Loss of Assembly of the Main Basement Membrane Collagen, Type IV, but Not Fibril-forming Collagens and Embryonic Death in Collagen Prolyl 4-Hydroxylase I Null Mice*

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Collagen prolyl 4-hydroxylases (C-P4Hs) catalyze the formation of the 4-hydroxyproline residues that are essential for the generation of triple helical collagen molecules. The vertebrate C-P4Hs I, II, and III are [α(I)]2β2, [α(II)]2β2, and [α(III)]2β2 tetramers with identical β subunits. We generated mice with targeted inactivation of the P4ha1 gene encoding the catalytic α subunit of C-P4H I to analyze its specific functions. The null mice died after E10.5, showing an overall developmental delay and a dilated endoplasmic reticulum in their cells. The capillary walls were frequently ruptured, but the capillary density remained unchanged. The C-P4H activity level in the null embryos and fibroblasts cultured from them was 20% of that in the wild type, being evidently due to the other two isoenzymes.

Collagen IV immunofluorescence was almost absent in the basement membranes of the null embryos, and electron microscopy revealed disrupted basement membranes, while immunoelectron microscopy showed a lack of collagen IV in them. The amount of soluble collagen IV was increased in the null embryos and cultured null fibroblasts, indicating a lack of assembly of collagen IV molecules into insoluble structures, probably due to their underhydroxylation and hence abnormal conformation. In contrast, the null embryos had collagen I and III fibrils with a typical cross-striation pattern but slightly increased diameters, and the null fibroblasts secreted fibril-forming collagens, although less efficiently than wild-type cells. The primary cause of death of the null embryos was thus most likely an abnormal assembly of collagen IV.

The collagen prolyl 4-hydroxylases (C-P4Hs)3 (EC 1.14.11.2), enzymes residing within the lumen of the endoplasmic reticulum (ER), catalyze the formation of 4-hydroxyproline by the hydroxylation of proline residues in -Pro-Gly- sequences in collagens and more than 20 other proteins with collagen-like sequences (1–3). Vertebrates have at least 28 collagen types, most of which form supramolecular assemblies such as fibrils and networks (3, 4). The C-P4Hs have an essential role in the synthesis of all collagens, as the resulting 4-hydroxyproline residues are necessary for the folding of the newly synthesized collagen polypeptide chains into stable triple helical molecules (1–3).

The vertebrate C-P4Hs are α2β2 tetramers in which the enzyme and chaperone protein-disulfide isomerase (PDI) serves as the β subunit (1, 2). Three isoforms of the catalytic α subunit have been cloned and characterized and shown to form [α(I)]2β2, [α(II)]2β2, and [α(III)]2β2 tetramers, with PDI, yielding the type I, II, and III C-P4Hs, respectively (5–9). C-P4H I is the main form in most cells, but C-P4H II is a major form in chondrocytes, osteoblasts, endothelial cells, and some other cell types (10, 11), whereas the mRNA for the α(III) subunit is expressed in many tissues but at much lower levels than the α(I) and α(II) mRNAs (8).

No mutations leading to heritable diseases have been characterized in any of the human C-P4H α subunit genes. To analyze the specific in vivo roles of C-P4H I and the ability of the other two isoenzymes to compensate for the lack of its activity, we generated mice with targeted inactivation of the α(I) subunit gene (P4ha1). These null mice died between E10.5 and E11.5, with an overall developmental delay and rupture of the basement membranes (BM). The level of C-P4H activity in the null embryos and fibroblasts cultured from them was about 20% of that in the wild type, this residual activity being apparently due to the presence of the other two isoenzymes. The BMs of the null embryos lacked collagen IV, whereas the distribution of other BM components was unaffected. In contrast to the basement membrane collagen, the embryos had collagen I and collagen III fibrils with a seemingly normal morphology. The cause of death of the null embryos was thus most likely an abnormal assembly of collagen IV, leading to rupture of the BMs.

