Glycosylation catalyzed by lysyl hydroxylase 3 is essential for basement membranes

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Summary

Lysyl hydroxylase 3 (LH3) is a multifunctional enzyme possessing lysyl hydroxylase (LH), hydroxylysyl galactosyltransferase (GT) and galactosylhydroxylysyl glucosyltransferase (GGT) activities in vitro. To investigate the in vivo importance of LH3-catalyzed lysine hydroxylation and hydroxylysine-linked glycosylations, three different LH3-manipulated mouse lines were generated. Mice with a mutation that blocked only the LH activity of LH3 developed normally, but showed defects in the structure of the basement membrane and in collagen fibril organization in newborn skin and lung. Analysis of a hypomorphic LH3 mouse line with the same mutation, however, demonstrated that the reduction of the GGT activity of LH3 disrupts the localization of type IV collagen, and thus the formation of basement membranes during mouse embryogenesis leading to lethality at embryonic day (E) 9.5-14.5. Strikingly, survival of hypomorphic embryos and the formation of the basement membrane were directly correlated with the level of GGT activity. In addition, an LH3-knockout mouse lacked GGT activity leading to lethality at E9.5. The results confirm that LH3 has LH and GGT activities in vivo, LH3 is the main molecule responsible for GGT activity and that the GGT activity, not the LH activity of LH3, is essential for the formation of the basement membrane. Together our results demonstrate for the first time the importance of hydroxylysine-linked glycosylation for collagens.

Key words: Lysyl hydroxylase, Glycosylation, Basement membrane, Transgenic mouse

Introduction

Collagens comprise a large protein family that is ubiquitously distributed throughout the body. Collagen biosynthesis involves many post-translational modifications some of which are unique to collagens and proteins with collagenous domains (Bateman et al., 1996; Kielty and Grant, 2002; Kivirikko et al., 1992; Myllyharju and Kivirikko, 2001). These include the hydroxylation of lysine residues, and glycosylation of hydroxylysine residues to galactosylhydroxylysine and glucosylgalactosylhydroxylysine residues (Kivirikko and Myllylä, 1979). The modified amino acids, located in the Y position of the repeating X-Y-Gly triplets in the triple-helical region of collagens, extend outward from the helix and thus form the surface of the molecules (Kielty and Grant, 2002). Therefore, these modifications are probably important for protein-protein and protein-cell interactions. The extent of hydroxylation of lysine residues and glycosylation of hydroxylysine residues is known to be age- and tissue-specific and especially high levels are found in embryonic tissues (Bateman et al., 1996; Cetta et al., 1982; Kielty and Grant, 2002; Kivirikko and Myllylä, 1979; Kivirikko et al., 1992). Furthermore, the number of hydroxylysine and glycosylated hydroxylysine residues varies among the various collagen types; for instance type IV collagen found in basement membranes (BM) and type VI collagen found in blood vessels have large numbers of these modifications (Ayad et al., 1998; Kivirikko et al., 1992). Hydroxylysine residues have an important function in the formation of collagen crosslinks. It is known that the hydroxylation of specific lysine residues governs the nature of the crosslinks formed between fibrillar collagen molecules and, as a consequence, the biomechanical properties of the tissues (Banse et al., 2002; Bateman et al., 1996; Kielty and Grant, 2002; Kivirikko et al., 1992; Myllyharju and Kivirikko, 2001; Robins and Brady, 2002). The function of the glycosylation of hydroxylysine residues is not fully understood. It has been suggested that glycosylations might have a role in collagen fibril formation (Notbohm et al., 1999), but the results are contradictory (Bätge et al., 1997). Studies of the glycosylated hydroxylysine residues in type II collagen in autoimmunity have shown that the glycosylated peptides are antigenic (Myers et al., 2004; Van den Steen et al., 2004). In addition, it has been reported that the galactosylation of Hyl1265 in the α1 chain of type IV collagen prevents the adhesion of melanoma cells (Lauer-Fields et al., 2003). Recent studies suggest that in adiponectin, the hydroxylation and glycosylation of lysine residues in the collagenous domain contribute to the insulin-sensitizing activity of the hormone (Wang, Y. et al., 2002).

Lysyl hydroxylase (LH, E.C. 1.14.11.4) is a member of the 2-oxoglutarate-dependent dioxygenase family (Bateman et al.,

1996; Kielty and Grant, 2002; Kivirikko et al., 1992; Kivirikko and Pihlajaniemi, 1998; Myllyharju and Kivirikko, 2001). Three isoforms (LH1, LH2, LH3) with LH activity have been characterized so far (Armstrong and Last, 1995; Hautala et al., 1992; Mercer et al., 2003; Myllylä et al., 1991; Passoja et al., 1998; Ruotsalainen et al., 1999; Valtavaara et al., 1997; Valtavaara et al., 1998). Nevertheless, the in vivo functions of the different isoforms are not yet clear. Large variations have been found in the mRNA levels of LH1 and LH2, but not of LH3, when analyzed from different human cell lines (Wang et al., 2000). LH isoforms have no strict sequence specificity but there is a clear preference for some sequences to be bound and to be hydroxylated by a certain isoform (Risteli et al., 2004). The significance of LH1 is clearly seen in patients with Ehlers-Danlos syndrome type VI, a heritable disorder linked to dysfunction of LH1 (Steinmann et al., 1993; Yeowell and Walker, 2000). LH2 has recently been shown to function as a telopeptide lysyl hydroxylase (Mercer et al., 2003; Uzawa et al., 1999; van der Slot et al., 2004) and its dysfunction has been indicated to cause Bruck syndrome in three families (Ha-Vinh et al., 2004; van der Slot et al., 2003). Recent in vitro findings have revealed that LH3 is a multifunctional enzyme (Heikkinen et al., 2000; Wang, C. et al., 2002b) possessing, in addition to LH activity, galactosyltransferase (GT, E.C. 2.4.1.50) and glucosyltransferase activities (GGT, E.C. 2.4.1.66). Hence, LH3 is able to catalyze the three consecutive reactions required for the formation of unique hydroxylysine-linked carbohydrate structures, galactosylhydroxylysine and glucosylgalactosyl-hydroxylysine, in collagens and other proteins with collagenous domains. The important amino acids for GT and GGT activity have been localized near the N-terminal end of the molecule (Heikkinen et al., 2000; Rautavuoma et al., 2002; Wang, C. et al., 2002b), whereas the amino acids crucial for LH activity are located in the C-terminal portion (Heikkinen et al., 2000; Pirskanen et al., 1996; Wang, C. et al., 2002a). The recently reported LH3-knockout study indicated that LH3 is essential for synthesis of type IV collagen during early development (Rautavuoma et al., 2004).

In order to investigate the significance of the lysine hydroxylation and hydroxylysine-linked glycosylations catalyzed by LH3 in vivo, two different targeting vectors were generated to create three different mouse lines with manipulated LH3. We knocked out LH3 completely, we mutated the LH activity but retained the glycosyltransferase activities of LH3, and we also dramatically reduced the mRNA level of mutated LH3. The different manipulations of LH3 had various effects on the basement membrane (BM). The hypomorphic and knockout mouse lines had an embryonic-lethal phenotype at E9.5-E14.5 due to abnormal type IV collagen localization causing fragmentation of the BM. By contrast, mice lacking the LH activity of LH3 but with nearly normal GGT activity levels developed normally but showed thinning of the epidermal lamina densa and disorganization of collagen fibrils. Our results demonstrate that the lethality of embryos in LH3-manipulated mice is solely due to a deficiency of GGT not LH; embryonic survival and formation of BM correlate with the GGT activity level.

