Severe Extracellular Matrix Abnormalities and Chondrodysplasia in Mice Lacking Collagen Prolyl 4-Hydroxylase Isoenzyme II in Combination with a Reduced Amount of Isoenzyme I*

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This work was supported by Health Science Council Grant 114344, Center of Excellence 2012–2017 Grant 251314 From the Academy of Finland and the S. Jusselius Foundation (to J. M.), Postdoctoral Researcher Grant 140144 from the Academy of Finland (to A. M. S.). This work was also supported, in part, by National Institutes of Health Grant RO1 AR065403 (to E. S.). Finally, we thank all members of our research group for their efforts and fruitful discussions.

Received for publication, April 30, 2015, and in revised form, May 20, 2015 Published, JBC Papers in Press, May 22, 2015, DOI 10.1074/jbc.M115.662635

Collagen prolyl 4-hydroxylases (C-P4Hs) I and II in growth plate development were studied.

*Background: Roles of collagen prolyl 4-hydroxylases (C-P4Hs) I and II in growth plate development were studied. Results: Proliferating chondrocytes undergo apoptosis in C-P4H-I+/−;C-P4H-II−/− growth plates, and the mice develop chondrodysplasia.

*Conclusion: Biomechanically impaired extracellular matrix is the primary cause of death in C-P4H-I+/−;C-P4H-II−/− growth plate chondrocytes.

*Significance: Sufficient C-P4H activity is essential for growth plate chondrocyte survival and proper skeletogenesis.

Most of the 4-hydroxyproline (4Hyp)5 in mammalian proteins is found in the -X-4Hyp-Gly- sequences of the 28 distinct collagen types and more than 20 proteins with collagen-like domains (1, 2). Collagen prolyl 4-hydroxylases (C-P4Hs, EC 1.14.11.2) residing within the lumen of the endoplasmic reticulum (ER) catalyze the formation of 4Hyp in collagens and collagen-like sequences (3, 4). The C-P4Hs have an essential role in the synthesis of all collagens, because the resulting 4Hyp residues are necessary for the folding of the newly synthesized col-
lagen polypeptide chains into stable triple-helical molecules. Nonhydroxylated collagen polypeptides cannot form functional molecules in vivo, and almost complete hydroxylation of the proline residues in -X-Pro-Gly- triplets is required for the generation of a molecule that is stable at human body temperature (1).

The vertebrate C-P4Hs are α2β2 tetramers with three catalytic α subunit isoforms that form [α(I)]2β2, [α(II)]2β2, and [α(III)]2β2 tetramers, the type I, II, and III C-P4Hs, respectively, with protein disulfide isomerase serving as the β subunit (5–9). The C-P4H α subunit mRNAs are expressed in many human and mouse cell types and tissues, expression of the α(III) mRNA generally being at a much lower level than those of the α(I) and α(II) mRNAs (5, 7, 8). At the protein level, C-P4H-I is the main form in most cells, whereas C-P4H-II is the predominant form in chondrocytes, osteoblasts, endothelial cells, and certain other cell types (10, 11).

Because no heritable human disease is known to be caused by mutations in any of the C-P4H α subunit genes, gene-modified mice can provide models to understand the individual roles of C-P4H isoenzymes. C-P4H α(I) subunit knockout mice (P4ha1−/−) generated by us are embryonic lethal at E10.5, whereas P4ha1+/− mice displayed no obvious anatomical or histological abnormalities (12). The P4ha1−/− embryos suffered from an overall developmental delay and rupture of the basement membranes caused by reduced production and abnormal deposition of collagen IV. The level of C-P4H activity in the null embryos and fibroblasts was ~20% of that in the wild type, this residual activity evidently being due to the presence of the other two isoenzymes (12).

The expression pattern of C-P4H-II (10, 11) suggests that it may play an important role in the development of the skeletal structures and capillaries. To study the in vivo role of C-P4H-II, we generated mice with targeted inactivation of the α(II) subunit gene (P4ha2). Surprisingly, the mice were viable and fertile and showed no obvious phenotypic abnormalities, suggesting functional compensation by the other two C-P4H isoenzymes. To study the combined effects of decreased C-P4H-1 activity and lack of C-P4H-II activity, we generated P4ha1+/−; P4ha2−/− mice. These compound mutant mice were found to be smaller than their littermates, and they had shorter long bones and developed kyphosis when older. A transient developmental defect was observed in the growth plates of the long bones, with hypocellular areas in the middle of the proliferative zone and loss of columnar organization of the proliferative chondrocytes. Moreover, in P4ha1+/−; P4ha2−/− growth plates, the extracellular matrix (ECM) was disorganized, less dense, and biomechanically impaired when compared with P4ha2+/− and control mice. The amount of 4Hyp in the collagen extracted from the growth plates and tibia of newborn P4ha1+/−; P4ha2−/− mice was significantly reduced, and a consequent decrease in the thermal stability of the growth plate collagen was measured, but no accumulation of collagen within the ER lumen or any signs of ER stress were detected. Some of these defects, albeit in a much milder form, were also detected in the P4ha2−/− mice.

