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Laura Sipilä

EXPRESSION OF LYSYL HYDROXYLASES AND FUNCTIONS OF LYSYL HYDROXYLASE 3 IN MICE

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EXPRESSION OF LYSYL HYDROXYLASES AND FUNCTIONS OF LYSYL HYDROXYLASE 3 IN MICE

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Abstract

Lysyl hydroxylase (LH, EC 1.14.11.4) catalyzes the post-translational hydroxylation of lysyl residues in collagens and other proteins with collagenous domains. The hydroxylysyl residues participate in the formation of collagen cross-links, and some of the hydroxylysyl residues are further glycosylated. Three lysyl hydroxylase isoforms LH1, LH2 and LH3, encoded by three individual genes have been characterized and one isoform, LH3 is a multifunctional enzyme containing lysyl hydroxylase, collagen galactosyltransferase (GT, E.C. 2.4.1.50) and glucosyltransferase (GGT, E.C. 2.4.1.66) activities *in vitro*.

In this thesis the genes for the mouse lysyl hydroxylases were each mapped to a different chromosome. In addition, the roles of the lysyl hydroxylase isoforms were characterized in mice by studying their expression during development and the distribution of LH2 and LH3 in adult mice. The results revealed a widespread expression of the mouse lysyl hydroxylases during embryonic development whereas LH2 and LH3 showed tissue- or cell-specific expression patterns in the adult. Alternative splicing of the gene for LH2 also showed developmental and tissue-specific regulation.

The different functions of LH3 were studied *in vivo* by generating three different LH3 manipulated mouse lines. Analysis of the mouse lines revealed that LH3 has lysyl hydroxylase and glucosyltransferase activities *in vivo*, and that, in particular, the glucosyltransferase activity of LH3 is essential for normal development. The loss of glucosyltransferase activity caused disruption of basement membranes leading to embryonic lethality while the absence of lysyl hydroxylase activity led to ultrastructural alterations in muscle and basement membranes and disorganization of collagen fibrils. The disruption of basement membrane was due to an intracellular accumulation of unglycosylated type IV collagen, whereas the ultrastructural alterations were related to the abnormal aggregation and distribution of underglycosylated type VI collagen. The results demonstrate that hydroxylysine-linked glycosylations are critical for the secretion of type IV collagen and its assembly into basement membranes, and for the assembly and distribution of type VI collagen.

Keywords: basement membrane, collagen, embryonic development, glycosyltransferase, lysyl hydroxylase, transgenic mice

To Mika, Leo and Enni

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Oulu, February 2007 Laura Sipilä

Abbreviations

BM	basement membrane
DAB	diaminobenzidine
DXD	aspartate-any amino acid-aspartate
cDNA	complementary DNA
E	embryonic day
ECM	extracellular matrix
EDS VI	Ehlers-Danlos syndrome type VI
EM	electron microscopy
ER	endoplasmic reticulum
EUCIB	European Collaborative Interspecific Backcross
FACIT	fibril-associated collagen with interrupted triple helices
GGT	galactosylhydroxylysyl glucosyltransferase
GT	hydroxylysyl galactosyltransferase
HE	hematoxylin-eosin
kDa	kilodalton
KDEL	lysine-aspartate-glutamate-leucine
LH	lysyl hydroxylase
mRNA	messenger RNA
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PLOD/Plod	procollagen-lysine, 2-oxoglutarate, 5-dioxygenase, gene name for human/mouse lysyl hydroxylase
RH	radiation hybrid
RT-PCR	reverse transcriptase polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
UDP	uridine diphosphate
X	any amino acid
Y	any amino acid

List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I Sipilä L, Szatanik, M, Vainionpää, H, Ruotsalainen, H, Myllylä R & Guénet J-L (2000) The genes encoding mouse lysyl hydroxylase isoforms map to Chromosomes 4, 5, and 9. Mamm Genome 11: 1132-1134.

II Salo AM, Sipilä L, Sormunen R, Ruotsalainen H, Vainio S & Myllylä R (2006) The lysyl hydroxylase isoforms are widely expressed during mouse embryogenesis, but obtain tissue-and cell specific patterns in the adult. Matrix Biol 25: 475-83.

III Ruotsalainen H*, Sipilä L*, Vapola, M, Sormunen R, Salo A, Uitto L, Mercer DK, Robins S, Risteli M, Aszodi A, Fässler R & Myllylä R (2006) Glycosylation catalyzed by lysyl hydroxylase 3 is essential for basement membranes. J Cell Sci 119: 625-635.