Results

Generation of LH3 mutant mouse lines

In this study three different mouse lines with manipulated *Plod3* genes were generated by using two different targeting vectors (Fig. 1A). In the LH3 knockout (Table 1) the *Plod3* gene was disrupted by an insertion of an *IRES-β-gal-polyA-Neo* cassette into exon 2 leading to the absence of LH3. In the hypomorphic mouse line, the Neo cassette was inserted into intron 18 and also the LH activity of the multifunctional LH3 was disrupted by a point mutation, Asp669Ala (Heikkinen et al., 2000), in exon 18 (Ruotsalainen et al., 2001). The LH activity of the point-mutated human (Heikkinen et al., 2000)

Table 1. Genotype distributions in offspring from heterozygous crosses and GGT activities of LH3 manipulated mouse lines

Stage of development	*n*	+/+		+/–		–/–		Number of viable –/–	% of expected –/–*	GGT activity of wt (%)[†]
		n	%	*n*	%	*n*	%			
Knockout										
E8.5	112	35	31	55	49	22	20	21	75	4[‡]
E9.5	246	49	20	147	60	50	20	37	60	2[‡]
E10.5	60	13	22	39	65	8	13	1	7	
E11.5	22	5	23	14	64	3	14	0	0	
P21	323	98	30	225	70	0	0	0	0	
Hypomorph										
E9.5	45	6	13	29	64	10	22	9	80	
E10.5	73	17	23	45	62	11	15	8	44	11±4.5
E11.5	44	8	18	29	66	7	16	6	55	12±4.2
E12.5	77	9	12	53	69	15	19	8	42	15±5.4
E13.5	101	26	26	57	56	18	18	11	44	20±14.6
E14.5	64	19	30	40	63	5	8	1	6	
P21	423	149	35	274	65	0[§]	0	0	0	
LH mutant										
E10.5/E13.5										83±32.8
P21	210	54	26	99	47	57	27	57		55–100[¶]

*Percentage of expected viable –/– calculated as 25% of *n*. [†]The enzyme activities were determined as dpm/μg soluble protein and given as percentage of age matched controls. The values are mean values ± s.d. based on five to seven measurements. [‡]Measurements were done once from eight pooled E8.5 embryos and twice from four pooled E9.5 embryos. [§]One –/– pup was born, but it died after 2 days. [¶]Mean value (%) ± s.d. of the GGT activities of adult mouse tissues: heart, 100.2±11.2, NS; lung, 54.5±11.3, $P<0.001$; spleen, 81.8±14.6, $P<0.01$; kidney, 91.5±17.6, NS; liver, 89.3±13.6, NS; testis, 79.9±14.8, $P<0.01$; skin, 79±21.6, NS; muscle 81±13.7, $P<0.1$. NS, not significant.

and mouse recombinant protein was undetectable in vitro, whereas the GGT and GT activities were unchanged. Several ES clones with the targeted mutations were obtained with both vectors, two of which were injected into blastocysts, and chimeric mice with germ-line transmission were obtained. Phenotypically normal heterozygous mice were interbred to produce homozygous mouse lines. No homozygous mice could be identified in either mouse line, indicating an embryonic-lethal phenotype (Table 1).

No homozygotes were born, therefore embryos at different developmental stages were analyzed. The LH3-null embryos showed similar growth retardation and embryonic death around E9.5 (Table 1) as previously described (Rautavuoma et al., 2004). In addition, most of the null embryos had dilated blood vessels, mainly in the region of sinus venosus (Fig. 2A). The number of homozygous embryos of the other mouse line (hypomorph) decreased with time (Table 1) demonstrating a hypomorphic phenotype. Hypomorphic embryos showed variation in the severity of the phenotype and the time of death varied from E9.5-E14.5. Interestingly, one homozygous pup was born but it died soon after birth. Some of the homozygous hypomorphic embryos showed growth retardation after E8.5 and dilation of blood vessels in the region of the sinus venosus (Fig. 2B) leading to death before E10.5. The rest of the homozygous embryos were characterized by slightly retarded overall growth and died before E14.5. In addition, an intracranial hemorrhage was observed consistently in the same region of the embryos at E12.5-E13.5 and some of the