EXPERIMENTAL PROCEDURES

Generation of Mouse Lines with an Inactivated P4ha1 Gene—The P1 mouse genomic library (Genome Systems) was screened with a mouse α(I) subunit cDNA (12) as a probe, and the genomic clone obtained was used to build the targeting con-
struct. It contained 1- and 5.5-kb genomic arms and a 4-kb lacZ-PGK-neo cassette inserted in-frame into the second exon of the P4ha1 gene after the translation initiation codon (Fig. 1). The NotI linearized targeting construct was electroporated into GS-1 embryonic stem cells (Genome Systems) selected with G418, and genomic DNA isolated from resistant colonies was screened by PCR. The PCR assay for the targeted P4ha1 gene used the primer pair 5′-AGTGATTAGGAGATCTCGGACACC-3′ from intron 1 and 5′-ACCCCTGCT-ATAAGAAACTGTGTTT-3′ from the 5′ end of the lacZ gene, which amplify a 2040-bp fragment, and that for the wild-type P4ha1 gene used the primer pair 5′-GCTAGAAACACAGAAGTAAAGGAAAGTTT-3′ from intron 1 and 5′-GTGAAATTCAGTAGACGGC-AGAAAGATGAACTC-3′ from exon 2, which amplify a 1500-bp fragment (Fig. 1). The PCR conditions were 95 °C for 2 min followed by 30 cycles of 95 °C for 1 min, 70 °C for 2 min and 72 °C for 3 min. Correct targeting was confirmed by Southern blotting analysis of BglIII-digested genomic DNA with a 340-bp probe from intron 1 of the P4ha1 gene (Fig. 1). Cells from correctly targeted clones were injected into C57BL blastocysts, and two separate mouse lines were generated by standard methods and backcrossed into C57BL. The genotypes of the mice were directly targeted clones were injected into C57BL blastocysts, and backcrossed into C57BL. The genotypes of the mice were determined by Southern blotting of genomic DNA isolated from resistant colonies with the primers 5′-GCCTGAGTGGC-AGTAAGAGAAA-3′ and 5′-CTGAAGACTG-AGGAAAGTGCATACTC-3′ from the 5′ end of the lacZ gene for the targeted P4ha1 allele, amplifying a 850-bp fragment (Fig. 1), and the primers described above for the wild-type allele. The animal experiments were approved by the Animal Research Committee of the University of Oulu, Finland. The analyses described below were carried out from mice that were backcrossed 5 times.

RNA Isolation and RT-PCR—Total RNA was isolated from the E10.5 embryos using TriReagent (Sigma Genosys). For RT-PCR, the RNA was transcribed into cDNA with SMART rapid amplification of cDNA ends cDNA Amplification Kit (Clontech), and a 730-bp product was amplified with the primers 5′-GCCCTGAGTGGCGCTGTTTTA-3′ and 5′-GTGAAATTCAGTAGACGGCATTTTACG-3′ from the 5′ end of the lacZ gene for the targeted P4ha1 gene, respectively. The PCR conditions were 95 °C for 2 min followed by 30 cycles of 95 °C for 1 min, 70 °C for 2 min and 72 °C for 3 min.

C-P4H Activity Assay—C-P4H activity was assayed by a method based on measurement of the formation of 4-hydroxy-[14C]proline in a [14C]proline-labeled protocollagen substrate consisting of nonhydroxylated pro-α chains of chick type I procollagen (13). E10.5 mouse embryos or fibroblasts cultured from them were homogenized in a 0.1 M NaCl, 0.1 M glycine, 0.1% Triton X-100, 10 mM dithiothreitol, 10 mM Tris buffer, pH 7.8, and centrifuged at 10,000 × g for 20 min. Samples of the Triton X-100-soluble fractions were used as the enzyme source, and their protein concentrations were determined by RotiQuant (Roth).

Histology and Immunofluorescence—Embryos were collected from pregnant heterozygous females 10.5 days postcoitum, and DNA was isolated from the fetal membranes for genotyping by PCR and Southern blotting as above. The embryos were either snap-frozen in isopentane cooled in liquid nitrogen to obtain cryosections or fixed in 10% neutral formalin and processed for paraffin sectioning. The paraffin sections (5 μm) were stained with hematoxylin-eosin or used for immunohistochemistry and the cryosections were used for immunofluorescence. The antibodies used were a monoclonal antibody against the rat laminin B2 chain (γ1) (Chemicon), polyclonal rabbit antibodies against mouse collagen IV and collagen I (Rockland), and a monoclonal rat antibody against mouse CD31 (Pecam-1, BD Pharmingen). Labeled secondary antibodies were Cy2 (Jackson ImmunoResearch) and Alexa Fluor (Molecular Probes) for immunofluorescence and biotinylated anti-rat IgG (Vector Laboratories) for immunohistochemistry. The immunohistochemical signal was amplified using the Tyramide Signal Amplification Kit (PerkinElmer Life Sciences) according to the manufacturer’s instructions and visualized with AEC reagent (Zymed Laboratories Inc.). Sections were counterstained with hematoxylin.