IV Sipilä L, Ruotsalainen H, Sormunen R, Vapola M, Wang C, Aszodi A, Fässler R & Myllylä R (2006) LH3-dependent post-translational modifications are required for normal secretion and assembly of type IV and VI collagens. Manuscript.

*These authors contributed equally to this work.

Contents

1 Introduction

Collagens are important structural proteins that provide the tissues with mechanical strength. Collagen biosynthesis involves many specific co- and post-translational modifications including hydroxylation of lysyl residues and their further glycosylation to galactosylhydroxylysine and glucosylgalactosylhydroxylysine. Hydroxylysyl residues have an important role in the formation of collagen cross-links, which are needed for stabilization of collagen structure, whereas the functions of hydroxylysine-linked carbohydrates are not known.

Lysyl hydroxylase (LH) catalyzes the hydroxylation of lysine residues in the Y-position of the repeating collagen Gly-X-Y-triplets. Three lysyl hydroxylase isoforms, LH1, LH2 and LH3, encoded by three individual genes have been characterized from different species. The importance of LH1 in collagen biosynthesis is clearly demonstrated by the heritable disorder, Ehlers-Danlos syndrome type VI, which is caused by mutations in the gene for LH1.

Since the functions of the two more recently found isoforms, LH2 and LH3 were not fully understood, this thesis study was initiated to differentiate and further characterize the different lysyl hydroxylase isoforms. While this work was in progress, LH2 was found to be alternatively spliced resulting in two different forms, LH2a and LH2b. Moreover, the longer form, LH2b, was shown to function as the telopeptide lysyl hydroxylase. New information was also gained regarding LH3; it was shown to be a multifunctional enzyme containing lysyl hydroxylase, hydroxylysyl galactosyltransferase and galactosylhydroxylysyl glucosyltransferase activities *in vitro*. Therefore, an additional goal was set to characterize the significance of the different enzymatic activities of LH3 *in vivo*.

In this study the genes for the mouse lysyl hydroxylases were mapped to chromosomes, and developmental expression of the different lysyl hydroxylase isoforms, specific tissue distributions of LH2 and LH3 and the occurrence of LH2 splice variants were characterized. In addition, the roles of the different enzymatic activities of LH3 were determined by generating three different LH3 manipulated mouse lines. Analysis of the mice revealed that LH3 has lysyl hydroxylase and glucosyltransferase activities *in vivo*, the latter being essential for normal development. The results thus provide new insights into the critical roles of the glycosylated hydroxylysines of collagens.

2 Review of the literature

2.1 Extracellular matrix

The extracellular matrix (ECM) is a dynamic network composed of heterogeneous macromolecules that can be grouped into collagens, other structural glycoproteins and proteoglycans. These molecules can share similar structural domains and they often show tissue-specific variation in structure and polymerization. The ECM is continually produced and remodelled and it can take many different shapes depending on the requirements of the tissue. The ECM provides cells with positional and environmental information and controls cell function by supporting adhesion of cells, transmitting signals through adhesion receptors, and binding, storing and presenting growth factors. The two most common architectural styles of extracellular matrix are basement membrane and connective tissue matrix (for reviews, see Aumailley & Gayraud 1998, Schwarzbauer 1999, Gustafsson & Fässler 2000).

2.1.1 Basement membranes

Basement membranes (BMs) are cell-associated sheet-like extracellular matrices underlying epithelial and endothelial cells and surrounding most muscle cells, fat cells and peripheral nerve axons. BMs are essential for tissue formation and repair and they provide mechanical stability and tissue compartmentalization by acting as barriers to cell penetration and filtration. Basement membranes also regulate biological activities such as cell growth, differentiation and migration (for reviews, see Timpl & Brown 1996, Erickson & Couchman 2000, Sasaki *et al.* 2004).

The fine structure and composition of basement membranes varies between tissues and also within the same tissue during different developmental periods and during repair. All basement membranes consist of two independent networks, one formed of collagen IV and one of structural glycoproteins, the laminins. The collagen IV network is highly cross-linked and is needed to maintain mechanical stability while laminin networks are mainly noncovalent and more dynamic. The two networks are connected by another

glycoprotein, nidogen/entactin, which stabilizes the network structures. Another component, the proteoglycan perlecan, interacts with the other components through its core protein or its heparan sulfate glycosaminoglycan chains. Other components of BMs such as agrin, BM40/SPARC/osteonectin, fibulins, fibronectin and type XV, XVIII and XIX collagens interact with one or more of the major constituents, and these interactions may be tissue-specific or developmentally regulated. Although type IV collagen and laminin can form networks through self-assembly, cell membrane receptors for basement membrane components, like β1-integrins and α-dystroglycan, are essential factors for the formation and stability of basement membranes *in vivo* (for reviews, see Timpl 1996, Timpl & Brown 1996, Erickson & Couchman 2000, Quondamatteo 2002, Yurchenco *et al.* 2004).