**Experimental Procedures**

**Generation of Mouse Lines with Inactivated P4ha1 and P4ha2 Genes**—Establishment of the knock-out mouse line for C-P4H-I by insertion of a lacZ-PKG-neo cassette into exon 2 of the P4ha1 gene (Fig. 1A) has been described previously (12). To generate a knock-out construct for the inactivation of C-P4H-II, the Sv) mouse cosmid library (Stratagene) was screened with a mouse α(II) subunit cDNA (6) as a probe, and BAC mouse library screening services (Genome Systems) were used to obtain 5′ genomic clones for the P4ha2 gene (13). The genomic clones obtained were used to build the P4ha2 targeting construct that contained 1.2- and 4-kb genomic arms and the 4-kb lacZ-PKG-neo cassette inserted in-frame into the third exon of the P4ha2 gene after the translation initiation codon (Fig. 1B). The NotI linearized targeting construct was electroporated into R1 embryonic stem cells (kindly provided by Dr. A. Nagy, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada) and selected with G418, and genomic DNA isolated from G418-resistant colonies was screened by PCR. Genomic insertion of the targeting construct was screened using a forward primer from intron 2 of the P4ha2 gene and a reverse primer from the 5′ end of the LacZ gene (Fig. 1B and Table 1). The wild-type allele was identified using the same forward primer and a reverse primer from exon 3 of the P4ha2 gene (Fig. 1B and Table 1). Correct targeting was confirmed by Southern blotting analysis of EcoRI-digested genomic DNA with a 450-bp probe from intron 2 of the P4ha2 gene (Fig. 1B). Correctly targeted ES cell clones were injected into C57BL blastocysts, and two separate mouse lines were generated by standard methods and backcrossed with C57BL mice. A forward primer from intron 2 and a reverse primer from intron 3, amplifying a 600-bp fragment, were used for genotyping the wild-type P4ha2 allele, and a primer pair from the LacZ gene, amplifying a 850-bp fragment, was used for the targeted P4ha2 allele (Fig. 1B and Table 1).

To generate P4ha1+/−; P4ha2−/−, P4ha1+/− and P4ha2−/− mice were first cross-bred to obtain double heterozygous P4ha1+/−; P4ha2+/− mice, which were then intercrossed with the P4ha2−/− mice. The genotypes of the offspring were analyzed by PCR assay using a forward primer from intron 1 of the P4ha1 gene together with a reverse primer either from exon 2 or from the LacZ gene, producing 1.5-kb and 850-bp fragments corresponding to wild-type and mutant alleles, respectively (Fig. 1A and Table 1). For the P4ha2 gene, a forward primer from intron 2 was used with a reverse primer from intron 3 or from the LacZ gene, producing a 1.9-kb fragment from the wild-type and a 2.0-kb fragment from the mutant allele, respectively (Fig. 1B and Table 1).

The analyses described in this study were carried out with mice that had been backcrossed at least 10 times. Growth of the gene-modified mice was monitored by measuring the body weights of the mutant mice and their littermates at P0 and then weekly between 5 and 20 weeks.

The animal maintenance and handling was performed according to a license approved by the Animal Ethical Committee and Animal Care and Use Committee of the University of Oulu. The animal experiments were performed according to a protocol approved by the Provincial State Office of Southern Finland.
Isolation and Culture of Primary Chondrocytes—For chondrocyte isolation, growth plates from the long bones of newborn mice were dissected and placed in Hanks’ buffered salt solution (Gibco) followed by digestion with 0.25% trypsin-EDTA (Life Technologies) for 30 min at 37 °C and 195 units/ml collagenase (Worthington Biochemical Corporation) in Hanks’ buffer for 2 h. Subsequently, the chondrocytes were plated at a density of $4 \times 10^4$ cells/well in 6-well plates and grown in monolayer cultures in high glucose DMEM (Gibco) supplemented with 5–10% fetal bovine serum (Hyclone), 100 units/ml penicillin, 0.1 g/ml streptomycin (BioWhittaker), and 50 μg/ml ascorbic acid (Wako). Depending on the experiment, the cells were cultured either in 21% oxygen (normoxia) or exposed to 1% O₂ (hypoxia) balanced with 5% CO₂ and 95% N₂ for 24 h in an InVivo2400 hypoxia workstation (Ruskinn Technologies). In all experiments, the medium was changed every second day.

Isolation of RNA and Quantitative Real Time RT-PCR—Newborn mouse growth plates were dissected immediately after the mice had been sacrificed, snap frozen in liquid nitro-
TABLE 2
Primers used in qPCR analyses

| Primer Location | Sequence (5’ → 3’) |
|-----------------|---------------------|
| P4ha1 forward   | CATCTGGGCCCCAGCAGACG |
| P4ha1 reverse   | CATCTGGGCCCCAGCAGACG |
| P4ha2 forward   | TTGGTTTCCCTCCAGCTCTTG |
| P4ha2 reverse   | TTGGTTTCCCTCCAGCTCTTG |
| P4ha3 forward   | CTGTTCATCGACCCAGGAGT |
| P4ha3 reverse   | CTGTTCATCGACCCAGGAGT |
| Col2a1 forward  | GCCCTGAAAGCCTGAGAGTC |
| Col2a1 reverse  | GCCCTGAAAGCCTGAGAGTC |
| Col9a1 forward  | ACACTTCCTGGCGCTTCAAGG |
| Col9a1 reverse  | ACACTTCCTGGCGCTTCAAGG |
| Aggrecan forward| ATGCCACATCATGACCCAGC |
| Aggrecan reverse| ATGCCACATCATGACCCAGC |
| Sox9 forward    | CGAGCCACGACTGACCCAGC |
| Sox9 reverse    | CGAGCCACGACTGACCCAGC |
| Bip forward     | ACGGCCAGCGACCACTTCCCTT |
| Bip reverse     | ACGGCCAGCGACCACTTCCCTT |
| Chop forward    | GAGAGCCAGCGACGGAGCAG |
| Chop reverse    | GAGAGCCAGCGACGGAGCAG |