Transmission and Immunoelectronic Microscopy—E10.5 embryos were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer and postfixed in 1% osmium tetroxide. Thin sections were cut with a Reichert Ultracut ultramicrotome. The embryos for immunoelectron microscopy (EM) were fixed in 4% paraformaldehyde in a 0.1 M phosphate buffer with 2.5% sucrose for 2 h and immersed in 2.3 M sucrose. They were then cut horizontally into three parts mounted on specimen stubs and frozen in liquid nitrogen. Thin cryosections were cut with a Leica Ultracut UCT microtome. For immunoling, the sections were incubated in 0.05 M glycine in 0.15 M NaCl and 0.02 M phosphate, pH 7.4 (phosphate-buffered saline, PBS), followed by incubation in 5% bovine serum albumin and 0.1% cold-water fish skin gelatin (Aurion) in PBS. The antibodies and protein A-gold conjugate were diluted in 0.2% bovine serum albumin cold-water fish skin gelatin in PBS, and the same solution was used for the washes. Sections were incubated with the collagen IV antibody and a polyclonal mouse collagen III antibody (Rockland) for 1 h followed by protein A-gold complex (size 10 nm) (14) for 30 min. The sections were embedded in methylcellulose and examined in a Philips CM100 transmission electron microscope. Images were captured with a CCD camera equipped with TCL-EM-Menu, version 3 (Tietz Video and Image Processing Systems GmbH), and the diameters of 204 collagen fibrils were measured in randomly chosen areas of the sections from wild-type and null samples with TCL-EM-Menu software.

Isolation and Culture of Mouse Embryonic Fibroblasts—E10.5 embryos were collected in sterile PBS and the fetal membranes used for genotyping. The embryos were minced with scissors and digested with 0.5% trypsin and 0.2% EDTA in PBS (Sigma) for 30 min at 37 °C, and the cells were cultured in Dulbecco's modified Eagle's medium (Biochrom) supplemented with 10% fetal calf serum (Sigma), 2 mM glutamine, 100 units/ml penicillin, 0.1 μg/ml streptomycin, 0.1 mM nonessential amino acids (BioWhittaker), and 50 μg/ml ascorbic acid phosphate (Wako) at 37 °C and 5% CO2.

SDS-PAGE and Western Blot Analysis—E10.5 embryos were homogenized in 0.5 M acetic acid and centrifuged at 10,000 × g for 20 min. Aliquots of the acetic acid-soluble fractions were analyzed by 5% SDS-PAGE under reducing conditions followed by Western blotting using a polyclonal antibody against collagen IV (Rockland) and a monoclonal antibody against α-tubu-
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Lack of C-P4H I Activity Leads to Embryonic Death between E10.5 and E11.5—The murine P4ha1 gene was disrupted by inserting the lacZ-PGK-neo cassette into its exon 2 in-frame with the translation initiation codon (Fig. 1A). Fifteen ES cell clones with the targeted mutation were identified by PCR and Southern blotting, two of which were injected into mouse blastocysts, resulting in high-level chimerism and germline transmission. The phenotypes of the novel lines were examined by PCR and Southern blotting (Fig. 1, A and B). The genotypes of time-staged embryos were determined by Roti-Quant (Roth). Medium samples from an equal number of cells were precipitated with 176 mg/ml ammonium sulfate for 24 h at 4 °C, centrifuged at 10,000 × g for 20 min. The protein concentrations in the soluble fractions were determined by Coomassie Blue staining or Western blotting with polyclonal antibodies against collagens I, III, or IV (Rockland) and ECL™ immunodetection. The thermal stability of the pepsin-resistant fi rbil-forming collagens in the embryo homogenates and medium samples was studied by digestion with a mixture of trypsin and chymotrypsin for 2 min at various temperatures (15), and the samples were analyzed by SDS-PAGE followed by Coomassie Blue staining.

