clinical heterogeneity dictated by the complex array of mutations. Recently, novel molecules and loci apart from classic type I collagens have been implicated in both murine [2] and human [3–5] alternative recessive forms of OI, thus expanding the genetic heterogeneity.

Type I collagen is the most common ubiquitously expressed fibrous protein in the extracellular matrix (ECM) of connective tissues with both biomechanical and physiological functions [6]. Type I collagen initially exists as a procollagen precursor with NH2- and COOH-terminal propeptide domains with distinct roles. Type I procollagen molecules consist of three polypeptide coiled subunit chains (two proc1(I) and one proc2(I) chain) that self-associate in the endoplasmic reticulum (ER), and require a highly coordinated post-translational regulation. The helical procollagens are deposited into the extracellular space, proteolytically cleaved, and then organized into highly ordered collagen fibrils covalently cross-linked to increase tensile strength and rigidity. Apart from its biomechanical properties, type I collagen stores key factors for remodeling maintenance, and acts as an adhesive substrate with cellular receptors and other macromolecular components along its major ligand binding regions [7]. These properties regulate complex intracellular signal transduction pathways for tissue remodeling and repair, immune response, polarization, migration, differentiation, and cell survival within various cellular contexts [8].

Based on detailed radiographic, molecular genetic and morphological analyses, structural collagen mutations are likely associated with lethal (type II) and moderate (types III

Editor: Greg Barsh, Stanford University School of Medicine, United States of America

Received May 14, 2007; Accepted November 30, 2007; Published February 1, 2008

A previous version of this article appeared as an Early Online Release on December 3, 2007 (doi:10.1371/journal.pgen.0040007.eor).

Copyright: © 2008 Lisse et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Abbreviations: ALP, alkaline phosphatase; BMC, bone mineral content; BMD, bone mineral density; ECM, extracellular matrix; ENU, N-ethyl-N-nitrosourea; ER, endoplasmic reticulum; OI, Osteogenesis imperfecta; UPR, unfolded protein response

* To whom correspondence should be addressed. E-mail: hrabe@helmholtz-muenchen.de

1 Current address: Bone and Extracellular Matrix Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland, United States of America

2 Current address: Department of Molecular Life Science, Division of Basic Medical Science and Molecular Medicine, Tokai University Medical School, Isehara-shi, Kanagawa, Japan
Author Summary

Osteogenesis imperfecta (OI) is a heterogeneous collection of connective tissue disorders typically caused by mutations in the COL1A1/2 genes that encode the chains of type I collagen, the principle structural protein of bone. Phenotypic expression in OI depends on the nature of the mutation, causing a clinical heterogeneity ranging from a mild risk of fractures to perinatal lethality. Here, we describe a new OI mouse model with a dominant mutation in the terminal C-propeptide domain of Col1a1 generated using the N-ethyl-N-nitrosourea (ENU) mutagenesis strategy. Heterozygous animals developed severe-to-lethal phenotypes that were associated with endoplasmic reticulum stress, and caspases-12 and –3 activation within calvarial osteoblasts. We provide evidence for endoplasmic reticulum stress–associated apoptosis as a key component in the pathogenesis of disease.

and IV) forms of OI [1]. Type II OI represents the most severe form of the disease accounting for ~20 % of cases, and the heterogeneous clinical and biochemical aspects have been described [9]. Most OI-II probands acquire new dominant mutations in COL1A1/2, and a low frequency recurrence risk can be the result of gonadal mosaicism in one of the parents [10]. OI-II-related mutations behave in a dominant negative manner as the mutant proα(I) proteins affect wild-type chain registration and formation due to the polymeric nature of assembly, impairing overall ECM stoichiometry and integrity. Typically in OI-II cells, abnormal trimers are poorly passaged through the secretory pathway, and are more vulnerable to degradation and overmodification, thus the cytoprotective unfolded protein response (UPR) is concordantly instigated [11–13].

Despite the observation of machinery involving BiP chaperones that might target most defective procollagens for subsequent proteasomal degradation in OI-II cells [13], the direct activation of apoptosis has not been shown to date. Here, we provide the first report on the isolation, cloning and characterization of the skeletal phenotype in a novel ENU-induced mouse line for human OI affecting the terminal C-propeptide region of Col1a1. Furthermore, we provide evidence of caspases-12 and –3-mediated apoptosis in Aga2 osteoblasts as a critical step toward increased cell death, thus broadening our molecular insight into OI.