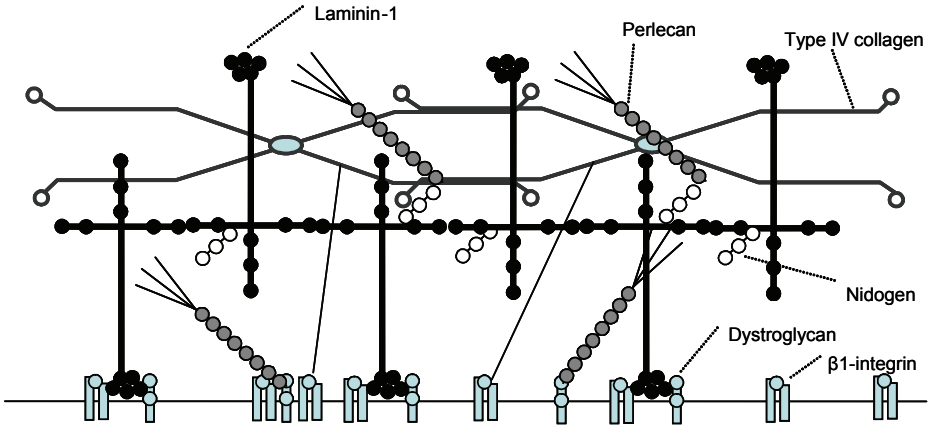

Fig. 1. Schematic model of embryonic basement membrane. For references, see text.

2.1.2 Connective tissue matrix

The connective tissue matrix is a fibrillar network that surrounds fibroblasts and other cells that produce it. The matrix contains collagen and elastin fibers and a hydrated gel composed of a glycosaminoglycan network. Most of the glycosaminoglycans are attached to proteins in the form of proteoglycans. The gel-like glycosaminoglycan structure resists compressive forces and controls the movement of molecules through the matrix. Collagen fibers provide the matrix with tensile strength, and the other structural glycoprotein, elastin, is needed for resilience. The matrix components are attached to the cell surface integrins through an important adhesive glycoprotein, fibronectin (for reviews, see Aumailley & Gayraud 1998, Schwarzbauer 1999, Alberts *et al.* 2002).

2.2 Collagens

Collagens constitute a large family of extracellular matrix proteins. Collagens play a dominant role in maintaining the structure of various tissues, but they also have many other important functions in cell adhesion and migration, chemotaxis and in regulation of tissue remodeling during growth, differentiation, morphogenesis and wound healing (for reviews, see Myllyharju & Kivirikko 2001, Kielty & Grant 2002, Gelse *et al.* 2003, Myllyharju & Kivirikko 2004).

All members of the collagen superfamily have at least one collagenous domain: a right-handed triple helix formed by three α chains composed of repeating Gly-X-Y sequences. All collagens also possess at least two non-triple-helical domains called non-collagenous domains. The triple helices can be homotrimers which are formed by three identical chains, or heterotrimers formed by two or three different chains. The α chains are each coiled into a left-handed helix and are then wound around a central axis in a right-handed manner to form a triple helix. A structural prerequisite for the assembly into a helix is a glycine residue, the smallest amino acid, in every third position of the polypeptide chains. The glycine residues are positioned in the center of the triple helix while the more bulky side chains of the other amino acids occupy the outer positions of the molecule, thus being able to participate in intermolecular interactions. The X-position of the triplet is often occupied by proline and the Y-position by 4-hydroxyproline, which is essential for the stability of the triple helix (for reviews, see van der Rest & Garrone 1991, Myllyharju & Kivirikko 2001, Kielty & Grant 2002, Gelse *et al.* 2003, Myllyharju & Kivirikko 2004).

2.2.1 Collagen subfamilies

The collagen superfamily so far includes 28 different proteins (Veit *et al.* 2006) consisting of 43 distinct α chains, and more than 20 additional proteins with collagen-like domains. Further heterogeneity within the superfamily is caused by alternative splicing and the use of different promoters in some genes. Most collagens form supramolecular assemblies and the superfamily can be divided into several subfamilies on the basis of these assemblies or other features (for reviews, see Kielty & Grant 2002, Myllyharju & Kivirikko 2004). The best characterized collagen types I-XIX and their division into subfamilies are summarized in Table 1.