RESULTS

Lack of C-P4H I Activity Leads to Embryonic Death between E10.5 and E11.5—The murine P4ha1 gene was disrupted by inserting the lacZ-PGK-neo cassette into its exon 2 in-frame with the translation initiation codon (Fig. 1A). Fifteen ES cell clones with the targeted mutation were identified by PCR and Southern blotting, two of which were injected into mouse blastocysts, resulting in high-level chimerism and germline transmission. Both mouse lines showed the same phenotype. The P4ha1+/− mice, which were born in normal Mendelian ratios, appeared normal and displayed no obvious anatomical or histological abnormalities. These heterozygous mice were interbred to produce homozygous mutants. Newborn mice and embryos were genotyped by PCR and Southern blotting (Fig. 1, B and C). No homozygous P4ha1−/− mice were identified among the pups, suggesting that the phenotype was lethal to the embryo (Table 1). The genotypes of time-staged embryos showed no significant deviation from normal Mendelian ratios until E10.5, after which the percentage of homozygous mutants decreased, indicating death between E10.5 and E11.5 (Table 1). The homozygous mutants were found to develop seemingly normally until E9, after which their development was retarded. At E10.5 they were pale, very fragile, and smaller than their littermates (Fig. 1E) but their hearts were still beating. At E11.5 null embryos were already dead but not yet resorbed (Table 1).

Our data thus indicate that implantation and initial embryonic development occur normally in the absence of C-P4H I activity, but this activity becomes essential after E10.5. Development of the extra-embryonic structures such as the yolk sac and placentae showed no obvious abnormalities in the homozygous mutants.

To confirm that the null mutants do not express the C-P4H α(I) subunit, the RNA isolated from E10.5 embryos was analyzed by RT-PCR. No α(I) subunit mRNA was detected in the homozygotes, whereas it was present in the wild-type and heterozygous embryos, its amount being reduced by approximately half in the latter (Fig. 1D).
The C-P4H Activity Level is Reduced by 80% in P4ha1−/− Embryos—The level of C-P4H activity was measured in E10.5 embryos and in cultured embryonic fibroblasts using Triton X-100-soluble proteins from the embryos and cell homogenates as sources of the enzyme and [14C]proline-labeled pro-collagen as a substrate (13). The amount of total C-P4H activity was found to be reduced in the homozygous mutant embryos and fibroblasts to about 20% of that in the wild-type embryos and cells, whereas the activity levels in the heterozygous samples were 55–74% of those in the wild type (Fig. 2).

Capillary Walls Are Disrupted in P4ha1−/− Embryos—Histological analysis of the E10.5 P4ha1−/− embryos indicated that their mesenchymal cell density was considerably lower than in the wild type (Fig. 3, A–D). Ultrastructural analysis by transmission EM showed that the contacts between the sparse mesenchymal cells in the P4ha1−/− embryos were rare and that these cells had fewer filopodia than the wild-type cells (Fig. 3, C and D). Furthermore, transmission EM analysis showed that most of the ER of the P4ha1−/− cells was dilated by comparison with that of the wild-type cells, indicating that the secretion of collagen may be impaired (Fig. 3, E and F).

As the P4ha1−/− embryos appeared pale, we also analyzed the capillary structures in the E10.5 embryos. Immunohistochemical staining with an antibody against PECAM-1, an endothelial cell marker, showed no apparent changes in capillary density between the wild-type and null embryos, indicating that no distinct differences in vascular architecture exist (Fig. 4, A and B). However, ultrastructural analyses by transmission EM showed that the capillary walls were frequently ruptured in the null embryos (Fig. 4, C and D) and red blood cells were only rarely detected in the lumen of the null capillaries.

Assembly of Collagen IV in BMs Is Affected in the Null Embryos—Collagen IV and laminin are the major BM components (16–18). Immunofluorescence studies with collagen IV and laminin antibodies showed intense, continuous staining of the BMs in the wild-type E10.5 embryos (Fig. 5, A and C), whereas staining for collagen IV was essentially absent from the BM zone of the E10.5 P4ha1−/− embryos and only faint, dispersed staining was seen in the adjacent cell layer (Fig. 5B). The BMs of the P4ha1−/− embryos stained for the laminin γ1 chain, but this staining seemed irregular and fragmented (Fig. 5D). Immuno-EM with the collagen IV antibody showed a continuous, distinct BM structure in the wild-type embryos that was evenly decorated with gold particles (Fig. 6A), but the P4ha1−/− embryos lacked a distinct BM structure and only a few scattered gold particles were seen outside the cells (Fig. 6B) or within the cell layer (not shown).