Table 1. Mode of Inheritance and Postnatal Lethality

<table>
<thead>
<tr>
<th>Breeding Pairs</th>
<th>Litter Size</th>
<th>Postnatal Progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>♂♂ × ♀♀</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results

Identification and Lethality of Aga2/+.

The original Aga2/+ mouse was identified in the Munich ENU dominant mutagenesis screen [14], and displayed an abnormal gait due to deformity of the hind limbs at five weeks of age among the F1 mutagenized progeny. Aga2/+ males have general reproductive success and the mutation segregated in a dominant manner with complete penetrance (Table 1). Aga2/+ females produced smaller litter sizes due to a reduction in body size. A large subset of Aga2/+ animals succumbed to postnatal lethality (Table 1), and featured severe bone deformities and fractures. Aga2/+ inter se mating yielded non-Mendelian ratios for dominant inheritance suggesting embryonic lethality. Various embryonic stages were investigated to determine gestational arrest in homozygotes, which was estimated to occur around embryonic day 9.5 post coitum (data not shown).

Positional Cloning and Characterization of the Aga2 Mutation

Genetic mapping of the Aga2 locus was performed following the standard outcross-backcross breeding strategy. The mutation was mapped to a 700 kb domain on Chromosome 11 (Figure S1). Within the Aga2 candidate region, two skeletal genes were identified, Col1a1 (procollagen type I, alpha1) and Chad (chondroadherin). Both candidate genes were sequenced and a novel T to A transversion mutation within intron 50 of Col1a1 was identified (Figure 1A). The Aga2 substitution generated a novel 3’ splice acceptor site and predicted a terminal frameshift beyond the endogenous stop (Figure 1C). On the transcript level, cDNA from homozygous embryos revealed a 16 bp expanded transcript, whereas in heterozygous embryos equal levels of two transcripts are present (Figure 1B). We further confirmed the mutation at the genomic level. The T to A exchange disrupted the endogenous MspI restriction site, generating a cleavage-resistant allele in Aga2/+ samples (Figure 1B).

The C-terminal portion of human, and murine as well as Aga2 mutant Col1a1 was aligned highlighting specific residues of importance (Figure 1C). Of interest, the most terminal conserved cysteine C244 (aa 1451) was ablated, and 48 endogenous amino acids including the stop were frameshifted, concomitantly predicting 90 new amino acids beyond the termination position. The Aga2 frameshift begins 56
amino acids away from the chain selectivity sequence [15]. Additionally, five new cysteines and a potential N-linked oligosaccharide (CHO) attachment site were introduced. The secondary structure of the Aga2 product was predicted to remove the hydrophobicity of the short loop formed by the last intra-chain disulphide bond (data not shown).

Aga2 Skeletal and Growth Phenotypes

Among all Aga2+/ animals, the gross skeletal phenotype was discernable starting between days 6 - 11 after birth. Heterozygous animals that survived to adulthood were classified as moderately-to-severely affected and displayed the hallmark dystrophic limb(s), long bone and pelvis fractures, reduced body size, and generalized decreases in DXA-based bone parameters (Figure 2 and Table S1; data were collected from the German Mouse Clinic dysmorphology primary screen [16]). Lethal animals developed thin calvaria, hemorrhaging at joint cavities and intracranial sites, scoliosis, provisional rib and long bone calluses and deformities, body size deficit, pectus excavatum, gasping, cyanosis, platyspondyly, edema of the eyes, greasy skin, and eczema (Figure 2 and data not shown). Comminuted fractures incurred signifying the severe brittleness of Aga2+/ bones accompanied by many repair blastemas characteristic of a type II OI phenotype.

The effect of the mutation on volumetric bone mineral density (vBMD) and content (vBMC) was evaluated in adult mice using in vivo peripheral quantitative computed tomography (pQCT; Table S2). In Aga2+/ distal femora, trabecular and cortical vBMD as well as cortical vBMC were substantially decreased compared to controls. In contrast, the trabecular vBMC was unchanged due to enlarged medullar areas (suggesting elevated resorption in Aga2+/). Collectively, these results suggest potential defects in mineralization and/or bone formation in Aga2+/ mice.
Clinical Chemical and Hormone Analysis