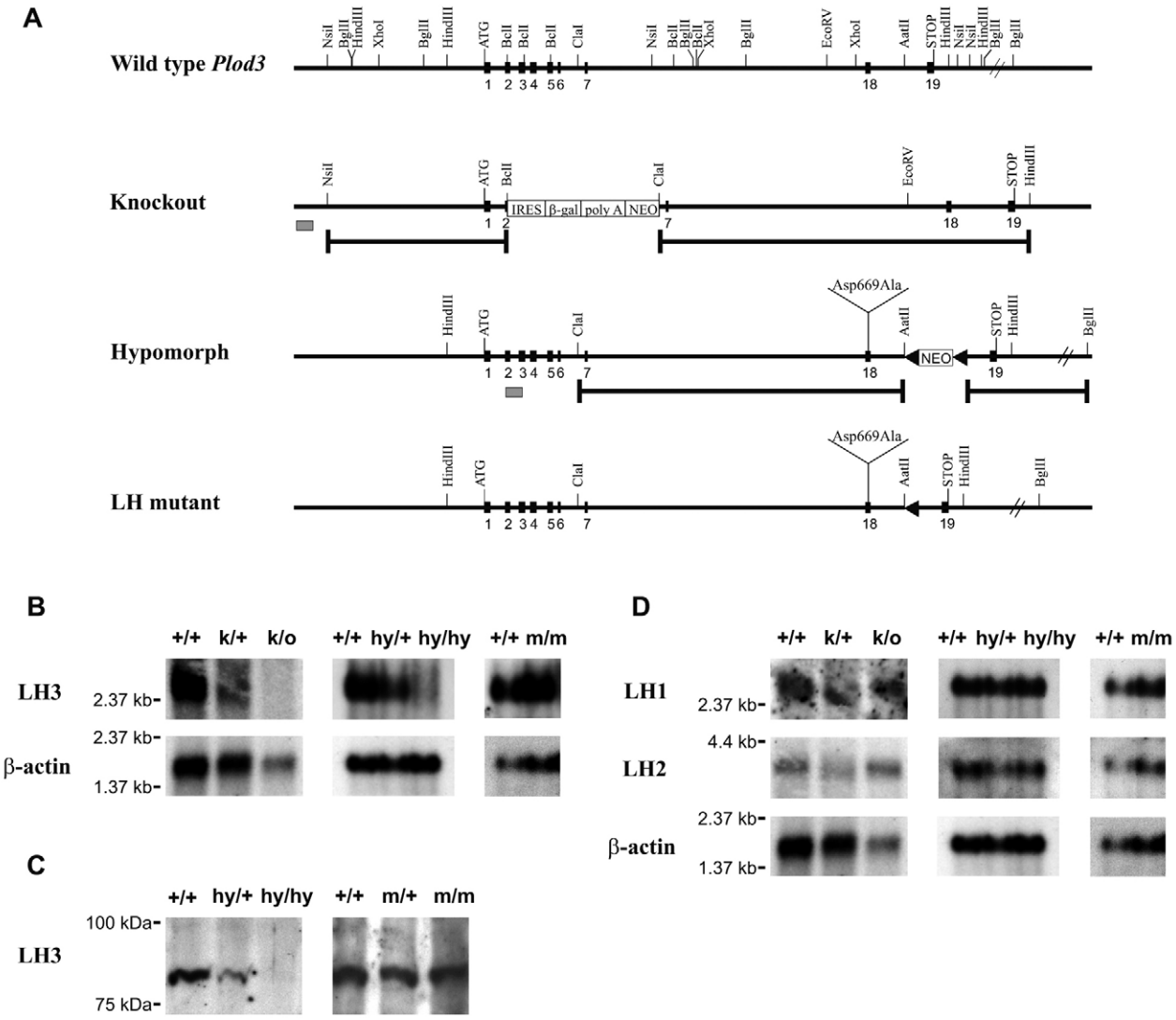

Fig. 1. Targeted manipulations of the *Plod3* gene by homologous recombination. (A) Targeting strategy showing the wild-type allele and the targeted *Plod3* alleles. Knockout (k/o) denotes the knockout of LH3, and hypomorph (hy/hy) and LH mutant (m/m) the LH-mutated LH3 with or without the Neo selection cassette, respectively. The homologous arms of the targeting construct are indicated as segments of a line. The positions of the 5′ external probe for Southern blot analyses are indicated as gray bars. Note that exons 8-17 are not shown. Northern analysis of LH3 (B) and LH1 and LH2 (D) levels in the embryos of each mouse line. The mRNA levels of LH3, LH2 and LH1 were determined from poly(A)$^+$ RNA isolated from E9.5-E13.5 embryos. LH3, LH2, LH1 and β-actin (loading control) mRNA were hybridized with radioactively labeled cDNA probes. (C) Western analysis of hypomorphic LH3 and LH mutant E13.5 embryos with LH3 antibody. The positions of molecular size markers are indicated.

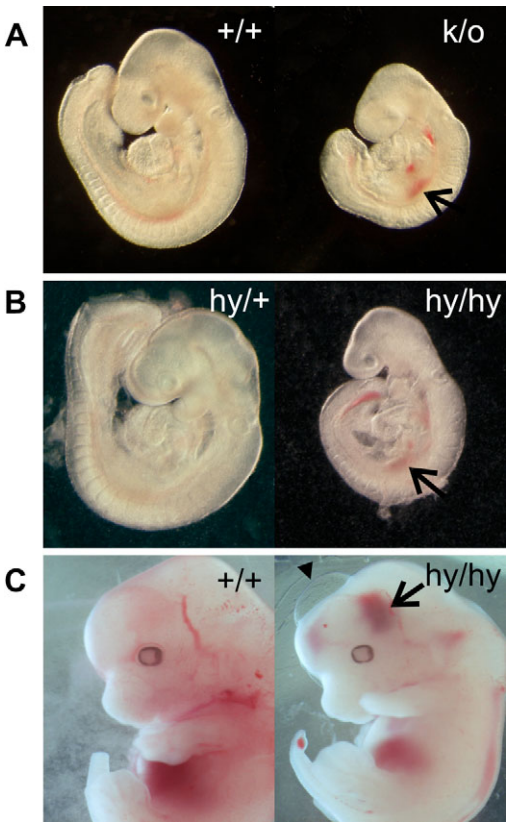

Fig. 2. E9.5 knockout (A, k/o) and hypomorphic (B, hy/hy) embryos, and E12.5 hypomorphic embryos (C, hy/hy) demonstrate retarded developmental size when compared with wild-type embryos (+/+). Arrows indicate blood vessel dilations in the region of the sinus venosus (A,B) or in the head (C). A blister in the hypomorphic embryo is marked with an arrowhead (C).

homozygous embryos developed blisters on the surface of the head (Fig. 2C), which were observed at E12.5 and thereafter. These findings were not seen in wild-type embryos.

The LH mutant mouse line with disrupted LH activity was generated by removing the Neo cassette from the hypomorphic mouse line. Heterozygous $LH3^{+/-}$, $NEO^{+/-}$ mice were bred with a mouse expressing Cre recombinase (Sakai and Miyazaki, 1997) and $LH3^{+/-}$, $NEO^{-/-}$ mice were interbred to obtain homozygous $LH3^{-/-}$, $NEO^{-/-}$ offspring (LH mutant). In the homozygous LH mutant mice, the presence of the mutation Asp669Ala in exon 18 was confirmed by sequencing (not shown). Analysis of the 260 newborn mice revealed a normal mendelian inheritance of the *Plod3* alleles (Table 1). Both heterozygous and homozygous LH mutant mice had a normal phenotype; no anatomical or behavioural abnormalities were found, when followed up to the age of 2 years. The fertility of heterozygous or homozygous mice did not differ from the wild-type mice either. In addition, there was no reduction in the litter size of the homozygous LH mutant couples and no differences in the body weights of the embryos.

LH3 mRNA and protein levels

The expression of LH3 in the embryos of all mouse lines was analyzed by northern blot. In knockout embryos, the LH3 mRNA level was undetectable in homozygotes, and in heterozygotes it was reduced when compared with the wild type. In the hypomorphic mouse line, both the mRNA (Fig. 1B) and protein (Fig. 1C) levels were reduced in the heterozygotes and markedly reduced (10% of the wild-type protein level) in homozygous embryos. In the LH mutant, the LH3 mRNA (Fig. 1B) and protein levels (Fig. 1C) were normal. The mRNA expression of LH1 and LH2 was also analyzed by northern blot. In the knockout mice with no LH3 mRNA there was a slight increase in the LH1 (1.6-fold) and LH2 (1.95-fold) mRNA levels in homozygous embryos at E9.5 when compared with controls (Fig. 1D). However, in the hypomorphic and LH mutant embryos at E11.5-E13.5 the mRNA expression of LH1 and LH2 were comparable to the wild type (Fig. 1D). Western blot data of LH2 from hypomorphic and LH mutant mouse lines support this finding (not shown). The effect of LH3 manipulations on the expression of the putative zinc-finger gene (*Znhit1*) located head to head with *Plod3* was also measured by northern analysis and no changes in the expression levels of *Znhit1* were observed (not shown). There are no other genes present within the *Plod3* gene.