The fibril-forming collagens types I, II and III represent the major products synthesized by connective tissue cells. Fibrillar collagens form large cross-striated fibrils that provide the body with mechanical strength. Collagen fibrils often contain more than one collagen type and the relative composition and amounts of different collagen types involved in the assembly of the fibrils govern the structure and organization of the matrix network. The FACIT (fibril-associated collagen with interrupted triple helices) collagens are found attached to the surfaces of collagen fibrils. FACIT collagens are probably involved in the stability and integrity of the extracellular matrix by interacting with other matrix molecules and with cells (for reviews, see van der Rest & Garrone 1991,Prockop & Kivirikko 1995, Myllyharju & Kivirikko 2004, Ricard-Blum & Ruggiero 2005).

Type IV collagen forms an open network structure which forms the insoluble scaffold of basement membranes while the short-chain collagens, type VIII and X form hexagonal networks. Type VI collagen is organized into a filamentous network which has cell-adhesion and matrix-binding properties suggesting that type VI collagen microfibrils may have a critical bridging function between cells and matrix. Type VII collagen is the major component of anchoring fibrils which anchor the basement membrane to the stroma. The membrane collagens contain ectodomains, which are released from the cell surface by shedding. Membrane collagens function as cell surface receptors and the shed ectodomains contribute to the regulation of cell behavior by binding to other matrix components. The multiplexin collagens contain sequences that produce endostatin fragments when released by proteolysis. The function of multiplexin collagens is not known, but they may be needed to maintain the structural integrity of basement membranes (for reviews, see Kielty & Grant 2002, Gelse *et al.* 2003, Myllyharju & Kivirikko 2004, Ricard-Blum & Ruggiero 2005).

2.2.2 Other proteins with collagenous domains

Several proteins contain at least one collagenous domain but have not been defined as collagens, since they are not a part of ECM. Collagen-like membrane proteins such as ectodysplasin, macrophage receptor with collagenous structure and macrophage scavenger receptors have structures similar to membrane collagens. The C1q component of complement, hibernation proteins, precerebellin, multimerins, elastin microfibril interface-located proteins and adiponectin, an abundant serum protein implicated in energy homeostasis, resemble short-chain collagens type VIII and X (for reviews, see Kielty & Grant 2002, Kishore *et al.* 2004, Ricard-Blum & Ruggiero 2005). Acetylcholinesterase and butyrylcholinesterase have a triple-helical collagen-like tail structure anchoring the enzyme to the basement membrane (Feng *et al.* 1999). In addition, several collectins (mannan-binding lectin, lung surfactant proteins A and D, conglutinin and collectin-43) and ficolins (H-, L- and M-ficolins), recognition molecules of the mammalian innate immune system, contain collagen-like sequences that enable their assembly into oligomeric structures (Lu *et al.* 2002).

2.2.3 Collagens are important for development and tissue structure

Collagens are a very heterogeneous group of proteins and some collagen types are restricted to specific tissues while the others show ubiquitous expression (for reviews, see Myllyharju & Kivirikko 2001, Gelse *et al.* 2003, Myllyharju & Kivirikko 2004). The distribution of different collagen types in the adult tissues and in the developing mouse embryo is summarized in Table 1. The expression of most collagens starts during early embryogenesis and the expression patterns of collagens during embryonic development are usually similar to adult patterns but in many cases broader. Especially cartilage collagens show wider distribution during embryogenesis suggesting they have additional roles in development (Cheah *et al.* 1991).

Table 1. Occurrence of collagen types I-XIX and their expression in mouse embryos