Several serum biochemical and hormonal markers were evaluated to detect possible bone metabolic disturbances (Table S3). Total alkaline phosphatase (ALP) and osteocalcin levels were significantly increased in both sexes in Aga2+/+ compared to littermate controls. In addition, circulating TRACP 5b (an osteoclast marker) was significantly elevated in Aga2+/+. No changes in inorganic phosphate levels were observed (data not shown). Aga2+/+ animals depicted a significant increase in PTH levels. Furthermore, Aga2+/+ mice yielded a distinct increase in calcitonin, which was more pronounced in females. Taken together, the significantly increased bone formation and resorption markers indicate that in Aga2+/+ mice metabolic bone turnover is elevated. This was further supported by histomorphometric analysis of bone formation and resorption showing increased numbers of endogenous osteoblasts and osteoclasts, an increased bone formation rate (BFR) and a reduced mineral apposition rate (MAR) in Aga2+/+ bone samples (Figures S2, S3 and Table S4).

Altered Cellular and Collagen Structures in Aga2+/+

Fibroblast and type I collagen features in Aga2+/+ connective tissues were evaluated by transmission and scanning electron microscopy (TEM and SEM). Aga2+/+ dermis contained more heterogeneous populations of fibroblasts with smaller nuclei, aberrant dilated electron-dense ERs, lysosomes and empty autophagic-like vacuoles interspersed throughout the cells (Figure 3A and 3B). Aga2+/+ ERs appeared contiguous with secretory vesicles signifying the formation of ER associated compartments. SEM studies on cortical nanostructure of Aga2+/+ samples depicted a less-parallel, less-densely packed network of collagen bundles when compared to controls (Figure 3C–3F).

Procollagen trafficking was assessed in immunofluorescence studies using dermal fibroblasts (Figure 4). To uncover mature type I collagen integrity we used an antibody that was directed against the triple helical domain and was also capable of detecting procollagens (Figure 4A). Wild-type cells depicted both intracellular and intact extracellular surface staining (Figure 4B). In Aga2+/+ cells, the intact type I collagen surface stain was largely reduced while intracellularly retaining procollagen molecules were stained (Figure 4C). Double immunofluorescence staining of proα1(I) chains was performed using antiserum LF-67 (Figure 4A), protein disulphide isomerase (PDI), and Golgi phosphoprotein 4 (GOLPH4) for detection of the ER and cis-Golgi apparatus, respectively. Merged stainings showed proα1(I) chain retention within the ER (Figure 4E) but not in the cis-Golgi (Figure 4G), when compared to controls (Figure 4D and 4F). It is speculated that aberrant procollagen molecules aggregate into vesicular structures (Figure 4E, merged green dots), which may be part of the proteasome. To further clarify the intracellular abnormalities double immunofluorescence staining of proα1(I) chains was performed using antiserum LF-41 and β-actin antibody for the cytoskeleton. The LF-41 epitope resides within the wild-type terminal C-propeptide.

Figure 2. Aga2+/+ Skeletal and Growth Abnormalities
(A) Lateral μCT views of 12-wk-old male skeletons highlight the axial vertebrae, pelvis (white trace), and hind limb (encircled) defects in Aga2+/+ mice.
(B) Cranial features of a 16-d-old lethal Aga2+/+ mouse.
(C) Skeletal radiograph of a 16-d-old lethal Aga2+/+ animal. Provisional-bony rib and long bone calluses and deteriorated pelvis depicted with arrows.
(D) Severe growth defects in Aga2+/+ animals.
(E) Skeletal preparations.
doi:10.1371/journal.pgen.0040007.g002

Apoptosis in Osteogenesis imperfecta
overlapping segment of the Aga2 frameshifted region and captures only molecules that contain chains with the normal C-propeptide (Figure 4A). Using antiserum LF-41, control fibroblasts depict dynamic intracellular procollagen and isoform trafficking to the infoldings of the plasma membrane (Figure 4H). Contrarily, Aga2/+ fibroblasts retained intracellular (wild-type) procollagen molecules at near perinuclear regions (Figure 4I, arrow). Surface staining in both wild type and Aga2/+ cells was limited given that the LF-41 antiserum was unable to discriminate mature, proteolysed type I collagen in the ECM. Based on our results, the mutation affected proα1(I) chain processing by blocking its ER-to-Golgi anterograde transport, thus inhibiting vesicular exocytosis to the matrix.