LH3 enzyme activities

As reported earlier, LH3 is a multifunctional protein that has LH, GT and GGT activities in vitro (Heikkinen et al., 2000; Rautavuoma et al., 2002; Wang, C. et al., 2002b). GGT activity was measured from all three mouse lines (Table 1) to determine how manipulations of LH3 affect the GGT activity in vivo. GGT activity measurements from E8.5 and E9.5 knockout embryos demonstrate that knocking out the LH3 gene has a dramatic effect on GGT activity. In the homozygous knockout embryos, GGT activity was nearly absent (2-4% of levels in wild-type embryos), and in the heterozygotes activity was about 50% of the level in the controls. In the tissues (heart, kidney, lung, muscle and skin) of adult heterozygous knockout mice, the GGT activity was also reduced to ~60-70% of the wild-type level. The hypomorphic mouse line showing variation in the phenotype and time of death also showed variation in the GGT activity. The GGT activity of homozygous hypomorphs, measured at E10.5-E13.5, was always considerably reduced, the values varying from 11% to 20% of the control levels and in the heterozygotes the activity was approximately 60% of normal levels. Remarkably, the survival of the hypomorphic embryos correlated directly with increasing GGT activity (Table 1). In the LH mutant mice, the GGT activity of homozygous embryos was 80% of that of the controls. In adult tissues the level of GGT activity was normal in liver, kidney, heart and skin, slightly reduced (80% of the control) in muscle, spleen and testis and reduced to 55% of the control level in lung (Table 1).

The LH activity of LH3 was also measured from the homozygous LH mutant embryos, to verify that the Asp669Ala mutation is sufficient to block in vivo the LH activity in the hypomorphic and LH mutant mice. UDP-hexanolamine agarose beads were used to partially purify LH3 from the crude tissue extracts and separate LH3 as a glycosyltransferase from the other LH isoforms, which do not have glycosyltransferase activities. UDP-hexanolamine is a sugar donor analogue of UDP-sugars and widely used in affinity purifications of glycosyltransferases (Nomura et al., 1998; Shah et al., 2000).

After the binding of LH3 to the affinity matrix, the LH activity of the LH mutant embryos was less than 9% of the wild-type level (not shown). This residual activity may be due to low levels of LH1, LH2 and/or other 2-oxoglutarate decarboxylating enzymes present in the partially purified tissue extract. The results indicate that the mutation abolishes the LH activity of LH3 in vivo as reported earlier in vitro (Heikkinen et al., 2000).

Immunohistochemistry of embryos

Type IV collagen is highly hydroxylated and glycosylated and expressed in early embryonic development. It is very likely that type IV collagen is affected by the manipulations of LH3, and therefore its expression was studied in E9.5 embryos by immunohistochemistry. Type IV collagen staining was abnormal in hypomorphic and knockout mouse lines having the embryonic lethal phenotype (Fig. 3A). In the knockout embryos, type IV collagen was located mostly inside the cells (insert in Fig. 3A, panel 2), not in the BM zone. The hypomorphic embryos had variable type IV collagen staining localized both inside the cells and in the BM. Similar abnormal type IV collagen localization was also seen in the Reichert's membrane in knockout mice and hypomorphs (not shown). In LH mutant embryos, type IV collagen staining was normal and localized to the BM (Fig. 3A, panel 4). These results clearly demonstrate that LH3, and especially the GGT activity of LH3, is essential for normal localization of type IV collagen in the BM. Staining for laminin was normal in all mouse lines

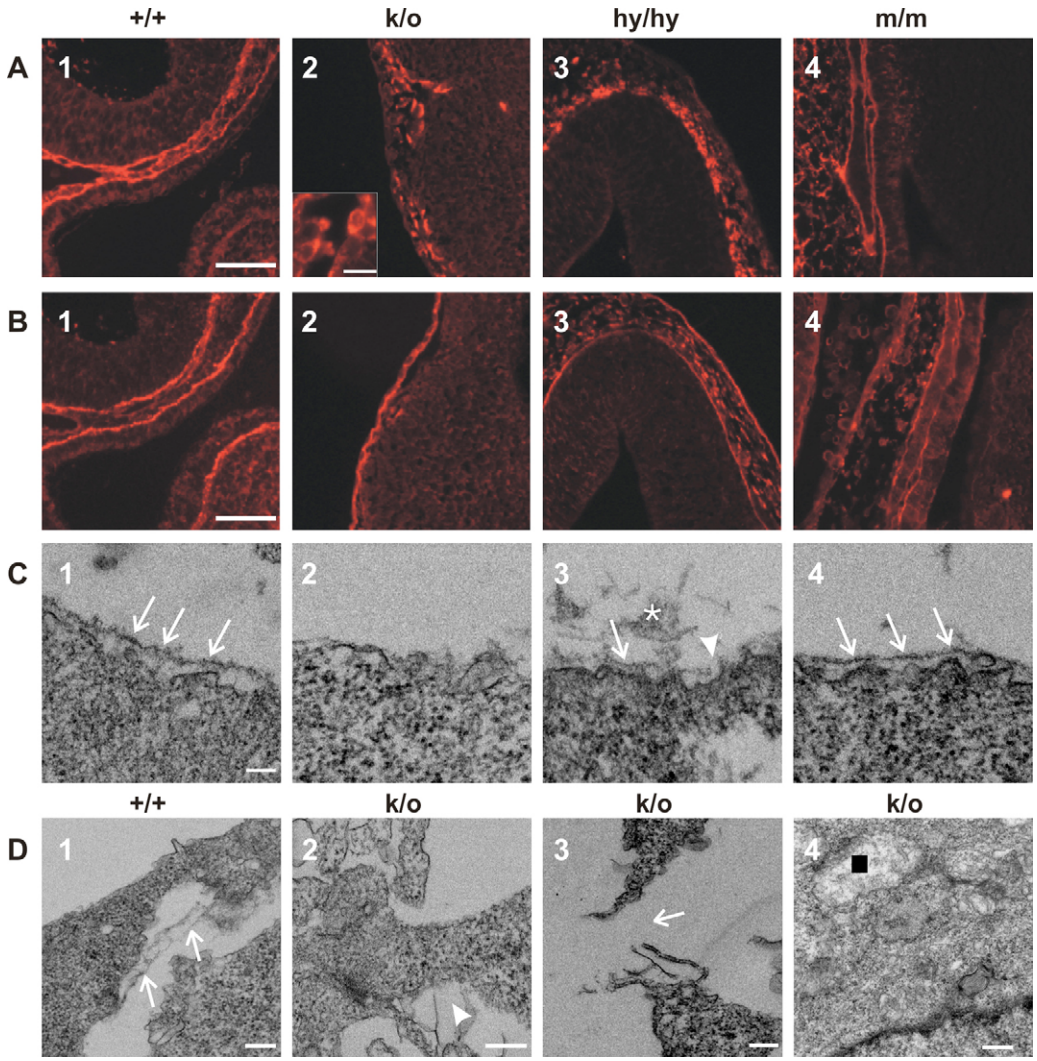

Fig. 3. Type IV collagen and laminin immunofluorescent staining of E9.5 wild-type (+/+) and homozygous embryos of knockout (k/o), hypomorphic (hy/hy) and LH mutant (m/m) mouse lines. In the knockout, the type IV collagen staining was mostly inside the cells (insert in A, panel 2). (C) EM figures of the BM (arrows) of the neural tube from wild-type, knockout, hypomorphic and LH mutant embryos at E9.5. In the knockout, the BM was absent (panel 2), whereas in the hypomorph it was discontinuous (panel 3, arrowhead) and amorphous material was also detected in the extracellular space (panel 3,*) when compared to the wild type (panel 1). The basement membrane of LH mutant was comparable to the wild-type (C4). (D) Fragments of BM were detected under the thin endothelial cells in wild-type (arrows in panel 1) and LH mutant (not shown) embryos at E9.5, but it was not detected in knockout (panel 2, arrowhead) and hypomorphic (not shown) embryos, leading to the ruptured cell layer (panel 3, arrow). The ER was dilated in homozygous knockout (panel 4, ■) and hypomorphic (not shown) embryos. Bars, 10 μm (A,B); 0.1 μm (C); 0.2 μm (D, panels 1-3); 1 μm (D, panel 4).

indicating that the absence of LH3 has no effect on the secretion or the localization of laminin (Fig. 3B).