We next studied the synthesis of collagen IV in the E10.5 embryos and in cultured fibroblasts isolated from them. Anal-
ysis of the acetic acid-soluble proteins of E10.5 embryos by SDS-PAGE followed by Western blotting with a collagen IV antibody showed that the amount of soluble collagen IV was distinctly higher, about 200%, in the null embryos relative to the wild type (Fig. 7A), even though the amount of collagen IV in the highly insoluble BMs was markedly decreased (above). Likewise, the amount of collagen IV was increased to about 170% in the soluble fraction of the cell layer and the culture medium of the null fibroblasts relative to the wild-type samples (Fig. 7B). These data indicate that the P4ha1/H11002/H11002 cells were capable of synthesizing and secreting collagen IV, but its assembly into insoluble supramolecular structures present in the BMs was affected.

Assembly of Collagen Fibrils Is Not Affected in the Null Embryos—Immunohistochemical staining of paraffin sections with an antibody against the main fibril-forming collagen, type I, surprisingly showed approximately similar staining patterns in the null and wild-type embryos (data not shown), the pattern
Nevertheless, have the ability to form triple helical molecules upon cooling of the cells and their homogenates, these trimers formed triple helices that were resistant to pepsin digestion at 4 °C. In contrast, all the pepsin-resistant chains present in the medium samples are highly likely to be derived from fully or almost fully hydroxylated triple helical molecules having a thermal stability of at least 37 °C, the amounts of these molecules being lower in the medium of the null fibroblasts than the wild-type cells.

The above suggestion was tested by analyzing the thermal stability of pepsin-digested samples of the embryo homogenates and the precipitated medium samples of cultured fibroblasts by a further digestion with a mixture of trypsin and chymotrypsin after heating to various temperatures (15). The \( T_m \) (midpoint of thermal transition from helix to coil) of the fibril-forming collagen present in the \( P4ha1^{-/-} \) embryo homogenate was about 38 °C, being 1–2 °C lower than that of the fibril-forming collagen in the corresponding wild-type sample, whereas the \( T_m \) of the fibril-forming collagen in the \( P4ha1^{-/-} \) culture medium was within experimental errors identical to that in the wild-type medium (Fig. 8).

**DISCUSSION**

4-Hydroxyproline is found in collagens and proteins with collagen-like sequences in all animal species (1) and additionally in many glycoproteins in plants (22). Hydroxylation of proline residues by the C-P4Hs is essential for the synthesis of all collagens because the resulting 4-hydroxyproline residues are required for the stability of the triple helix (1, 2, 21). C-P4Hs and C-P4H-like enzymes have been characterized from vertebrates, nematodes, flies, plants, and even a virus (1–3, 22). These enzymes typically have several isoenzymes, for example, *Cae norhabditis elegans* has four genes encoding C-P4H catalytic subunits, *Drosophila melanogaster* has about 20 genes encoding C-P4H \( \alpha \) subunit-like polypeptides, and *Arabidopsis thaliana* has six such genes (2, 3, 23–25).

The *C. elegans* C-P4H that is needed for the synthesis of its cuticle collagens is a PHY-1/PHY-2/(PDI)2 tetramer (2, 3), the catalytic subunits, and the complete subunit-like polypeptides, and *Arabidopsis thaliana* has six such genes (2, 3, 23–25).

**FIGURE 7.** SDS-PAGE analysis of collagen IV and fibril-forming collagens in the E10.5 embryos and in cultured fibroblasts isolated from them. Genotypes of the samples are indicated below the lanes. A, equal amounts of acetic acid-soluble proteins from wild-type, heterozygous, and \( P4ha1^{-/-} \) embryos were analyzed by 5% SDS-PAGE under reducing conditions followed by Western blotting with a collagen IV antibody and ECL immunodetection. B, equal amounts of Triton X-100-soluble proteins from wild-type, heterozygous, and \( P4ha1^{-/-} \) fibroblasts (indicated by \( C \) on top of the lanes) and their culture medium (indicated by \( M \) on top of the lanes) were analyzed as in A. C–E, equal amounts of Triton X-100-soluble proteins from wild-type and \( P4ha1^{-/-} \) fibroblasts (indicated by \( C \) on top of the lanes) and their culture medium (indicated by \( M \) on top of the lanes) were digested with pepsin and analyzed by 8% SDS-PAGE under reducing conditions followed by Coomassie Blue staining (C) or Western blotting with collagen I (D) or collagen III (E) antibody and ECL immunodetection.