Primers used in qPCR analyses

Amino Acid and Thermal Stability Analyses of Collagen—Lyophilized mouse tibial bone and fresh growth plates from newborn mice were transferred to prepyrolyzed hydrolysis tubes (6 × 60-mm; Duram), which were then inserted into a 50-ml hydrolysis vial containing 1 ml of 5.7 M hydrochloric acid solution (Sigma-Aldrich). Each sample tube and the hydrolysis vial were purged with nitrogen to remove the air (oxygen), and the vacuum-phase hydrolysis was then carried out at 165 °C for 50 min. The tubes containing the hydrolyzed samples were removed to a desiccator connected to a vacuum pump, and the samples were dried overnight. The hydrolysate from each tube was dissolved in 50 μl of 20 mM HCl and then filtered with an Ultrafree-MC (0.45 μm; Millipore). Amino acid derivatization was performed with a Waters AccQ-Tag chemistry package according to the manufacturer’s instructions. The derivatized amino acids were analyzed with a Shimadzu Prominence HPLC with its fluorescence detector set at 250/395 nm.

The thermal stability of the pepsin-resistant fibril-forming collagens was analyzed from newborn mouse growth plates. Protein was extracted from the growth plates in 4 M guanidine hydrochloride using TissueLyser LT (Qiagen) with 5-mm steel beads. The remaining pellet was washed with H2O and digested with pepsin in 0.5 M acetic acid for 48 h. After neutralization, soluble collagens were subjected to digestion with a mixture of trypsin and chymotrypsin for 2 min at various temperatures (19). The samples were analyzed by 8% SDS-PAGE followed by Coomassie Blue staining.

Atomic Force Microscopy—For atomic force microscopy (AFM) analysis, tibiae were harvested from newborn animals and snap frozen in liquid nitrogen, and 30 μm sections were prepared using a cryostat. Cartilage slices were collected on microscope slides for AFM imaging and mechanical testing of the proximal tibial growth plate. AFM measurements were car-
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ried out using a NanoWizard AFM (JPK Instruments) in combination with an inverse Axiovert 200 optical microscope (Carl Zeiss MicroImaging). For reduced influence of ambient noise, the optical microscope was placed on a Micro 60 active isolation table (Halcyonics), and the whole setup was placed into a 1-m³ soundproof box. To precisely position the cantilever within the proliferative zone of mouse samples, the microscope was equipped with a CCD camera (The Imaging Source Europe). The AFM had a maximum lateral scan range of 100 × 100 μm² and a vertical range of 15 μm. For contact mode imaging and indentation measurements, silicon nitride MLCT microcantilevers with pyramidal tip (Bruker) and a nominal spring constant of 10 mN/m were used. The force constant of all cantilevers was determined individually using the thermal spring constant of 10 mN/m were used. The force constant of each indentation measurement, silicon nitride MLCT cantilever was determined individually using the thermal spring constant of 10 mN/m were used. The force constant of the cantilever (Eq. 1)

\[ F = \frac{2}{\pi} \times \tan \alpha \times \frac{E}{(1-\nu^2)} \times \delta^2 \]  

where \( F \) is the force which is required to push the tip into the sample, \( \delta \) is the indentation depth, \( E \) is the Young’s modulus, \( \nu \) is the Poisson ratio which was assumed to be 0.5, and \( \alpha \) represents the tip half-opening angle (17.5° for the cantilevers used) (21). The contact point was determined manually for each force curve, and the fit range was limited to a maximum indentation depth of 500 nm. For generating histograms and fitting Gaussian distributions to the histograms, the data analysis software Origin 8.0 (OriginLab Corporation) was used.

Transmission Electron Microscopy—Proximal tibial epiphyses from newborn control and P4ha gene-modified mice were microdissected and fixed in a 1% glutaraldehyde and 4% formaldehyde in 0.1 M phosphate buffer. The samples were postfixed in 1% osmium tetroxide, dehydrated in acetone, and embedded in Epon LX 112 (Ladd Research Industries). Thin sections (80 nm) were cut with a Leica Ultracut UCT ultramicrotome and stained with uranyl acetate and lead citrate. The samples were examined in a Philips CM100 TEM with a Morada CCD camera (Olympus Soft Imaging Solutions GMBH) for image capture or in a Tecnai G2 Spirit with Veleta and Quemesa CCD cameras (FEI Company).

Statistical Analyses—The statistical analyses were performed using Student’s two-tailed t test. The area under the curve of the growth curves was calculated using the method of summing measures (22, 23). The data are shown as the means ± S.D. Values of \( p < 0.05 \) were considered statistically significant (*, \( p < 0.05 \); **, \( p < 0.01 \); ***, \( p < 0.001 \)).

| TABLE 3 | Offspring of P4ha1+/−;P4ha2+/− × P4ha1+/−;P4ha2−/− breeding |
|---|---|---|
| P4ha1; P4ha2 | % | n |
| +/+; +/− | 30 | 143 |
| +/+; +/+ | 30 | 142 |
| +/+; −/− | 28 | 130 |
| +/−; +/− | 12 | 55 |
| Total | | 470 |

Results

Generation of C-P4H-II null (P4ha2−/−) Mice and Mice in Which Homozygous Inactivation of C-P4H-II Is Combined with Heterozygous Inactivation of C-P4H-I (P4ha1+/−;P4ha2−/−)—We have previously shown that homozygous targeted disruption of the P4ha1 gene coding for the C-P4H-I α(I) subunit (Fig. 1A) leads to embryonic death between E10.5 and E11.5, whereas heterozygous P4ha1+/− mice are born in the expected Mendelian ratios, are fertile, have a normal life span, and display no obvious phenotypic abnormalities (12). To study the in vivo roles of C-P4H-II, we established a mouse line lacking C-P4H-II activity by disrupting the P4ha2 gene through insertion of a lacZ- PGK-neo cassette into exon 3 in-frame with the translation initiation codon (Fig. 1B). Correctly targeted ES cells were injected into mouse blastocysts, and heterozygous mice were generated by routine methods. Cross-breeding of the P4ha2+/− mice produced all three genotypes in the expected Mendelian ratio (data not shown). The homozygous P4ha2+/− mice were viable and fertile with no obvious phenotypic abnormalities.