Transmission electron microscopy of embryos

The basement membranes of E9.5 embryos of all mouse lines were analyzed in more detail by transmission electron microscopy (EM). In the wild-type embryos and also in the homozygous LH mutant embryos, normal continuous BM of the neural tube was observed (Fig. 3C, panels 1 and 4). In the knockout, the BM of the neural tube was absent (Fig. 3C, panel 2). In the hypomorphic embryos, the BM was abnormal, but the severity of the defects varied. The BM was generally discontinuous and in some places clearly fragmented (Fig. 3C, panel 3).

Because dilated blood vessels were seen in the homozygous knockout and hypomorphic embryos, the vessels of these embryos were analyzed by EM and compared with those of wild-type embryos. The endothelial BM of the blood vessels was not fully developed in the wild-type embryos at E9.5, but fragments of BM structures were clearly detected underlining the thin endothelial cells (Fig. 3D, panel 1), whereas the BM was absent in the knockouts (panel 2). In hypomorphic embryos the endothelial BM was usually absent, but in some spots there was some amorphous material (not shown). Furthermore, ruptures of the endothelial cell layer were observed in both mouse lines (Fig. 3D, panel 3 and not shown) indicating that in the absence of the BM, the endothelial cells of the homozygous hypomorphic and knockout embryos do not withstand the increasing mechanical stress. The knockout embryos also showed dilation of the ER (Fig. 3D4) and increased apoptosis. The ER of homozygous hypomorphic embryos was also dilated and showed accumulation of proteins.

Histological and immunohistochemical analysis of LH mutant newborns and adults

In the LH mutant mouse line, where the LH activity of LH3 had been abolished but GGT activity was nearly normal, no apparent external abnormalities were observed when the animals were followed for 2 years. The kidney, heart, liver, muscle, spleen and testis had no abnormalities in the HE staining (not shown). Lung tissue with reduced GGT activity and skin having nearly normal GGT activity were analyzed in more detail. HE staining revealed that the morphology of the lung and skin of the newborns and 15- to 17-week-old adult mice was comparable to that of the controls, as was the immunohistochemical staining of type IV and VI collagens and laminin (not shown). In addition, no changes were observed in the staining pattern of type I and III collagens in the homozygous adult lungs (not shown).

Transmission electron microscopy of LH mutant newborns and adults

The loss of LH activity in LH mutant mice does not cause dramatic changes in the tissue morphology at the light-microscopic level or in the distribution of type IV and VI collagens. However, skin and lung tissues were analyzed more closely by EM. The most distinct deformity was observed in the epidermal BM, which showed a significant reduction (P<0.0001) in the thickness of the lamina densa from 31.2±7.1 nm in the control animals to 23.1±6.4 nm in the homozygous

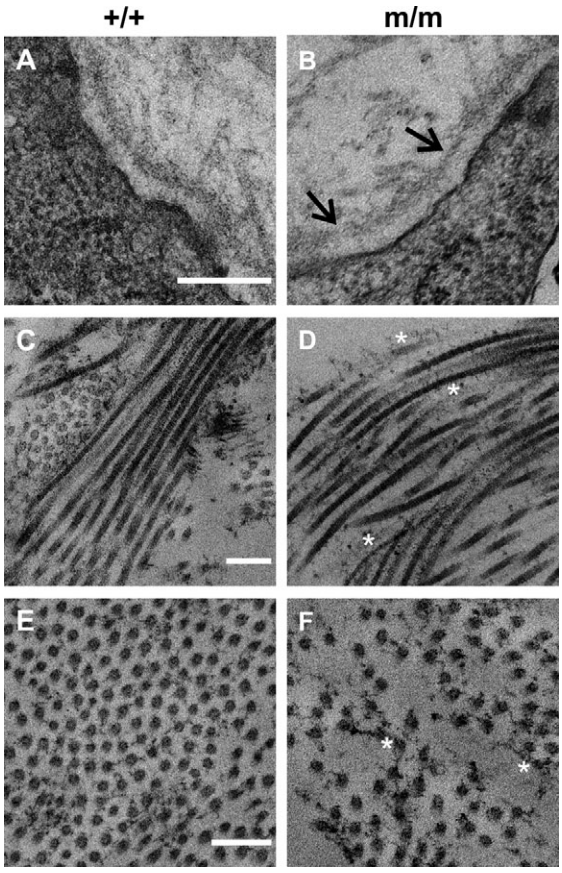

Fig. 4. EM analysis of the LH mutant newborn skin. The BM of the homozygous (m/m) newborn skin showed thinner lamina densa (B, arrows) than wild-type (+/+) littermates (A). The structure of collagen bundles was looser and less organized in the homozygotes (D,F) than in the wild type (C,E). Collagen fibrils in the homozygotes were covered with diffuse material (* in D and F). Bars, 0.2 μm.

LH mutant newborns (Fig. 4A,B). The changes were more evident in the newborn than in the older mice (not shown). Possibly the alterations seen in the newborn mice are partially compensated for by other proteins/functions in the adults. In the homozygous LH mutant newborn skin and lung (not shown), the collagen fibrils were more disorganized and loosely packed in collagen bundles compared with wild-type tissues (Fig. 4C-F). Strikingly, in the homozygous mice, the collagen fibrils were covered by diffuse material, which was not seen in control animals (Fig. 4C-F).

Hydroxylysine residues and hydroxylysine-aldehyde-derived crosslinks of collagens of LH mutant mice

The effect of the elimination of the LH activity of LH3 was studied by determining the number of hydroxylysine residues in collagen fractions, and the number and quality of crosslinks derived from hydroxylysine aldehyde in vivo. Fractions of the fibrous collagens (type I, II and III) and type IV and V collagens were extracted from the kidneys and lungs of adult LH mutant mice by pepsin digestion combined with a series of salt precipitations. The hydroxylysine content was calculated as a hydroxylysine/4-hydroxyproline (Hyl/4-Hyp) ratio

Table 2. Analysis of hydroxylysine residues of collagenous proteins and hydroxylysine aldehyde derived crosslinks in LH mutant mice

A. Hydroxylysine residues

Sample	Protein fraction*	Ratio of Hyl/4-Hyp†,‡	% of wild type
Kidney, wt	I,II,III	0.19±0.02	–
Kidney, –/–	I,II,III	0.20±0.06	103
Kidney, wt	IV,V	0.78±0.20	–
Kidney, –/–	IV,V	0.55±0.18	71
Lung, wt	I,II,III	0.20±0.01	–
Lung, –/–	I,II,III	0.24±0.04	118
Lung, wt	IV,V	0.60±0.10	–
Lung, –/–	IV,V	0.42±0.14	70