Collagen type	Occurrence[1]	Expression in mouse embryo[2]		References
		From[3]	Distribution	
Fibrillar collagens				
I, III, V	Most connective tissues, especially bone (not III), dermis, tendon, ligament, cornea	E8.5, E9.5	Developing connective tissues, mesoderm, sclerotomes, dermatomes, blood vessels, later developing bone (not III), joints, tendon	(Niederreither *et al.* 1995, Andrikopoulos *et al.* 1992, Wenstrup *et al.* 2004)
II, XI	Cartilage, vitreous body	E9.5 E11.5	Cartilage, somites, notochord, mesenchymal and epithelial cells, heart, bone, brain, eye	(Cheah *et al.* 1991, Sandell *et al.* 1994, Yoshioka *et al.* 1995)
FACIT collagens				
IX	Cartilage, vitreous body	E10.5	Eye, heart, lung, cartilage, bone	(Liu *et al*. 1993)
XII	Tissues containing collagen I	E9.5	Similar to type I collagen	(Oh *et al.* 1993, Bohme *et al.* 1995)
XIV[4]	Tissues containing collagen I			
XVI	Most tissues, especially heart	E11.5	Heart, spinal neural fibers, skin	(Lai & Chu 1996)
XIX	BM zones of differentiating muscle cells	E9.5	Myotome, limb, esophagus, stomach, later skin, brain	(Sumiyoshi *et al.* 2001)
Basement membrane collagen				
IV	Basement membranes	E3	Inner cell mass, all BMs	(Leivo *et al.* 1980)
Microfibrillar collagen				
VI	Most connective tissues	E11.5	Subepidermal mesenchyme, dorsal aorta, meninges, muscle, lung, perichondrium, vertebrae, later most connective tissues	(Dziadek *et al.* 1996)
Collagen forming anchoring fibrils				
VII[4]	Skin, oral mucosa, cervix			
Hexagonal network-forming collagens				
VIII	Descemet's membrane, subepithelial and subendothelial ECM	E11.5	Embryonic heart, cranial mesenchyme, placental capillaries, brain, heart, lung	(Sage & Iruela-Arispe 1990, Iruela-Arispe & Sage 1991)
X	Hypertrophic cartilage	E13.5	Hypertrophic cartilage	(Kong *et al.* 1993)
Transmembrane collagens				
XIII	Cell-matrix and cell-cell interaction sites	E9.5	Central and peripheral nervous system, heart	(Sund *et al.* 2001a)
XVII[4]	Skin hemidesmosomes			
Multiplexin collagens				
XV	Many tissues, especially heart, skeletal muscle, in BM	E10.5	Capillaries, skeletal muscle, peripheral nerves, in BMs	(Muona *et al.* 2002)
XVIII	Vascular and epithelial BMs	E8.5	Vascular and epithelial BMs	(Miosge *et al.* 2003)

[1]determined from different species, for references, see the table and (Myllyharju & Kivirikko 2001, Gelse *et al.* 2003), [2]determined by *in situ* hybridization or immunofluorescence, [3]developmental stage when mRNA or protein first detected, [4]developmental expression not known

2.2.3.1 Mouse models for studying the function of collagens

Mouse models are useful for defining the significance and function of large, insoluble proteins like collagens (for a review, see Myllyharju & Kivirikko 2004). The importance of the correct expression of collagens for normal development and tissue architecture is clearly demonstrated by several collagen knockout mice (Table 2). Some of the knockout mice have proven the importance of collagens for the stability of tissue structure. For instance, type IV collagen has been shown to be necessary for stabilization of basement membranes (Pöschl *et al.* 2004), and the importance of fibril-forming collagens for the mechanical support of the blood vessels is clearly established in type I and III collagen knockout mice (Löhler *et al.* 1984, Liu *et al.* 1997).

In addition, analyses of knockout mice have revealed new information about the functions of certain collagen types. For example, studies of type V collagen knockout mice demonstrated the significance of type V collagen in the regulation of collagen fibrillogenesis (Wenstrup *et al.* 2004). Furthermore, studies of type XV and XVIII collagen knockout mice have provided new information about the function of these collagens as structural components stabilizing the structure of muscle and basement membranes (Eklund *et al.* 2001, Fukai *et al.* 2002, Utriainen *et al.* 2004). Several collagen knockout mice are viable and fertile, and many of them serve as models for human disorders (Table 2), which can be used to analyze the consequences of mutations in collagen genes (for reviews, see Byers 2000, Myllyharju & Kivirikko 2004).

2.2.3.2 Collagen-related human disorders

Mutations in collagen genes cause a wide spectrum of rare or common diseases. So far, more than 1300 mutations have been characterized in human collagen genes. Most of the mutations are single base substitutions that convert glycine to a more bulky amino acid and thus prevent the folding or cause an interruption in the triple helix, but mutations in different parts of the collagen chain can cause different phenotypes. A continuous triple helix is particularly important for the fibril-forming collagens whereas collagens containing interruptions in the triple helix can tolerate additional interruptions. If the mutant chains form triple helixes, the mutant molecules can have kinks or other abnormalities that can reduce the amount of supramolecular assemblies or alter their structure or function (for reviews, see Byers 2000, 2001, Myllyharju & Kivirikko 2004).

The presence of only one abnormal α chain in a collagen molecule containing two normal chains can lead to degradation of all three chains. Consequently, mutations that lead to the production of a structurally abnormal polypeptide which forms a triple helix with normal chains usually cause more severe phenotypes than mutations that prevent triple helix formation (for reviews, see Prockop *et al.* 1990, Prockop & Kivirikko 1995). Most of the structurally abnormal mutant and normal unassembled α chains are degraded intracellularly by a proteasomal pathway associated with the ER, or in lysosomes (Lamande *et al.* 1995, Ripley & Bienkowski 1997, Fitzgerald *et al.* 1999, Gotkin *et al.* 2004) while some of the unfolded proteins are degraded after secretion (for a review, see Prockop *et al.* 1990).