Altered Ex Vivo Osteogenic Activity in Aga2/+ Calvarial Culture System

OB metabolism and function were characterized utilizing the primary calvarial culture system. We determined the growth curve of Aga2/+ primary OBs and observed a limited saturation density within the stimulated samples (Figure 5A) that was accompanied by a relative increase in ALP levels until day 26 (Figure S4A). Also, Aga2/+ total protein and mitochondrial reductase activity levels were significantly increased (Figure S4B). Consistent with our immunofluorescence studies there was a clear reduction in the total amount of secreted acid-soluble collagens tested in Aga2/+ media (Figure S4B). Of note, unstimulated Aga2/+ OBs depicted no significant change in cellular protein and metabolic activity levels (data not shown).

To address the functional defects in Aga2/+ OBs, nodular formation, growth and binding capacities were studied over time (Figure S4C). The formation of bone-like nodules was persistently reduced between days 9 and 16 in Aga2+. In addition, the average area of individual Aga2/+ nodules was reduced compared to controls. Lastly, a 3.2 fold comparative increase in nodular dye-binding capacity was observed in Aga2/+ (Figure S4C). Taken together, these results demon-

**Figure 3.** Electron Microscopical Findings in Dermal Fibroblasts and Cortical Bone

(A,B) TEM micrographs of wild-type and Aga2/+ fibroblasts. The rough endoplasmatic reticulum (rER) is engorged in Aga2/+ cells (bars, 2 μm for main, 1 μm for inset). Increased intracellular empty vacuoles (arrow heads) and lysomes (L) were present in Aga2/+.

(C–F) SEMs of femoral endosteal surfaces reveal differences in organization of the collagen matrix. (C,D) Outer cortical surface. (E,F) Inner cortical surface.

doi:10.1371/journal.pgen.0040007.g003
strate clear functional and metabolic disturbances in Aga2+/+ OBs.

Initiation of ER Stress-Induced Apoptosis in Aga2+/+ OBs

To show involvement of key regulators of ER stress response pathways, we performed qPCR studies using primary calvarial OBs (Figure 5B). At days 16 and 26 in culture (11 and 21 days after induction, respectively), expression of the molecular chaperones genes Hspa5 (also known as BiP/GRP78) and Serpinh1 (also known as Hsp47) was upregulated; 1.5 – 2.2 folds above control. The expression of Ddit3 (also known as Gadd153/chop), a transcription factor involved in the induction of apoptosis, was ~3.7 folds higher in Aga2+/+ cells at day 26 compared to control levels. To confirm the induction of cytoprotective unfolded protein response (UPR) in Aga2+/+ cells, Western analysis of BiP protein expression was performed using primary calvarial OBs. As shown in Figure 5C BiP protein levels were increased compared to controls.

Within the context of ER-induced stress we investigated cell apoptosis. The Aga2 mutation led to a combined relative increase in the number of early-late stage TUNEL-positive picnotic OBs compared to control (Figure 6E). At 16 days in culture, Aga2+/+ preparations depicted a 10 % relative increase in the number of OBs, which contained aberrant caspase-12-immunoreactivity (Figure 6A and 6B; 3.3 ± 0.9 % +/- and 12.9 ± 1.9 % Aga2+/+; p = 0.0007 Student t-test). For specificity, proteolytic processing of procaspase-12 was evident only in Aga2+/+ samples via Western analysis (Figure 6G). Caspase-3/7 activation was independently confirmed depicting a significant 9 % comparative increase in activity in Aga2+/+ OBs (Figure 6F). Lastly, activated caspase-3 immunoreactivity was observed within femoral periosteum (Figure 6C and 6D). Aga2+/+ tissue contained elevated relative numbers of activated caspase-3-positive OBs (28.9 ± 2.7 % +/- and 42.6 ± 4.1 % Aga2+/+).
Figure 5. Anomalies in Aga2+/− Osteoblast Growth and Induction of Cytoprotective Unfolded Protein Response

(A) Growth curve analysis of +/- and Aga2+/− primary calvarial culture system. (B) UPR, qPCR analysis of stimulated calvarial OBs. Values represent means ± SEM, where n = 4–5 experiments (*p < 0.01 Student t-test). (C) Western analysis of BiP using primary OBs. doi:10.1371/journal.pgen.0040007.g005