**FIGURE 8.** Analysis of the thermal stability of fibril-forming collagens in the E10.5 embryos and in the culture medium of fibroblasts isolated from them. The thermal stability of the pepsin-resistant fibril-forming collagens present in wild-type and \( P4ha1^{-/-} \) embryo homogenates (A) and in the culture medium of fibroblasts isolated from them (B) was studied by digestion with a mixture of trypsin and chymotrypsin at increasing temperatures as indicated below the lanes and the samples were analyzed by SDS-PAGE followed by Coomassie Blue staining. Genotypes of the samples are indicated above the panels.

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being in agreement with previous analyses of the distribution of collagen I protein and mRNA during mouse embryogenesis (19, 20). EM analysis showed that collagen fibrils with a normal cross-striated appearance were assembled in the extracellular space of the \( P4ha1^{-/-} \) embryos and they could be labeled with an antibody against collagen III (Fig. 6C). The only apparent difference between the sparse collagen fibrils in the E10.5 wild-type and null embryos was that their diameters were slightly increased in the null embryos, the mean value being 22.46 ± 3.4 nm (S.D.), whereas in the wild-type embryos was 17.40 ± 3.0 nm, the difference being statistically significant (\( p < 0.0005 \)) (Fig. 6, E and F). The distribution of fibril diameters was approximately symmetrical in both genotypes (Fig. 6, E and F).

Synthesis of triple helical fibril-forming collagens in cultured fibroblasts isolated from the wild-type and null E10.5 embryos was studied by digesting samples of Triton X-100-soluble fractions of the cells and the culture medium with pepsin followed by SDS-PAGE analysis with Coomassie Blue staining (Fig. 7C). The amounts of cellular pepsin-resistant fibril-forming collagens were increased to about 130% in the null fibroblasts relative to the wild-type cells, whereas the amounts of secreted pepsin-resistant collagens were reduced to about 60% (Fig. 7C). Similar results were obtained when the synthesis of particular fibril-forming collagens, types I and III, was analyzed by SDS-PAGE followed by Western blotting (Fig. 7, D and E). Nonhydroxylated and underhydroxylated collagen chains are known to form trimers that are correctly assembled at their C-propeptide but do not form triple helices at 37 °C and are not secreted, due to effective quality control (21). Such trimers accumulate within cells and are subsequently degraded. These trimers nevertheless have the ability to form triple helical molecules upon cooling. Most of the pepsin-resistant chains present in the \( P4ha1^{-/-} \) fibroblast samples may therefore represent underhydroxylated chains present in trimers that accumulated inside cells until hydroxylated to the extent that they could form triple helices at 37 °C. Nevertheless, upon cooling of the cells and their homogenates, these trimers formed triple helices that were resistant to pepsin digestion at 4 °C.
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C-P4H isoenzymes, we generated mice lacking C-P4H I, which is regarded as the major isoenzyme (2, 8, 10, 11). Our data demonstrate that P4ha1−/− mice are not viable but die during embryogenesis, between E10.5 and E11.5, due to impaired assembly of collagen IV, leading to rupture of the BMs. In contrast, the assembly of collagen fibrils in the P4ha1−/− mice was seemingly normal.

The C-P4H activity level in the P4ha1−/− embryos and cultured null fibroblasts was about 20% of that in the wild type, this residual activity being apparently due to the C-P4H isoenzymes II and III. Nevertheless, this activity was not sufficient for normal development after early embryogenesis. Interestingly, although the C-P4H I deficiency severely affected the deposition of collagen IV into the BMs, seemingly normal collagen fibrils were formed in the null embryos, the only apparent deviation being a slight increase in their diameters. Cultured null fibroblasts also secreted fibril-forming collagens, although less efficiently than wild-type cells. This suggests that C-P4H II and III levels in the null embryos and cells were sufficient to hydroxylate the fibril-forming collagens to such an extent that their triple helices has only been detected in mice lacking the collagen-specific ER-resident molecular chaperone Hsp47, suggesting that this chaperone is important for the quality control of the triple helices of the wild-type fibril-forming collagen molecules present in the null embryos (30, 31). Hsp47 null mice essentially lack collagen fibrils, however (30), and the aberrant collagen molecules secreted by their fibroblasts self-assemble into abnormally thin and frequently branched fibrils (31). These findings support the conclusion that the fibril-forming collagens secreted by the P4ha1−/− cells had an essentially correctly aligned triple helical conformation, as seemingly normal fibrils were present in the null embryos. Furthermore, the Tm of the fibril-forming collagen molecules present in the P4ha1−/− tissues was about 38 °C, being only 1–2 °C lower than that of the corresponding wild-type molecules, whereas the Tm of these molecules secreted by the P4ha1−/− fibroblasts was within experimental errors identical to that of the molecules secreted by the wild-type cells.