Table 2. Collagen knockout mouse models and corresponding human disorders

Gene[1]	Phenotype[2]	Defects	References	Human disease[3]
Col1a1	L, E12-14	Vascular defects	(Löhler *et al.* 1984)	OI, EDS VIIA, VIIB
Col2a1	L , birth	Chondrodysplasia, intervertebral disc defects	(Li *et al.* 1995, Aszodi *et al.* 1998)	Chondodysplasias, osteoarthritis
Col3a1	L, birth	Vascular and skin defects	(Liu *et al.* 1997)	EDS IV, arterial aneurysms
Col4a1/a2	L, E10.5-11.5	BM defects	(Pöschl *et al.* 2004)	Porencephaly[4]
Col4a3	L, 12-16w	Renal failure, progressive glomerulonephritis	(Cosgrove *et al.* 1996)	Alport syndrome
Col5a1	L, E10.5	Cardiovascular defects, lack of collagen fibrils	(Wenstrup *et al.* 2004)	EDS I, EDS II
Col6a1	V, F	Mild muscular dystrophy	(Bonaldo *et al.* 1998)	Bethlehem myopathy, Ullrich muscular dystrophy
Col7a1	L, 2w	Skin blistering	(Heinonen *et al.* 1999)	Dystrophic epidermolysis bullosa
Col8a1/a2	V, F	Corneal defects	(Hopfer *et al.* 2005)	Corneal endothelial dystrophy
Col9a1	V, F	Degenerative changes in articular cartilage	(Fässler *et al.* 1994)	Multiple epiphyseal dysplasia, osteoarthritis, intervertebral disc disease
Col10a1	V, F	Mild skeletal changes	(Kwan *et al.* 1997)	Schmid metaphyseal chondrodysplasia
Col11a2	V, F	Hearing loss, moderate chondrodysplasia	(McGuirt *et al.* 1999, Li *et al.* 2001)	Chondrodysplasias, nonsyndromic hearing loss, osteoarthritis
Col12a1^{T}	V, F	Disruption of matrix structure of periodontal ligament and skin	(Reichenberger *et al.* 2000)	
Col13a1^{T}	L, E10.5-13.5	Cardiovascular and placental defects	(Sund *et al.* 2001b)	
	V, F	Progressive myopathy	(Kvist *et al.* 2001)	
Col15a1	V, F	Skeletal myopathy, cardiovascular defects	(Eklund *et al.* 2001)	
Col18a1	V, F	Ocular abnormalities, tendency to form hydrocephalus	(Fukai *et al.* 2002, Utriainen *et al.* 2004)	Knobloch and pigment dispersion syndromes
Col19a1	V, F	Esophagus abnormalities	(Sumiyoshi *et al.* 2004)	

[1]gene knocked out, except [T]transgenic, [2]abbreviations used: L, lethal; E, embryonic day; w, weeks V, viable; F, fertile, [3]human diseases with mutations in the same gene, abbreviations used: OI, osteogenesis imperfecta; EDS, Ehlers-Danlos syndrome, for references see (Byers 2000, Myllyharju & Kivirikko 2001, 2004) except [4] (Gould *et al.* 2005)

2.2.4 Collagen biosynthesis

Collagen biosynthesis involves many co- and posttranslational modifications many of which are unique to collagenous proteins. The posttranslational processing of fibril-forming collagens takes place in two stages. Synthesis of the proα chains together with intracellular co- and posttranslational modifications results in the formation of triple-helical procollagen molecules, which are converted into collagens by extracellular processing (for reviews, see Kivirikko 1995, Kielty & Grant 2002, Gelse *et al.* 2003, Canty & Kadler 2005).

2.2.4.1 Intracellular processing

Procollagen molecules are translocated into the lumen of the rough endoplasmic reticulum (ER) with the help of N-terminal signal peptides. After the removal of the signal peptide by signal peptidase, prolyl 4- and 3-hydroxylases and lysyl hydroxylase hydroxylate some proline and lysine residues to 4-hydroxyproline, 3-hydroxyproline and hydroxylysine. Some of the hydroxylysine residues are further glycosylated to galactosylhydroxylysine and glucosylgalactosylhydroxylysine by hydroxylysyl galactosyltransferase and galactosylhydroxylysyl glucosyltransferase, respectively. Most of these modifications occur co-translationally and they continue, after the release of the polypeptide chain from the ribosomes, until triple helix formation starts (for reviews, see Kivirikko 1995, Kielty & Grant 2002, Canty & Kadler 2005).