To study the combined effects of decreased C-P4H-I activity and lack of C-P4H-II activity, we generated P4ha1+/−;P4ha2−/− mice. Genotyping (Fig. 1C) of the offspring from the P4ha1+/−;P4ha2+/− × P4ha2−/− matings (\( n = 470 \)) indicated that the number of P4ha1+/−;P4ha2−/− pups was lower than expected for normal Mendelian inheritance (12% versus 25%) (Table 3). The heterozygous or homozygous inactivation of the P4ha1 and P4ha2 genes, respectively, in the P4ha1+/−;P4ha2−/− pups and their littermates obtained from the P4ha1+/−;P4ha2+/− × P4ha2−/− matings was verified at the mRNA level by RT-PCR (data not shown) and qPCR analysis of RNA isolated from cultured primary growth plate chondrocytes (Fig. 1D) or crude growth plates (data not shown). No statistically significant changes were detected in the expression level of the P4ha3 gene coding for the α(III) subunit of C-P4H-III (8), although there was a trend for a slight increase in the P4ha2−/− and P4ha1+/−;P4ha2−/− mice, indicating potential partial compensation (Fig. 1D).

The amount of total C-P4H activity was measured using Nonidet P-40-soluble lysates from the chondrocyte homogenates as sources of the enzyme and [14C]proline-labeled type I procollagen as a substrate. The amount of total C-P4H activity was reduced in the P4ha1+/−;P4ha2−/− chondrocytes to ~35% of that in the wild-type cells, the C-P4H activity of the P4ha2+/−, P4ha1+/−;P4ha2−/− and P4ha1+/−;P4ha2+/− chondrocytes being 55–75% of that of the wild type (Fig. 2).

P4ha1+/−;P4ha2−/− Mice Display Impaired Skeletal Growth and Kyphosis Postnatally—Neither P4ha1+/−;P4ha2−/− (Fig. 3A) nor P4ha2−/− mice had any overt skeletal phenotype at birth, and their weights were similar to those of the control

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littermates (data not shown). To measure their growth rate, the mice were weighed weekly from 5 to 20 weeks. The weights of the $P4ha1^{+/+}$; $P4ha2^{-/-}$ mice were significantly lower than those of the $P4ha2^{-/-}$ mice and control littermates throughout the measurement period (Fig. 3B) and a similar difference persisted throughout their life. The weights of the $P4ha2^{-/-}$ mice did not differ significantly from the control littermates (Fig. 3B). The reduced postnatal body weight of the $P4ha1^{+/+}$; $P4ha2^{-/-}$ mice was accompanied by moderate shortening of the long bones. The hind limbs were measured by \( \mu \)CT to determine potential differences in the femoral and tibial lengths in newborn (P1) and 12-week-old $P4ha1^{+/+}$; $P4ha2^{-/-}$ mice relative to $P4ha2^{-/-}$ and control littermates. At 12 weeks, both the femurs and tibias were significantly shorter in the $P4ha1^{+/+}$; $P4ha2^{-/-}$ mice, whereas no differences were observed between the $P4ha2^{-/-}$ and control mice (Fig. 3C). Also, the ratio of the femoral to tibial length was slightly, but signifi-
controls, that the growth defect is initiated already during embryonic growth plates of the long bones of limb buds isolated from E13.5 limb buds of E17.5 onwards, being most obvious in the newborn samples represents a higher magnification of the upper panel. The hypocellular area is indicated by an asterisk.

Next, TUNEL assay was performed on tibial sections of newborn mice to establish whether the hypocellularity in the inner region of the developing growth plate was a consequence, at least in part, of cell death. Chondrocytes normally proliferate and do not undergo cell death in the proliferative/prehypertrophic zones of the growth plate. TUNEL-positive chondrocytes were detected only in the peristeme and at the chondro-osseous junction but not in the proliferative/upper hypertrophic zones of the P4ha1+/−;P4ha2−/− mutant mice (Fig. 6A). In contrast, dying chondrocytes were observed in the inner region of both the proliferative and hypertrophic zones of the P4ha1+/−;P4ha2−/− growth plates, indicating that cell death transiently decreased in the 12-week-old P4ha1+−;P4ha2−/− mice relative to the P4ha2+/− and control mice (83% in P4ha1+−;P4ha2−/−, P4ha2+/− mice versus 85–86% in P4ha2+/−, P4ha1+−;P4ha2+/−, and P4ha1+−;P4ha2+/−, p < 0.01, n = 5–7 per genotype) (Fig. 3C). The distorted femur/tibia length ratio was present already at newborn (P1) mice (79% versus 82–83% in the controls, p < 0.05, n = 6 per genotype) (Fig. 3C). This suggests that the growth defect is initiated already during embryonic development.