Discussion

ENU mutagenesis is a powerful approach to identify mouse models for human skeletal disorders by generating novel phenotypes. Human mutations affecting the α1/2(I) C-propeptide coding and non-coding regions have been identified less often than other types of mutations and reflect strong variability in clinical outcomes. Here, we provide the first report on the cloning and phenotypic description of the novel mouse line Aga2, which bear a chemically induced genetically stable mutation affecting the terminal C-propeptide domain of Col1a1. We provide evidence that abnormal proc1(I) chains accumulated intracellularly and were poorly secreted extracellularly. Furthermore, we show for the first time that this was associated with the induction of an ER stress-specific unfolded protein response (UPR) with caspases 12 and 3 activation and apoptosis of osteoblasts both in vitro and in vivo. The Aga2 line provides a unique tool for understanding the underlying mechanisms of the incurable debilitating human disease Osteogenesis imperfecta (OI) and the efficacy of novel therapeutic strategies.

Aga2+/− Skeletal Phenotypes

Aga2+/− mice feature phenotypic variability ranging from postnatal lethality to moderate-to-severe changes including increased bone fractures, fragility, deformity, osteoporosis, and disorganized trabecular and collagen structures. Compared to the lethal transgenic lines [17,18], the Aga2+/− phenotype is stronger and associated with increased lethality, but comparable to the BritIV knock-in (Col1a1<sup>G310D</sup>) line [19]. The biochemical serum and histomorphometric results of Aga2+/− mice indicated an elevated bone turnover that is described similarly for OI patients [20–23]. Our ex vivo studies showed that Aga2+/− OBs deposited less collagen matrix, and were overly active, affecting nodule growth and function.

Aga2+/− proc1(I) Chain Defects and Human Case Correlations

The procollagen triple helix proceeds from the carboxyl to amino end, and this association is modulated by correct folding via intra-chain disulphide bonds in the C-propeptide region before chain association and inter-chain disulphide linkage [24,25]. In certain C-propeptide OI-II lethal conditions, the mutated proc1(I) chains can associate with normal chains to form secreted triple helical procollagen molecules despite alterations in endogenous disulphide bonds [12,26], while in other lethal cases chain formation with the mutated propeptide is entirely or largely precluded [27,28]. Based on these results, the lethal OI phenotypes are heterogeneously derived from structural and/or cellular metabolic defects.

At the biochemical level, we report significant increased retention of aberrant procollagen molecules within Aga2+/− cells, compromising cellular metabolism and the ECM. Moreover, we show that the majority of accumulated chains were wild type in nature. Gel analysis of cDNA showed significant splicing of the new Aga2 splice site. Although cryptic mRNA transcripts were readily available for translation, unstable mRNA decay and/or anomalies in transport from the nucleus to the cytoplasm due to secondary structure may have mediated low-level translation of mutant chains. The presumptive elongated chain with newly introduced cysteins and disruption of a crucial beta sheet due to the Aga2 mutation likely destabilized protein conformation, thus affecting preferential chain assembly and proteolysis. Also, a N-linked oligosaccharide unit was introduced capable of altering intracellular processing and secretion of procollagen as well [29].

Our findings were consistent with observations made by Willing et al. [30] who investigated a human OI family that harbored a mutation in the 3′ end of COL1A1, which ablated the intra-chain disulfide cysteine as in the Aga2 condition. Fitzgerald et al. [13] reported that the fate of mutated unassembled C-propeptide chains from the proband described by Willing et al. were intracellularly degraded in the proteasome. Similar to Aga2, it was unclear if the mutated chains ever incorporated into procollagen molecules causing protein suicide and the expected downstream effects [31], or involved an alternative mechanism to influence the clinical outcome.

ER Stress and Degradation within Aga2+/− Cells

Many disorders result from the cell’s inability to export mutated proteins and enzymes from the ER, including OI [32,33]. Depending on the nature of the mutation, incorrectly folded proc1(I) molecules are managed via multipartite...
highly regulated sorting pathways in order to retain ER homeostasis and to prevent the secretion of abnormal proteins. The cytoprotective unfolded protein response (UPR) involves the activation of complex regulatory ER stress signaling mechanisms that can either repress protein synthesis or upregulate ER-resident chaperons and other translation regulators [34]. BiP/Grp78, a central regulator of ER function, was shown to bind to proα(I) chains from cell strains, which harbored unique C-propeptide mutations that inhibited chain association [11] and mediated intracellular ER-degradation [12]. Contrarily, mutations that inhibit folding in the triple helical domain of type I procollagen chains do not result in abnormal BiP binding or induction even though the aberrant molecules are retained within the ER [35].