B. Crosslinks

Sample	Hydroxylysyl pyridinoline (residues/molecule)§	Lysyl pyridinoline (residues/molecule)§	Total hydroxylysine cross-links (residues/molecule)§
Skin, wt	0.0080±0.0008	0.0021±0.0019	0.0100±0.0014
Skin, –/–	0.0040±0.0010¶	0.00043±0.0001	0.0044±0.0011¶
Bone, wt	0.2106±0.0411	0.0235±0.0041	0.2340±0.0373
Bone, –/–	0.2022±0.0500	0.0233±0.0034	0.2255±0.0473

*The fraction obtained by salt precipitation contains the collagen types indicated. †The values are given as a mean ± s.d. value of three to six measurements. ‡Hyl, hydroxylysine; 4-Hyp, 4-hydroxyproline. §The values are given as a mean ± s.d. value of three measurements. ¶These values were significantly (P<0.05) reduced compared with the wild type.

because 4-hydroxyproline is quite constant in collagens, and is therefore used as a measure of total collagenous protein (Bateman et al., 1996; Kielty and Grant, 2002; Kivirikko et al., 1992). Hydroxylysine residues were reduced by approximately 30% in the fraction of type IV and V collagens whereas normal levels were observed in the fraction of type I, II and III collagens in both the kidney and lung extracts of LH mutant mice (Table 2). The results of the cross-link measurements demonstrated that the number of crosslinks derived from hydroxylysine aldehyde (hydroxylysyl pyridinoline and lysyl pyridinoline) is reduced in the skin of LH mutant mice. However, there were no significant differences in the values of bone crosslinks (Table 2). The amount of 4-hydroxyproline in skin and bone was normal indicating no changes in the quantity of the collagenous proteins in these tissues.

Discussion

To study the roles of different enzyme activities catalyzed by LH3 in vivo, we used homologous recombination to generate three mouse lines each with a different modification of the *Plod3* gene (Table 3). The LH3 knockout was generated to study the effect of abolishing all three activities catalyzed by LH3. While this work was in progress, another LH3 knockout (Rautavuoma et al., 2004) was reported. Both these knockouts showed embryonic lethality at E9.5 because premature aggregation of type IV collagen led to lack of normal basement membranes, ER dilatation and increased apoptosis. In addition, our LH3 knockout showed dilation of blood vessels, which was not detected in the other knockout. Remarkably, LH3 expression and GGT activity were absent in our LH3 knockout, whereas in the LH3 knockout published by Rautavuoma and co-workers (Rautavuoma et al., 2004) GGT activity was decreased to 15% of the control activity and a truncated LH3 transcript was produced from the targeted LH3. These differences probably explain the slight phenotypic divergence between these two knockouts. Together these data indicate that the multifunctional LH3 is indispensable for normal embryonic development.

To differentiate the effects of total loss of the LH3 activities from selective loss of only the LH activity of LH3, a point mutation was introduced to the *Plod3* gene to inactivate the LH activity. Two different mouse lines carrying this mutation were generated. The viable homozygous LH mutants were externally normal and showed normal mendelian ratios. The homozygous LH mutants had normal LH3 mRNA and protein levels demonstrating that the point mutation in *Plod3* does not affect the expression level of LH3. In the hypomorphic mouse line, LH3 expression and protein levels were significantly reduced. Because the mutation does not change the expression of LH3 the changes in the LH3 expression levels in this mouse line are due to the inserted Neo cassette. It is not known how the presence of the Neo cassette inside the *Plod3* gene lowers the LH3 mRNA and protein levels generating this hypomorphic condition, however, a similar phenomenon has been demonstrated for the fibronectin gene (Georges-Labouesse et al., 1996). Owing to this hypomorphic phenomenon, this mouse line had an embryonic lethal but variable phenotype. The embryos died between E9.5 and E14.5. Embryos dying at E9.5 showed similar growth retardation and dilation of blood vessels as the knockout embryos. However, many embryos survived longer and showed growth retardation, intracranial haemorrhage and sometimes blistering of the skin.

The role of GGT activity catalyzed by LH3 was studied by comparing GGT activities of each mouse line. We have previously observed that the LH3 protein level correlates with the GGT activity measured in mouse tissues (unpublished data), and this was confirmed in the present study (Table 3). The absence of LH3 mRNA in the lethal homozygous

Table 3. LH3 manipulations in mice

Mouse	LH3 in vivo: mRNA*	LH3 in vivo: GGT activity as a % of the wild type	BM defect	Consequence in mouse
Knockout	Not detectable	2-4%	Lack of BM	Lethality at E9.5
Hypomorph with LH mutation	Significantly reduced	11-20%	Fragmentation of BM	Lethality at E9.5-E14.5
LH mutant	Normal	55-100%†	Thinning of epidermal lamina densa	Normal development, lung and skin abnormalities

*See Fig. 1. †GGT activity in the adult tissues (see Table 1).

knockout embryos leads to a deficiency of GGT activity. Similarly, heterozygous knockout embryos had reduced LH3 mRNA levels and reduced GGT activity. The hypomorphic embryos with an embryonic-lethal phenotype at E9.5-E14.5 had dramatically decreased LH3 mRNA expression leading to dramatically reduced GGT activity (11-20% of the wild-type level). Strikingly, the survival of the embryos correlated directly with the increasing GGT level. In the LH mutant embryos, the LH3 mRNA level and the GGT activity were nearly normal, and no abnormalities were observed in the embryonic development, indicating that lack of LH activity of LH3 does not cause any dramatic changes that affect development or survival. Interestingly, in some tissues of the LH mutant mice, the GGT activity was slightly reduced (55-80% of the wild-type level). Together the data indicate, that embryonic lethality is associated with the highly reduced GGT activity (<20%), not with the lack of LH activity of LH3. The concomitant reduction of LH3 expression and GGT activity and the almost undetectable GGT activity values in knockout mice indicates that the multifunctional LH3 is the main molecule, if not the only one, responsible for GGT activity.

GT, another glycosyltransferase activity associated with LH3 in vitro (Wang, C. et al., 2002a) was also reduced in both mouse lines with the embryonic-lethal phenotype (data not shown). However, the specificity of the GT assay is not high enough to accurately measure GT activity in crude tissue extracts (Anttinen, 1977; Kivirikko and Myllylä, 1982), and thus a more specific assay is needed to confirm these results.