The P4ha1+−;P4ha2−/− mice, but not the P4ha2−/− or control mice, developed kyphosis at a later stage. Kyphosis, which is observed in many mouse models of chondrodysplasia upon aging, was clearly observed in the mutant mice (Fig. 5). This phenotype was clearly detectable in the newborn samples (Fig. 5). At later developmental stages, however, a hypocellular area in the middle of both the columnar proliferative and hypertrophic zones was observed in the developing growth plates of the long bones of P4ha1+−;P4ha2−/− mutant mice (Fig. 5). This phenotype was clearly detectable from E17.5 onwards, being most obvious in the newborn P4ha1+−;P4ha2−/− mice, but had virtually resolved by the postnatal age of 7 days (Fig. 5). Hypocellularity in the inner region of the P4ha1+−;P4ha2−/− mutant growth plates was clearly observed also in in situ hybridization analysis with riboprobes that detect the cartilaginous marker Col2a1, Col10a1, and osteopontin (Spp1) mRNAs (Fig. 6A). Hypocellularity was also present in the growth plates of E17.5 and newborn P4ha2−/− mice, although the defect was much milder than in the P4ha1+−;P4ha2−/− mice (Figs. 5 and 6A).

FIGURE 4. Normal mesenchymal condensations in the P4ha1+−; P4ha2−/− limb buds. Hematoxylin and eosin staining (top panel) and in situ hybridization with a Col2a1 mRNA probe (lower panel, darkfields) on histological sections of E13.5 limb buds isolated from P4ha1−/−;P4ha2−/−, and P4ha1−/−;P4ha2−/− mice.

FIGURE 5. Transient hypocellularity in the middle of the columnar proliferative and hypertrophic zones of the developing growth plates of P4ha1−/−;P4ha2−/− and P4ha2+−/− mice. Hematoxylin and eosin staining of histological sections of the proximal epiphysis of tibia isolated from E17.5, newborn (NB), P7, and P21 P4ha1+−;P4ha2−/−, P4ha2+−/−, and P4ha1−/−;P4ha2−/− mice. The lower panel of the newborn samples represents a higher magnification of the upper panel. The hypocellular area is indicated by an asterisk.
FIGURE 6. Cell death in the middle of the columnar proliferative and prehypertrophic zones of the developing growth plates of \( P4ha1^{+/+};P4ha2^{-/-} \) mice. A, in situ hybridization of newborn growth plate samples with probes for \( Col2a1 \), \( Col10a1 \), and \( Spp1 \) mRNAs; darkfields are shown. B, TUNEL staining of histological sections of the distal epiphysis of femurs isolated from newborn mice. C, proliferation rates of round and columnar proliferative chondrocytes in E18.5 growth plates (\( n = 2 \) per genotype). D, proliferation rates of columnar proliferative chondrocytes in 3-week-old mice (\( n = 3 \) per genotype).
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could play a role in the hypocellularity. The number of TUNEL-positive cells was low, typically 3–4 dying cells per section (Fig. 6B), which is in good accordance of the cell death rate reported earlier in α10 and β1 integrin-deficient cartilage (23, 24). Notably, dying cells were limited to the most hypoxic region of the developing growth plate.

To investigate the possibility that an impairment of chondrocyte proliferation may also contribute to the inner hypocellularity of the developing P4ha1+/−;P4ha2+/− growth plates, BrdU incorporation assay was performed on histological sections of the proximal epiphysis of tibiae isolated from E18.5 P4ha1+/−;P4ha2+/−, P4ha2+/−, and control P4ha1+/+; P4ha2+/− mice. No significant difference in the proliferation rate of round proliferative chondrocytes was observed in the mutant epiphysis versus the control (Fig. 6C). As expected, the proliferation rate of the columnar proliferative chondrocytes in the growth plate was significantly higher than that of the round proliferative chondrocytes in P4ha1+/−;P4ha2+/− control specimens (Fig. 6C). Proliferation rate of the columnar chondrocytes appeared to be mildly reduced in the P4ha1+/+; P4ha2+/− and P4ha2+/− mutants relative to the controls, but the difference was on the limit of statistical significance (Fig. 6C). We also analyzed the proliferation rate of columnar proliferative chondrocytes in 3-week-old mice. No statistically significant difference in the proliferation rates was observed between the genotypes (Fig. 6D). Therefore, the inner hypocellularity is not caused by differences in the proliferation rate.

Taken together, our findings indicate that severe deficiency in C-P4H activity in embryonic and perinatal cartilages causes increased apoptosis of the proliferating chondrocytes, resulting in an inner cell death phenotype with hypocellular areas in the developing growth plate. This phenomenon, however, is transient because hypocellularity cannot be detected at later stages of postnatal life. Interestingly, chondrocyte proliferation and hypertrophic differentiation were not affected by the impairment of C-P4H activity. Consistent with this conclusion, no differences were observed in the expression levels of the Col2a1, Col9a1, Aggrecan, and Sox9 mRNAs analyzed by qPCR (Fig. 7).

Mutations in ECM constituents have been shown to affect cell proliferation, apoptosis, migration, and differentiation via two major mechanisms, ER stress caused by impaired secretion and intracellular accumulation of the mutant molecules and impaired biomechanical properties caused by abnormal assembly of the ECM (25–27). We next analyzed the contribution of these molecular mechanisms to the observed growth plate phenotype of the P4ha1+/−;P4ha2+/− mice.