In Aga2+/−, the presence of dilated ERs and accumulation of aberrant procollagens in the ER strongly support intracellular trafficking defects and degradation within the proximal region of the secretory pathway. We speculate that aberrant procollagen molecules aggregated into vesicular structures (Figure 4E), which are part of the proteasome. Degradation did occur within more distal secretory compartments by the presence of lysosome-like structures [36]. In Aga2+/− OB cultures treated with ascorbate, BiP levels were slightly upregulated implicating its constitutive involvement during ER stress. Previous studies from Chessler et al. suggested that in skin fibroblasts from OI patients with mutations in the C-terminal propeptide BiP peaked about 48 hours after exposure to ascorbate and that longer exposure resulted in decreasing levels [11]. As cells studied here were

Figure 6. Initiation of Apoptosis in Aga2+/−

(A,B) Caspase-12 immunofluorescence of 16-d-old stimulated OBs.
(C,D) Activated caspase-3 labeling of 12-wk-old distal femur depicting dying cells within the periosteum (white arrows) and cortical bone (red arrows). Nuclei were stained with DAPI.
(E) Quantification of TUNEL stained 16-d-old stimulated calvarial OBs.
(F) Caspase-3/7 OB activity was measured via a fluorometric substrate assay.
(G) Western analysis of procaspase-12 using primary OBs. Results are presented as mean ± SEM (*p = 0.0002 ANOVA, and **p = 0.04 Student t-test), where n = 4 independent experiments. Bars, 50 μm in (A) and (B), 100 μm in (C) and (D) main panels, 20 μm in (C) and (D) insets. Arbitrary fluorescent unit (AFU), bone marrow (Bm), cortical bone (Cx), low-present isoform (IS), periosteum (Ps).

doi:10.1371/journal.pgen.0040007.g006
Ex Vivo and In Vivo Evidence for Increased ER Stress-Induced Apoptosis

Many diseases are associated with either inhibition or increase of apoptosis [34,38], but to date the active involvement of apoptosis during OI pathogenesis is inconclusive. Caspase-12 is a specific mediator of the ER stress-induced UPR within skeletal tissue [39]. In primary Aga2+/−OBs, removal of the procaspase-12 adaptor protein-binding domain was evident, and the processing of procaspase-12 demonstrated the ER stress origin of mutated pro-C(II) initiator signals. In addition, the presence of increased activated caspase-3 and TUNEL-positive OBs in Aga2+/−primary culture and periosteum also implicated a tendency toward apoptosis commitment and cell death, respectively.

Although several models have been proposed for the direct activation of caspase-12 the entire process has yet to be described. The Bcl2 family-proteins are well-established components of the apoptotic machinery, some of which are associated with ER stress pathways. For example, Bim (Bcl2-interacting mediator of cell death) activates caspase-12 via translocating from the dynein-Rich ER compartment upon stress [40]. BiP is known to inhibit caspase-mediated cell death by forming complexes with procaspases 7 and 12, and BiP disassociation facilitates procaspase activation [41]. Gadd153/CHOP is a key transcription factor in the regulation of cell growth, differentiation and ER stress-induced apoptosis. Gadd153 induces death by promoting ER client protein load and oxidation via translocation of target genes, which mediate apoptosis, presumably leading to caspase cascades [34]. In our studies, Gadd153 was highly induced over time, and was presumably triggered by mutated procollagens and the modulation of ER stress signaling. Of interest, the anti-apoptotic component Bcl2 is down regulated during ER stress conditions attributed to Gadd153 upregulation, leading to enhanced oxidation and apoptosis [42]. Thus, given the upregulation of Gadd153 within Aga2+/−OBs, Bcl2 was likely down regulated, exacerbating and contributing to cell death. Further studies are necessary to elucidate the pro- and anti-apoptotic components that modulated the severe clinical outcome in Aga2+/−animals. In addition to severe long bone deformities we observed immunological, blood, heart, lung and energy metabolic defects pointing to a systemic effect of the Col1a1 mutation. Further experiments will be necessary to elucidate the influence of the systemic defect on bone and early lethality (unpublished data).