Before the formation of the triple helix, the C-terminal domains of α chains associate through a process directed by their structure and the formation of disulfide bonds catalyzed by protein disulfide isomerase. Furthermore, the oligosaccharyl transferase complex adds N-linked carbohydrate groups to specific asparagine residues within the C-propeptide. A nucleus of the triple helix forms in the C-terminal region, and the triple helix is then propagated towards the N-terminus in a zipper-like fashion. The efficient formation and folding of the procollagen chains depend on the presence of peptidyl-prolyl cis-trans-isomerase and collagen-specific chaperone Hsp47 (for reviews, see Kivirikko 1995, Kielty & Grant 2002, Gelse *et al.* 2003, Canty & Kadler 2005).

The triple-helical procollagen molecules are transported from the ER to the Golgi complex for further processing of N-linked carbohydrates. Procollagen subsequently moves through the cell without ever leaving the lumen of the Golgi cisternae, and after transport to the plasma membrane, the procollagens are secreted into the extracellular space. Triple helix formation is an absolute requirement for the normal rate of collagen secretion. If it is prevented, misfolded polypeptides will first accumulate within the ER and will then be in part degraded and in part secreted as a non-functional protein at reduced rate (for reviews, see Kivirikko 1995, Kagan 2000, Canty & Kadler 2002, 2005).

2.2.4.2 Extracellular processing

The processing of procollagen to collagen takes place in the extracellular space where the N- and C-propeptides are cleaved by procollagen N-and C-proteinases. The collagen molecules then form fibrils spontaneously by self-assembly, and covalent cross-links are formed within and between collagen molecules (for reviews, see Kivirikko 1995, Kagan 2000, Canty & Kadler 2002, 2005).

The processing and assembly of non-fibrillar collagens feature the same steps as the fibril-forming collagens, but with some differences. All non-fibrillar collagens contain interruptions in the triple helix, and many collagen molecules also contain N- and/or C-terminal non-collagenous domains that are not cleaved. The assembly of type VI collagen tetramers is unique as it occurs intracellularly. The folding of the triple helix in type XIII and XVII collagens proceeds from the N- to the C-terminus, in opposite orientation to that of the fibrillar collagens. In addition, some collagens also have additional processing steps such as the addition of glycosaminoglycan side chains (for reviews, see Myllyharju & Kivirikko 2001, Kielty & Grant 2002, Ricard-Blum & Ruggiero 2005).

2.3 Hydroxylysine

The hydroxylysyl residues of collagen molecules have two important functions: the hydroxyl groups serve as attachment sites for carbohydrate units, either galactose or glucosylgalactose and they are essential for the stability of the intermolecular collagen cross-links. The hydroxylysine appears almost exclusively in the Y position of the X-Y-Gly-triplets, although the short non-triple-helical sequences at the ends of the α chains contain one sequence of -X-Hyl-Ser or -X-Hyl-Ala in some collagens (Kivirikko & Myllylä 1980).

2.3.1 Hydroxylysine in collagens

The number of hydroxylysyl residues per 1000 amino acids varies from 5 to 70 among different collagens. The lowest amounts have been found in collagen types I and III, where 17-38 % of all lysyl residues are hydroxylated. The level is highest in the type IV and VI collagens having 70-89 % of all lysines hydroxylated. Additional variations in the hydroxylation of lysyl residues can be seen within the same collagen type in different tissues and even in the same tissue in different physiological and pathological states. The differences in the extent of lysyl hydroxylation are dependent on the lysyl hydroxylase activity but also on the time needed for the formation of the collagen triple helix. Furthermore, the variation is affected by differences in the total amount of lysyl residues incorporated into the collagen chain, distribution of these residues between the X and Y positions and sometimes incomplete hydroxylation of lysyl residues in the Y position (for reviews, see Kivirikko *et al.* 1992, Kivirikko & Pihlajaniemi 1998).

The amount of hydroxylysine is especially high in embryonic collagens and the extent of hydroxylation falls with increasing age (Miller *et al.* 1967, Barnes *et al.* 1974). The hydroxylysine content is also increased during bone repair when compared with normal adult bone (Glimcher *et al.* 1980), and in keloid (Uzawa *et al.* 1998). Moreover, increased lysyl hydroxylation have been detected in several diseases, such as osteoarthritis (Bank *et al.* 2002), osteoporosis (Bailey *et al.* 1992, Knott *et al.* 1995) and in chondrodysplasias (Murray *et al.* 1989). Lysines are also overhydroxylated in osteogenesis imperfecta (Tenni *et al.* 1993, Bank *et al.* 2000), which is often caused by mutations that delay the formation of triple helix (for a review, see Byers 2001).