P4ha1+/−;P4ha2+/− Mouse Chondrocytes Do Not Display Any Detectable Signs of Uncompensated ER Stress—Collagen molecules that are not correctly folded are typically not secreted but are retained within the ER and degraded via an ER-associated lysosomal pathway. On the other hand, excess intracellular accumulation of unfolded/misfolded collagen may result in uncompensated ER stress that elicits an unfolded protein response. It has been proposed that unfolded protein response may play a key role in the pathogenesis of chondrodysplasias (25, 28, 29). The cell death phenotype observed in the P4ha1+/−;P4ha2+/− mutant growth plates was most pronounced in the inner, hypoxic region of the developing growth plate and logically reflects the fact that C-P4H requires molecular oxygen in its reaction (3, 4). Hypoxia-inducible factor (HIF)-1 is the key mediator of cellular adaptation to hypoxia and an essential survival factor for growth plate chondrocytes in vivo and inactivation of HIF-1 in cartilage leads to a severe chondrocyte death phenotype in the growth plate (30, 31). Notably, HIF-1 up-regulates C-P4Hs in chondrocytes cultured in hypoxia to ensure sufficient hydroxylation of collagens in hypoxic conditions (14, 31), and inactivation of HIF-1 in the growth plate leads to reduced hydroxylation, intracellular retention of type II collagen, and induction of ER stress (31). To assess whether ER stress was triggered in P4ha1+/+;P4ha2−/− or P4ha2−/− mutant growth plate chondrocytes, we investigated the relative expression levels of Bip and Chop with qPCR in whole, crude growth plate samples isolated from newborn mice. No induction of either marker was detected in the mutant samples relative to controls (Figs. 8, A and B). Furthermore, C-P4H deficiency had no effect on Chop mRNA level in isolated growth plate chondrocytes cultured in 21% O2, nor did it have any statistically significant additive effect on the induction of Chop when the cells were cultured in 1% O2 for 24 h (Fig. 8C). Consistent with these findings, electron microscopic analyses of proliferative chondrocytes in the growth plate of the P4ha1+/+;P4ha2−/− and P4ha2−/− mice showed normal cell morphology and well-organized ER without signs of collagen accumulation within the ER lumen (Fig. 8D). Collectively, no signs of uncompensated ER stress were detected in the P4ha1+/+; P4ha2+/− or P4ha2−/− mutant chondrocytes either in vivo or in vitro, and uncompensated ER stress can thus not be the underlying cause of the observed cell death phenotype.

Altered Structural and Biomechanical Properties of the ECM in Vivo Are Accompanied with Impaired Thermal Stability of Collagen Fibrils Extracted from P4ha1+−/+;P4ha2+/− Cartilage—We next asked the question whether the reduced C-4PH activity and the cell death phenotype observed in the growth plates of the P4ha1+/−;P4ha2−/− mutant mice is associated with abnormalities in the ECM. Crude growth plate samples from newborn P4ha1+/+;P4ha2−/− and P4ha2+/− mice and control P4ha1+/+;P4ha2+/− and P4ha1+/−;P4ha2+/− mice were analyzed by amino acid analysis to determine the 4Hyp level. The degree of proline hydroxylation, 4Hyp/(4Hyp + Pro), was significantly reduced in the growth plate in both the P4ha1+/+;P4ha2−/− and P4ha2−/− mice relative to the control mice, the reduction in the P4ha1+/+;P4ha2−/− mice being more severe, 13–16% (Fig. 9A). Because of the reduced amount of 4Hyp, the thermal stability of collagen in these mice is likely to be reduced. The thermal stability of extracted and pepsin-digested fibrillar growth plate collagen (mainly type II collagen) was determined by digestion with a mixture of trypsin and chymotrypsin after heating to various temperatures. The Tm (midpoint of thermal transition from helix to coil) of the growth plate collagen was reduced in both the P4ha1+/+;P4ha2−/− and P4ha2−/− mice relative to the controls (Fig. 9B), the reduction being ∼2 °C in the former and 1 °C in the latter. We also analyzed the growth plate collagen for possible overglycosylation of hydroxylysines. SDS-PAGE analysis showed no retardation in the mobility of collagen extracted from the mutant
growth plates relative to the control (Fig. 9C), indicating that there is no major delay in the secretion of type II collagen in the mutant growth plate chondrocytes.

To analyze structural and biomechanical properties of the developing growth plates in detail, we employed histochemical stainings, transmission electron microscopy, and indentation-type AFM to analyze growth plate ECM in detail. Masson’s trichome staining (Fig. 10A) and picrosirius red staining visualized by polarized light resulting in birefringence from collagen (Fig. 10B) showed abnormal accumulation of collagenous material in the central growth plate, the area where chondrocyte death occurs, in the \( \text{P4ha1}^{-/-};\text{P4ha2}^{-/-} \); \( \text{P4ha1}^{-/-};\text{P4ha2}^{-/-} \); \( \text{P4ha1}^{-/-};\text{P4ha2}^{-/-} \); \( \text{P4ha1}^{-/-};\text{P4ha2}^{-/-} \) mice. In transmission electron microscopy analysis, a regular meshwork-like structure formed by collagen fibrils with a uniform thickness was observed in the control (\( \text{P4ha1}^{+/-};\text{P4ha2}^{+/-} \)) mice (Fig. 10C). In contrast, in \( \text{P4ha1}^{+/-};\text{P4ha2}^{-/-} \) mice, this characteristic arrangement was partially lost, and several longer and thicker fibrillar collagen molecules were present (Fig. 10C). AFM height images at the central region of the growth plate revealed flattened chondrocytes nicely organized into columns in the control newborn \( \text{P4ha1}^{+/-};\text{P4ha2}^{+/-} \); \( \text{P4ha2}^{+/-} \); \( \text{P4ha2}^{+/-} \); in \( \text{P4ha2}^{+/-} \) mice and also in the \( \text{P4ha2}^{+/-} \) mice, whereas chondrocytes were more sparse, rounded, and less organized in the \( \text{P4ha1}^{+/-};\text{P4ha2}^{-/-} \) mice (Fig. 10D). Furthermore, high resolution deflection images of the interterritorial matrix demonstrated a collagen network that was less dense and organized in the \( \text{P4ha1}^{+/-};\text{P4ha2}^{-/-} \) mice compared with control and \( \text{P4ha2}^{+/-} \) mice (Fig. 10D). Indentation measurements using nanometer-scaled pyramidal...
tips within the interterritorial matrix resulted in a bimodal nanostiffness distribution (Fig. 10E), where the first low modulus peak likely depicts the proteoglycan moiety, and the second peak represents the collagen fibrils (32). The frequency distributions of the elastic moduli were comparable between the control and \textit{P4ha2}/H11002 mice (Fig. 10E). In contrast, the interterritorial matrix of \textit{P4ha1}/H11001;\textit{P4ha2}/H11002 mice was significantly softer evidenced by the shift of both nanostiffness peaks. The mean values of the peaks were reduced by \(40–50\%\) relative to control, indicating that both the proteoglycan and collagen networks are softer in the \textit{P4ha1}/H11001;\textit{P4ha2}/H11002 mutant growth plates (Fig. 10E). The range of the stiffness values was also reduced in the \textit{P4ha1}/H11001;\textit{P4ha2}/H11002 mice (9.07–70.56 kPa) relative to control (14.26–107.62 kPa) and \textit{P4ha2}/H11002 (16.02–119.36 kPa) mice, further suggesting biomechanical impair-