The molecular diversity of apoptosis in OI is unknown, and it is unclear if the apoptotic program is a phenomenon of type 1 procollagen mutations that severely affect chain assembly and retention caused by triple helical versus C-propeptide structural defects, or by both. Recently, Forlino et al. [43] evaluated phenotype heterogeneity within the BritIV knock-in OI mouse line and identified increased Gadd153 transcript and protein levels within calvaria of lethal animals. In this study we isolated and characterized a unique mouse model for OI, and provided evidence that the Aga2 C-propeptide C(II) mutation induced ER-mediated osteoblast apoptosis that affected cellular function and metabolism, which we now suggest to be a key component, among others, that influenced disease severity in Osteogenesis imperfecta.

Materials and Methods

Antibodies and kits. Activated caspase-3 (R&D Systems, Germany), caspase-12 (BD Pharmingen, Germany), anti-α-tubulin (Sigma, Germany), β-actin, type I collagen, and BiP/GRP78 (all Abcam, UK), and fluorescently labeled secondary antibodies (Molecular Probes, Germany) were commercially purchased. Antisera LF-41 and -67 ([44] were generous gifts provided by Dr. L. Fisher (NIH, USA). The serum biochemical analysis has been previously described [45]. The mouse TRACP 5b (IDS Ltd), osteocalcin ELISA (B1T), and mouse/rat intact PTH as well as calcininin immunoradiometric assay (Immunodiagnostics, Germany) kits were commercially purchased. All measurements were performed based on the manufacturers’ recommendations.

Animal housing and handling. Mouse husbandry was conducted under a continuously controlled specific-pathogen-free (SPF) hygiene standard according to the Federation of European Laboratory Animal Science Associations (FELASA) protocols. Standard rodent diet and water were provided ad libitum. All animal experiments were conducted under the approval of the responsible animal welfare authority.

Linkage analysis and Aga2 genotyping. The F1 dominant ENU mutagenesis screen was conducted on the inbred strain C57He/BjFe and has been previously described [14]. Aga2 was backcrossed to wild-type C3H mice for five generations, and then outcrossed to C57BL/6J (F2) and backcrossed to the outcrossed strain (F3). Phenotype-positive N2 progeny were tail-genotyped with microsatellite markers. Recombination fractions and map distances were calculated using Mapmanager QTiX. All primers for sequencing are available upon request. For genotyping, a PCR fragment containing the entire intron 30 of Col1a1 was previously used [46]. The positions of the primer pair were 5'-gggagctcttggttatt-3' and rev-5'-ggaggtgctggttggttg-3'. The product was then cleaved using MspAI for gel analysis.

Skeletal and radiological analysis. Skeletal preparations were generated as previously described [46]. Simple and compound fractures were grossly examined. Compression fractures were evaluated using a dissecting microscope. For μCT scans (Tomoscope 10010m; VAMP, Germany), image reconstruction and visualization were performed using the ImpactCB and ImpactView software (VAMP, respectively). μCT analysis was performed as previously described [37].

Electron microscopy, histology, and histomorphometry. SEM was performed as previously described [48]. For TEM, lower dorsal skin sections, where 6-20 repeated measurements were made per section using four non-sequential sections, were performed as previously described [49]. Automatic color thresholding was applied to stacked image sets. Labels were traced with minimal operator bias. 6 - 20 repeated measurements were made per section using four non-sequential sections, where n (4 - 6) equals the number of animals examined.

Western analysis. Dermal fibroblasts were cultured as previously described [53]. For caspase-12 and BiP detection, cells were lysed in RIPA buffer with protease inhibitors. Supernatants were analyzed using the NuPAGE Novex gel system (Invitrogen, UK).

Primary OB culture and cellular assays. OBs were prepared from 3-day-old pups with similar appearance as previously described [54]. Cells were plated at 2 × 10⁴ cells/cm² for all experiments. OB differentiation was initiated via media exchange (i.e. ø-MEM w/10% FCS, 2 mM glutamine, 1 % pen-strep, 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate). A minimum of four calvarialgenotype was conducted presenting an independent experiment (n) with four repeated measurements. DNA, total protein, ALP and MTT (kit) measurements were performed in 24-well plates as previously described [55]. Caspase-3/7 activity was monitored using a fluorogenic.
substrate (AC-DEVD-AMC, ANXXOR, Germany) at excitation 380 nm and emission 460 nm. Mineral content was quantified by establishing an alizarin red standard curve and measuring dye release using 10 % hexadecylpyridinium in 6-well plates at 570 nm. For nodule characterization, samples were analyzed using the ImageJ software.