Owing to the decreased GGT activity in the hypomorphic embryos, the glycosylation of hydroxylysine residues is most likely incomplete, if not fully absent, and the embryos die around E9.5-14.5. It has been suggested that these hydroxylysine-linked carbohydrate units and the asparagine-linked oligosaccharides have a role in the assembly and stability of the tetramerization domain of type IV collagen, and thus are important for the structure-function relationship of collagens in the BM (Langeveld et al., 1991). In agreement with this suggestion, the homozygous hypomorphic embryos revealed a discontinuous type IV collagen distribution detected as fragmented BM staining and inside the cells, whereas the BM localization of laminin was comparable to the wild-type littermates. The Reichert's membrane showed similar abnormalities in the localization of type IV collagen, thus forming an insufficient barrier between maternal and embryonic environments, probably inducing embryonic death. The ultrastructural analysis of hypomorphic embryos revealed that the BM was abnormal but the severity of the defects varied. In the worst cases the BM was heavily fragmented, whereas in some cases it was only discontinuous. Also, dilatation of ER could be seen in the homozygous hypomorphic embryos and the dilatation correlated with the BM abnormality, suggesting that the type IV collagen is accumulated in the ER. The hypomorphic embryos with higher GGT activity survived longer and also had less-severe defects in the BM and ER suggesting that the correct BM localization of type IV collagen depends on the glycosylation of hydroxylysines. Furthermore, in the homozygous hypomorphic embryos, dilation of blood vessels was often seen at E9.5 and hemorrhage was observed in the intracranial region at E12.5-13.5. The formation of dilated blood vessels is probably due to the absence of endothelial BM, and in the worst cases the endothelial cell layer ruptures as shown in Fig. 3D, panel 3. The early BM formed by laminin does not withstand the increasing mechanical stress during the rapid morphological changes in the developing embryo and the cell-cell contacts are not sufficient to stabilize the tissue structures. Interestingly, it has been described that type IV collagen defects in the lethal knockout of type IV collagen α1/α2 chains (Pöschl et al., 2004), and collagen chaperone Hsp47 (Marutani et al., 2004; Nagai et al., 2000) cause similar BM findings and also dilation of blood vessels. In the Hsp47-knockout mouse, defective processing of collagen caused dilatation of ER (Marutani et al., 2004; Nagai et al., 2000) which was also seen in our knockouts and hypomorphs. Also the mutated multifunctional LH (Norman and Moerman, 2000; Wang, C. et al., 2002a; Wang, C. et al., 2002b) of *Caenorhabditis elegans* showed similar intracellular aggregation of type IV collagen and larval lethality (Norman and Moerman, 2000). The findings indicate that type IV collagen is not necessary for the deposition of BM components during early development but it has an essential role in maintaining the structural integrity of continuous BM at developmental stages associated with increasing mechanical demands. Our results demonstrate that defective glycosylation of hydroxylysines in type IV collagen is as fatal as abnormal folding or total loss of type IV collagen, and thus reveal for the first time the importance of glycosylation of hydroxylysines in collagens. It is important to note that some hypomorphic embryos as well as the knockout embryos died at least 1 day earlier than the type IV collagen α1/α2 and Hsp47-knockout mice (Marutani et al., 2004; Nagai et al., 2000; Pöschl et al., 2004) indicating that the glycosylation of hydroxylysine residues is also important for proteins other than type IV collagen. These other proteins can be thus considered potential targets for LH3 enzyme activities, especially for GGT.

The LH mutant mice had a normal appearance during embryonic development and adulthood. Nevertheless, some ultrastructural abnormalities were detected, best demonstrated by the thinning of lamina densa of the epidermal BM, when the tissues were studied in more detail. Although the lack of LH activity and slightly reduced GGT activity affected the structure of the BM, the defect was milder than the fragmentation of the BM seen in the hypomorphic mouse line. It is possible that the changes seen in the LH mutants are also due to the slightly reduced GGT activity, not solely to the lack of the LH activity of LH3. The reduction in collagen lysine hydroxylation and probably also in glycosylation changes the behavior of collagen molecules, which was seen as looser packing and as a slight disorganization of the collagen fibrils in the skin and lung. An increased amount of material associated with collagen fibrils was observed both in the skin and lung. The changes in the collagen lysine modifications probably affect the interactions with other proteins bound to collagens, such as type VI and XII collagens, decorin, fibromodulin, tenascin-X and others, that affect the fibrillogenesis and fibril organization (Keene et al., 2000; Minamitani et al., 2004; Nareyeck et al., 2004; Svensson et al., 1999).

Biochemical analysis indicated a reduction in the hydroxylation of lysyl residues in collagens in LH mutant mice demonstrating the effect of the point mutation in vivo. The reduction was especially seen in the type IV and V collagen fraction, suggesting that these collagens are important targets

of the LH activity of LH3 in vivo. The cross-link analysis of adult homozygous LH mutant mice indicated a reduction in the hydroxylysine aldehyde-derived crosslinks in the skin, but not in bone. However, the lack of LH activity of LH3 does not lead to the absence of the hydroxylysine-derived crosslinks in the mutant mouse, and hence, LH3 is not the main molecule responsible for these types of crosslinks in vivo. This finding is in agreement with the data suggesting that LH2 functions in this role, as a telopeptide lysyl hydroxylase (Mercer et al., 2003; Uzawa et al., 1999; van der Slot et al., 2003; van der Slot et al., 2004). The reduction in hydroxylysine-derived crosslinks may be related to collagen fibril changes found in the skin. In addition, the data also indicate that LH1 and LH2 cannot fully compensate for the lack of LH activity of LH3. This is further supported by normal or slightly increased LH1 and LH2 mRNA levels detected in the mice with mutated LH activity of LH3 (LH mutant) or with the significant reduction in LH3 levels (hypomorph/knock out).

Lysyl hydroxylase 3 (LH3) is a fascinating enzyme, which has three catalytic activities in vitro. Based on analyses of three different mouse lines that were designed to delineate the effects of these catalytic activities during mouse development we conclude that: (1) LH3 has LH and GGT activities in vivo and LH3 is the main molecule responsible for the GGT activity; (2) the reduction of GGT activity of LH3 disrupts the formation of BMs during mouse embryogenesis leading to lethality at E9.5-E14.5; (3) the survival of embryos and formation of BM correlate with increasing GGT activity level; (4) the lack of LH activity of LH3 affects the structure of BM and the collagen fibril organization in newborn skin and lung, but does not disturb embryonic development. Together these data indicate that glucosylations catalyzed by LH3 are important for collagens, especially for type IV collagen, and are thus essential for the formation of functional BM during embryogenesis.

Materials and Methods

Generation of LH3 mutant mice

Genomic clones carrying the *Plod3* gene were isolated from a mouse BAC library (Ruotsalainen et al., 2001). Two different constructs were generated to manipulate the LH3 activities in vivo (Fig. 1A). R1 ES cell culture, electroporation and the selection and isolation of G418-resistant clones were carried out as described elsewhere (Fässler and Meyer, 1995). ES cells were screened by Southern blot hybridization with an external probe after *Eco*RV or *Hin*dIII digestion (Fig. 1A). ES cells carrying the disrupted allele were microinjected into C57BL/B6 blastocysts to generate chimeras, which were subsequently mated with C57BL/B6 mice. ES cells carrying mutated LH activity of LH3 and Neo cassette in the *Plod3* gene (as described below) were generated in R.F.'s laboratory, and the LH3 knockout ES cells at the Transgenic Facility of Biocenter Oulu, Finland. All mouse lines were subsequently generated at the Transgenic Facility of Biocenter Oulu, Finland.

The targeting vector for knocking out the LH3 contained 2.8 kb and 7 kb genomic arms and an *IRES-β-gal-polyA-Neo* cassette inserted into the *Plod3* gene between the *Bcl*I site of exon 2 and the *Cla*I site of intron 6 (Fig. 1A). The mice were genotyped by PCR with primers from exon 6, exon 7 and from the neomycin (Neo) cassette.