The reasons for the slightly increased collagen fibril diameters in the null embryos are currently unknown. Although the Tm of the triple helices of the wild-type fibril-forming collagen molecules is around 40 °C, these helices also contain regions with a slightly lower thermal stability leading to sites of local “breathing” (32). As the Tm of the triple helices of the corresponding P4ha1−/− molecules present in the null embryos was slightly lower than that of the wild-type molecules, the possibility is not excluded that the P4ha1−/− triple helices were slightly more breathing than the wild-type triple helices. This might explain the slightly increased diameters of the fibrils formed by such molecules.

The quality control for the secretion of collagen IV appears to be much less tight than that for the secretion of the fibril-forming collagens. Although mutations in mouse and C. elegans collagen IV and lack of Hsp47 have been shown to lead to intracellular accumulation of unfolded collagen IV chains (30, 33–36), a number of studies have demonstrated that various cells can secrete nontriple helical collagen IV chains in culture (e.g. Refs. 37–40) and in vivo (41). Secretion of such chains is markedly increased when the functioning of the C-P4Hs is impaired, e.g. due to lack of ascorbate or the presence of an iron chelator, in which cases the secreted nontriple helical collagen IV chains are markedly underhydroxylated (38, 40). As the C-P4H activity levels in the null embryos and cultured null fibroblasts were only about 20% of that in the wild-type, collagen IV molecules secreted by P4ha1−/− cells are likely to be underhydroxylated and have an abnormal conformation and may therefore be unable to assemble into the highly ordered insoluble network structures (42–44) that contribute to BMs but remain soluble instead.

BM comprise two intermingling polymeric networks formed by laminin and collagen IV, respectively, other BM components such as nidogen, perlecain, and collagen XVIII being integrated into these networks, presumably through various noncovalent interactions (16–18). Data on the phenotypes of null mice lacking various BM components (18) suggest a hierarchy in BM formation. BM-like matrices with an apparently normal deposition of laminin are seen in collagen IV null mice that die between E10.5 and E11.5 because of structural deficiencies in their BMs (45). In contrast, laminin-1 null mice die within 1 day of implantation, at E5.5 (46, 47). It has therefore been suggested that laminin is sufficient for the deposition and initial assembly of BM-like matrices during early development, but that the collagen IV network becomes fundamental for their integrity, stability, and function under conditions of increasing mechanical demands during later development (45). The phenotype of the P4ha1−/− mice is highly similar to that of the collagen IV null mice supporting the fundamental role of collagen IV in BM stability.

Inactivation of the gene for one of the three collagen lysyl hydroxylase (LH) isoenzymes, LH3, also leads to a lack of collagen IV in the BMs, the LH3 null mice dying even earlier than the P4ha1−/− and collagen IV null mice, at E9.5 (48, 49). LH3 is unique among the LH isoenzymes in that it also possesses hydroxylsyl glycosyltransferase activities (50–52). Lack of LH3, and in particular its glucosyltransferase activity, was found to lead to premature aggregation of collagen IV, due to the absence of hydroxylsyne-linked carbohydrates, which thus seem to play an important role in the proper assembly of the collagen IV network (48, 49). It is possible that the formation of abnormal extracellular collagen IV aggregates is more harmful to the functioning of the early BM-like matrices than a complete lack or diminished amount of collagen IV, which could explain the earlier death of the LH3 null embryos than of the collagen IV null embryos or P4ha1−/− embryos.

Synthesis of collagen IV in the mouse already occurs at the blastocyst stage, whereas fibril-forming collagens I and III are...
first detected at E8 (19, 20). Mice lacking the major fibril-forming collagen, type I, die between E12 and E14, which coincides with the onset of a substantial increase in the transcription of its genes in wild-type embryos (53–55). Lack of collagen III leads with the onset of a substantial increase in the transcription of its first detected at E8 (19, 20). Mice lacking the major fibril-forming collagen, type I, die between E12 and E14, which coincides with the onset of a substantial increase in the transcription of its genes in wild-type embryos (53–55). Lack of collagen III leads with the onset of a substantial increase in the transcription of its