**Indirect immunofluorescence and TUNEL analysis**. Cells were cultured on glass coverslips coated with poly-L-lysine. For immunohistochemical analysis, sodium citrate (10 mM, pH 6.0 in microwave) and proteinase K (10 µg/ml, 37 ºC) antigen retrieval methods were performed, and the Vectastain ABC kit (Vector Labs, USA) utilized. The TUNEL assay was performed with an ALP-conjugated kit (Roche, Germany). Independent preparations (n = 4) were analyzed with repeated measurements. For cell culture, 300 - 400 cells were counted per image field. For in vivo caspase-3 analysis, a region 0.4 mm from the growth plate was analyzed, and 400 - 480 total cells were evaluated within set regions (i.e. magnification and field size).

**qPCR**. RNA and cDNA were prepared as previously described [56]. qPCR was performed using the TaqMan and QuantiTect ready-to-use primers (Qiagen, Germany). Independent preparations (n = 4) were analyzed using the Prism 4 (GraphPad, USA) and SigmaStat v. 3.1 (Systat Software, Germany) software.

**Supporting Information**

**Figure S1.** Genome-Wide Linkage and High-Resolution Haplotyping Analysis of the Aga2 Locus
(A) Genome-wide linkage analysis revealed Aga2 linkage on mouse distal Chromosome 11.
(B) Haplotype analysis identified the most probable Aga2 candidate region between D11Mit288 and D11Lis2. Log of odds ratio (LOD), centimorgan (cM), megabase (Mb), and (black) heterozygous (C3HeB/FeJ & C57BL/6 alleles), (white) homozygous (C57BL/6 allele). Data depicted from Mouse Genome Informatics (MGI; www.informatics.jax.org) and HUGO Gene Nomenclature Committee (HGNC; http://www.genenames.org).

**Figure S2.** In Vivo Bone Formation and Resorption in 16-Wk-Old Male Mice
(A,B) Bone formation and mineral accretion were monitored by dual calcein (red arrows) and tetracycline (white arrows) in vivo labeling (dL). Tibial secondary cancellous (c, inset) and endocortical (not shown) regions were characterized. Bar, 100 µm for main in (A) and (B) combined. Bracketed bars within the corner boxes represent mean ± SEM of sample dL lengths (17.9 ± 0.6 µm ± d, **p < 0.01; and 11.7 ± 1.0 µm ± d, Aga2+). (C,D) Undecalcified tibial sections in control and Aga2+ respectively, depict increased resorptive bays with multinucleated OCs (black arrows) at the osteogenic zone, and extended hypertrophic zone of the growth plate (Gp; horizontal arrow) in Aga2. Bar, 100 µm combined; stained with von Kossa and counterstained with Paragon. Bone marrow (Bm), trabecula (Tb).

**Figure S3.** Quantitative Analysis of Physiological Bone Growth and Remodeling in 16-Wk-Old Male Tibiae
(A) Percentage of secondary trabecular surface encompassed with OBs.
(B) MAR calculated within both secondary cancellous (c) and endocortical (EC) bones, while BFR was calculated for mid-cancellous bones.

**References**

**Acknowledgments**
We would like to especially acknowledge M. Schulz, R. Seeliger, S. Witsch, E. Holstrup, L. Jemsen, and H. Wehnes for their excellent technical assistance. TSL would like to acknowledge M. Rosemann, J. Favor, M. Atkinson, G. Moeller, W. P. Liao, E. Drew, S. J. De Armond technical assistance. TSL would like to acknowledge M. Schulz, R. Seeliger, S. Witsch, E. Holstrup, L. Jemsen, and H. Wehnes for their excellent technical assistance. TSL would like to acknowledge M. Rosemann, J. Favor, M. Atkinson, G. Möller, W. P. Liao, E. Drew, S. J. De Armond (UCSF), J. Marini (NIH), P. Salmon (SkyScan, Belgium), S. Ferrari (University of Geneva), U. Kornak (Max Planck Institute, Berlin), and S. Rieger for critical discussions and support of the manuscript.

**Author contributions**. TSL and MHdA designed the experiments and wrote the paper. TSL, FT, WH, BR, GH, and MH performed the experiments. TSL, FT, IQ, HF, WH, GKHP, KA, BR, SHR, and MHdA analyzed the data. EW provided reagents/materials/analysis tools.

**Funding**. This work was supported by National Genomforschungszentrum (NGFN 01GR0430) and EU ANABONOS (LSH2-2002-2.1.4-3) grants to MHdA.

**Competing interests**. The authors have declared that no competing interests exist.