**Discussion**

Hydroxylation of proline residues by C-P4Hs is crucial for the stability of all collagens, and therefore for the formation of a
proper ECM in all tissues. To date, little information has been available on the specific in vivo functions and mutual compensatory capacities of the three vertebrate C-P4H isoenzymes. We have shown earlier that $P4ha1^{-/-}$ mice with a residual $\sim 20\%$ C-P4H activity contributed by the C-P4Hs II and III die between E10.5 and E11.5, which underscores the indispensable function of C-P4H-I as the major C-P4H isoform (12). It has been shown previously that C-P4H-II is the major isoenzyme in mouse chondrocytes representing $\sim 70$ and $80\%$ of the C-P4H activity in cells isolated from rib cartilage of 7-day-old and 1–2-month-old mice, respectively (10). We thus expected that knock-out of $P4ha2$ would seriously compromise the development of cartilage and endochondral bones. Surprisingly, the $P4ha2^{-/-}$ mice are healthy, have a normal life span, and display no overt skeletal phenotype. However, homozygous inactivation of $P4ha2$ in combination with heterozygous ablation of $P4ha1$ resulted in postnatal chondrodysplasia that was characterized by reduced body size and severe kyphosis. C-P4H deficiency severely impaired collagen quality, properties of the cartilaginous ECM, and survival of growth plate chon-
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drocytes, demonstrating that a proper amount of C-P4H activity is an absolute requirement for normal endochondral bone development.

To date, human skeletal disorders have not been associated with mutations in the P4HA1 or P4HA2 genes (33). Our present data show that C-P4H-I can to a large extent compensate for the lack of C-P4H-II in skeletogenesis, suggesting that any osteochondrodysplasia in humans would require mutations in both genes. Based on our analysis of P4ha1−/− (12), P4ha2−/−, and P4ha1−/−;P4ha2−/− mice, human P4HA1 homozygous loss of function mutations are expected to be lethal, whereas heterozygous mutations predictably would not lead to any skeletal abnormalities. Similarly, inactivating mutations of P4HA2 are likely not to result in severe skeletal disorders, although we cannot exclude that such mutations may predispose for degenerative changes of the articular cartilage seen in osteoarthritis and osteoporosis because the P4ha2−/− mice displayed certain, but very mild, defects in the growth plates.

The developing growth plate possesses a central hypoxic region, and HIF-1 deficiency has been shown to cause massive cell death in this region (30). This indicates that HIF-1 is a survival factor for hypoxic chondrocytes, but the molecular mechanisms that mediate this action are less certain. We have recently reported that hypoxia increases the amount of C-P4H isoenzymes I and II in chondrocytes in a HIF-1-dependent fashion (14). Because O2 is a necessary cosubstrate for C-P4Hs, severe hypoxia impairs C-P4H activity (14). This effect is aggravated by HIF-1 deficiency (14), which indicates that a HIF-1-dependent increase in C-P4H levels is biologically important for chondrocytes, which need to actively synthesize collagen in a hypoxic tissue environment. The inner cell death phenotype observed in the P4ha1−/−;P4ha2−/− mice resembles that observed in the HIF-1 null growth plates (30) but is markedly milder. Moreover, the phenotype is manifested in a short time window at around birth and virtually disappears postnatally, although the end result is a mild dwarfism that persists in adult mutants. It is therefore unlikely that C-P4Hs are the only, or even the most important, mediators of the survival role of HIF-1 in growth plate chondrocytes. In agreement with this, we have recently shown that an increase in O2 availability to the growth plate by up-regulating VEGF-A in chondrocytes, and consequently increasing the number of blood vessels in the soft tissue surrounding the avascular cartilaginous mold, could contribute to the survival of hypoxic chondrocytes (17). It has also been suggested that HIF-1 may reduce oxygen consumption in the growth plate chondrocytes by inducing pyruvate dehydrogenase kinase 1 and thus inhibiting the mitochondrial tricarboxylic acid cycle (31).