To disrupt the LH activity of LH3 (hypomorphic and LH mutant mouse lines), Asp669 was mutated to Ala669 in exon 18 by in vitro mutagenesis as described (Heikkinen et al., 2000). The targeting construct (Fig. 1A) consisted of a 6.1 kb 5′ homologous region harboring the mutated exon 18, a floxed Neo cassette in intron 18 and a 4.7 kb 3′ homologous fragment. Progeny were genotyped by PCR with primers from intron 18 and the Neo cassette. To confirm the point mutation in the *Plod3* gene, exon 18 was amplified and sequenced. To establish the mouse strain lacking the Neo cassette (LH mutant), $LH^{+/-}$, $NEO^{+/-}$ mice were intercrossed with a transgenic Cre-deletor mouse line (Sakai and Miyazaki, 1997). The offspring were genotyped by PCR with primers from intron 18. The removal of the *cre* locus was verified by PCR with primers from the *cre* gene (Sakai and Miyazaki, 1997). The animal experiments were approved by the Animal Care and Use Committee of the University of Oulu, Oulu, Finland.

RNA isolation and northern blot analysis

Poly(A)$^+$ RNA was extracted from pooled E9.5-11.5 embryos with an Oligotex Direct mRNA mini kit (Qiagen). Total RNA was extracted from single E12.5-E13.5 embryos with the Trizol Reagent (Invitrogen) and poly(A) RNA was purified with Dynabeads oligo d(T) (Dynal). Poly(A)$^+$ RNA was fractionated in a 0.8% agarose gel containing 0.22 M formaldehyde and transferred to a nylon membrane (Ausubel et al., 1989). The filter was hybridized with [^{32}P]dCTP-labeled cDNA fragments of mouse LH1, LH2 and LH3 (Ruotsalainen et al., 1999), and with a cDNA clone of zinc-finger HIT-1 (BC026751). Actin cDNA was used as a control probe to normalize the quantities of mRNA. Quantification of mRNA levels was performed with ImageQuant 5.2 software (Molecular Dynamics).

Protein isolation and western blotting

Mouse tissues or pooled E13.5 embryos were homogenized using a Teflon glass homogenizer in 0.2 M NaCl, 0.1 M glycine, 0.5% Triton X-100, 50 mM Tris-HCl pH 7.4. The homogenate was centrifuged at 15,000 ***g*** for 20 minutes, and the supernatant was collected for analysis. The proteins bound to Concanavalin-A-Sepharose were eluted in SDS sample buffer and separated under reducing conditions by 7.5% SDS-PAGE. The proteins were transferred to a PVDF membrane, which was incubated with polyclonal anti-LH2 and anti-LH3 antibodies (unpublished). Horseradish-peroxidase-conjugated anti-rabbit IgG (P.A.R.I.S.) was used and visualized by an ECL+ detection system (Amersham Biosciences). Quantification of LH3 protein levels in the hypomorphic mouse line was performed with ImageQuant 5.2 software (Molecular Dynamics).

LH activity measurements

LH activity was measured from recombinant mouse LH3, and from mouse embryos. Expression of mouse LH3 cDNA corresponding to amino acids 28-741 was carried out by the BAC-TO-BAC^{TM} expression system (Life Technologies), and the supernatant of transfected insect cell extracts was used in the LH activity assay (Valtavaara et al., 1997). Five E13.5 embryos were homogenized into a buffer containing 20 mM Tris-HCl, pH 7.8, 0.1 M glycine and 15 mM $MnCl_2$. The 15,000 ***g*** supernatant was mixed with 50 μl uridine 5′ diphosphohexanolamine agarose beads (Sigma) to affinity purify LH3. The beads were washed with 20 mM Tris-HCl, pH 7.8, 0.1 M glycine, 15 mM $MnCl_2$ and 0.3 M NaCl. The beads in 20 mM Tris-HCl, pH 7.8, 0.1 M glycine, 1% Igepal CA-630 (Sigma) buffer were used in the lysyl hydroxylase activity assay, which was based on decarboxylation of 2-oxo[1-^{14}C]glutarate (Kivirikko and Myllylä, 1982). The specific activity of the 2-oxo[1-^{14}C]glutarate was 74×10^5 dpm/μmol and the synthetic peptide IKGIKGIKG was used as a substrate in the reaction.

GGT activity measurements

The pooled E8.5 and E9.5 embryos or single E10.5-E13.5 embryos or adult mouse tissues were homogenized and the supernatant was used for the enzyme assay based on the transfer of ^{3}H-labeled sugar from UDP-glucose (139 Ci/mol) to galactosyl hydroxylysyl residues in a calfskin gelatine substrate (Kivirikko and Myllylä, 1982; Myllylä et al., 1975).

Measurement of hydroxylysyl residues and hydroxylysyl aldehyde derived crosslinks of collagenous proteins

Collagenous proteins were extracted from mouse tissues by using pepsin digestion, and a series of salt precipitations were used to obtain a fraction of fibrous collagens (type I, II and III) and a fraction containing type IV and V collagens (Miller and Rhodes, 1982), which were hydrolyzed by acid hydrolysis. The free phenylthiocarbamyl amino acid derivatives were separated on a reversed-phase column and analyzed with an amino acid analyzer.

The cross-link measurements were carried out from skin and bone. The samples were reduced with sodium borohydride. After acid hydrolysis the collagen content was measured (hydroxyproline assay), and hydroxylysyl aldehyde derived crosslinks (hydroxylysyl pyridinoline and lysyl pyridinoline) were determined by RP-HPLC as described previously (Mercer et al., 2003).

Histology, immunohistochemistry and transmission electron microscopy

The embryos and tissues were fixed in 4% paraformaldehyde in PBS or in 10% neutral formalin overnight and embedded in paraffin, and 5 μm thick sections were stained with hematoxylin and eosin (HE).

For immunohistological staining, the embryos and tissues were fixed in 95% ethanol/5% acetic acid overnight and processed for paraffin embedding. Sections were stained with polyclonal rabbit anti-mouse collagen type I α2 chain, polyclonal rabbit anti mouse collagen type III, rabbit anti-mouse collagen type IV (Chemicon) and type VI (Rockland) or rabbit anti-mouse laminin (Sigma). The primary antibody was detected with fluorescent-labeled secondary antibody, Alexa Fluor 594-conjugated goat anti-rabbit IgG (Molecular Probes).

The samples for transmission electron microscopy (EM) were fixed in 1% glutaraldehyde, 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4), post-fixed in 1% osmium tetroxide, dehydrated in acetone and embedded in Epon Embed 812. The thin sections were cut with a Reichert Ultracut ultramicrotome and examined in

a Philips CM100 transmission electron microscope. The thickness of the whole epidermal BM and lamina densa separately were measured from the images captured with a CCD camera equipped with TCL-EM Menu version 3 from Tietz Video and Image Processing Systems. The measurements were taken from the skin of five homozygous LH mutant and wild-type newborn mice, 127 and 124 measurements, respectively. Statistical analysis was performed using the Student's *t*-test.

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