C-P4Hs control an essential post-translational modification of collagens that is vital for proper folding of collagens in the ER (3, 4). Therefore, impaired C-P4H activity might lead to intracellular accumulation of underhydroxylated collagen polypeptides, resulting in uncompensated ER stress, particularly in hypoxic chondrocytes. Interestingly, it has been recently shown that chondrocyte-specific inducible inactivation of HIF-1 at E15.5 leads to intracellular accumulation of ECM-like material and ER stress 2 days later (E17.5) accompanied by 40 and 60% reduction in P4ha1 and P4ha2 mRNA levels, respectively, and a 9% reduction in the amount of 4Hyp in the E17.5 growth plates (31). In contrast, in the present study, 50 and 100% reduction in P4ha1 and P4ha2 mRNA levels, respectively, and an up to 16% reduction in the amount of 4Hyp in the growth plates of newborn P4ha1−/−;P4ha2−/− mice did not lead to any signs of uncompensated ER stress. The apparent discrepancy between the two studies could be, at least in part, due to the different timing of induction of the HIF-1 loss and concomitant reduction in the C-P4H amount in chondrocytes. In the study by Bentovim et al. (31), the process was markedly more acute as the analyses were carried out at E17.5, 48 h after inactivation at E15.5, whereas in our study the inactivation of P4ha genes was present throughout the development, which may allow either activation of potential compensatory mechanisms or balancing collagen gene expression with the reduced amount of C-P4H activity to not produce huge amounts of severely unhydroxylated collagen polypeptides. Furthermore, we show that C-P4H deficiency does not cause up-regulation of Bip and Chop mRNA levels in normoxic chondrocytes and does not increase the up-regulation of Chop mRNA in hypoxic chondrocytes. Likewise, we did not observe any signs of uncompensated ER stress or intracellular accumulation of collagen in EM samples of newborn P4ha1−/−;P4ha2−/− growth plates. Taken together, these data indicate that uncompensated ER stress is not the cause of the inner cell death phenotype found in the C-P4H deficient growth plates.

In our study, we show that the quality of the collagen extracted from the P4ha1−/−;P4ha2−/− and P4ha2−/− growth plates is clearly impaired when compared with the controls. A decrease in 4Hyp content in these tissues was associated with a decrease in the Tm of type II collagen in the mutant samples relative to the controls, the decrease being more marked in the P4ha1−/−;P4ha2−/− than in the P4ha2−/− mice. Histochemical, EM, and AFM analyses revealed abnormally organized collagen matrix in the central growth plate in newborn P4ha1−/−; P4ha2−/− mice. The matrix was also significantly softer throughout. Abnormal shape, organization, differentiation, and apoptosis of chondrocytes have been observed in certain gene-modified mouse lines that lack or have mutant forms of certain collagens or their binding receptors. For example, mice that lack collagen II are perinatal lethal and show complete disorganization of the growth plate and loss of collagen birefringence at E15 and chondrocyte apoptosis at E19 (34, 35). Transgenic mice harboring a collagen II deletion mutant that in human causes spondyloepiphyseal dysplasia are neonatal lethal and have a severe reduction in collagen II fibrils in the growth plate, where chondrocytes are irregularly organized and do not undergo normal differentiation (36). Mice lacking collagen IX develop a severe degenerative joint disease resembling osteoarthritis upon aging (37). In a later study, it was reported that in newborn collagen IX null mice, the long bones are shortened, and growth plates have abnormal organization and reduced proliferation of chondrocytes and hypocellularity particularly in the central region (38). The α1β1, α2β1, α10β1, and α11β1 integrins bind collagens (39). Mice with a chondrocyte-specific deletion of the β1 subunit suffer from severe skeletal chondrodysplasia, and the mutant growth plate chondrocytes are disorganized (23). Based on expression patterns of the collagen-
binding integrins in developing mouse cartilage, it has been suggested that α10β1 could be the major integrin for collagen binding in mouse chondrocytes (24). The phenotype of mice with a ubiquitous depletion of the α10 subunit is milder than that of the mice with chondrocyte-specific deletion of the β1 subunit, suggesting that the other collagen-binding integrins can partially compensate for its absence (24). The phenotype of the α10 knock-out mice resembles that of the P4ha1−/−; P4ha2−/− mice with growth retardation of the long bones, abnormal arrangement of growth plate chondrocytes, and an elevated number of apoptotic chondrocytes. Based on the above data and our observations, it is most likely that the abnormal ECM present in the P4ha1−/−;P4ha2−/− mutant growth plates contributes via aberrant mechanosensing and signaling cues to the observed cell death phenotype and, more generally, is the main cause of the chondrodysplasia in these mice.

Author Contributions—J. M. conceived the study. J. M. and E. S. coordinated the study, designed the experiments, and wrote the paper. E. A., A. M. S., and R. K. contributed to writing the paper. E. A., O. P., T. H., and R. Soininen generated the gene-modified mouse lines (Fig. 1). The data was generated and analyzed by E. A. (Figs. 2–8), A. M. S. (Figs. 2, 3, and 6–10), and R. K. (Figs. 4–6). M. F. and J. T. contributed to the μCT analyses (Fig. 3, C and D). I. M. and R. Sormunen performed the electron microscopy (Figs. 8D and 10C). C. P., H. C.-S., and A. A. performed and analyzed the atomic force microscopy experiments (Fig. 10, D and E). K. I. K. contributed to the design of the study and writing of the manuscript. All authors reviewed the results and approved the final version of the manuscript.

Acknowledgments—We thank Minna Siurua, Liisa Åjälä, and Anu Myllymäki and the personnel of the Biocenter Oulu Transgenic and EM Core Facilities co-funded by the University of Oulu and Biocenter Finland, and the University of Oulu Laboratory Animal Centre for excellent technical assistance.

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