2.3.2 Hydroxylysine in other proteins

Hydroxylysine residues are also found in a few other proteins with collagen-like domains. The collagen-like sequences of subcomponent C1q and acetylcholinesterase have high contents of hydroxylysine, and hydroxylysyl residues have also been detected in lung surfactant proteins A and D, mannose-binding protein, conglutinin, collectin-43, ficolins, macrophage scavenger receptors and macrophage receptor (for reviews, see Kivirikko *et al.* 1992, Kivirikko & Pihlajaniemi 1998). Interestingly hydroxylysine residues have also been found in non-collagenous proteins, although their biological significance is not known. For example, anglerfish somatostatin-28, a peptide hormone, has one hydroxylysine in a –Trp-Hyl-Gly-sequence (Andrews *et al.* 1984). Hydroxylysine has also been detected in human tissue plasminogen activator and the human CD4 receptor at surface-accessible Lys-Gly sites (Molony *et al.* 1995).

2.3.3 Glycosylated hydroxylysine

Some of the hydroxylysine-linked carbohydrates are present as the monosaccharide galactose and some as the disaccharide glucosylgalactose (for reviews, see Kivirikko & Myllylä 1979, Kivirikko 1995). The structure of the disaccharide with its peptide attachment is 2-O-α-D-glucopyranosyl-O-β-D-galactopyranosylhydroxylysine, involving an unusual α1→2-O-glycosidic bond between glucose and galactose (Spiro 1967).

The extent of glycosylation of hydroxylysines and the ratio of the monosaccharide to the disaccharide varies markedly between different collagen types and even within the same collagen type in various physiological and pathological states. A similar heterogeneity exists with age, the collagen molecules of embryonic tissues having more of these modifications than adult tissues (Kivirikko & Myllylä 1979). The amount of glycosylated hydroxylysines is also increased in some osteogenesis imperfecta cases (Tenni *et al.* 1993), in osteoporosis (Lo Cascio *et al.* 1999), osteosarcoma (Lehmann *et al.* 1995) and lipodermatosclerosis (Brinckmann *et al.* 1999).

Type I and III collagens have very few glycosylated hydroxylysines, the monosaccharide being the predominant form, whereas about 80% of the hydroxylysines are glycosylated, mostly by the disaccharide in type IV collagen (Spiro 1969). Moreover, in type VI collagen all lysyl residues in the Y position of Gly-X-Y triplets are

hydroxylated and glycosylated (for a review, see Ayad *et al.* 1998). The extent of glycosylation of hydroxylysyl residues in collagens is affected by the glycosyltransferase activities. The time available before the formation of the triple helix also influences the glycosylation. Therefore, the slower helix formation in type II and especially type IV collagens contribute to the higher glycosylation stage when compared with type I with quicker helix formation (for a review, see Kivirikko & Myllylä 1979).

2.3.4 Function of glycosylated hydroxylysines

The function of the hydroxylysine-linked carbohydrates is not fully understood. As they form the most extrusive groups in the collagen molecule, they might regulate the packing of collagen molecules into supramolecular assemblies (for a review, see Kivirikko 1995). However, when the effect of overglycosylation on fibril diameter and formation has been studied *in vitro*, the results are inconsistent. Excess hydroxylation and glycosylation of lysines seems to affect the fibril formation and morphology of type II collagen leading to thinner fibrils with no interfibril interaction (Notbohm *et al.* 1999), but the results on fibril formation of type I collagen are contradictory (Torre-Blanco *et al.* 1992, Bätge *et al.* 1997). Furthermore, type I procollagen with a high degree of lysine hydroxylation and hydroxylysine glycosylation was cleaved by type I procollagen N-proteinase at a reduced rate, suggesting that the extent of collagen glycosylation may also affect the interaction with this enzyme (Torre-Blanco *et al.* 1992).

It has been suggested that glycosylation of hydroxylysines may affect the interactions of collagens with other matrix macromolecules (Tenni *et al.* 1993). The immunodominant epitope in natural human collagen II-derived peptides contains glycosylated hydroxylysines (Van den Steen *et al.* 2004) and the immune response to type II collagen is dependent on the degree of this glycosylation suggesting that glycosylation of type II collagen has a role in the induction of arthritis (Myers *et al.* 2004). In particular, T-cell recognition of a specific galactosyl hydroxylysyl residue in a type II collagen derived peptide is important for the development of collagen induced arthritis (Corthay *et al.* 1998, Bäcklund *et al.* 2002). Furthermore, vaccination with the galactosylated peptide prevented the development of arthritis while the nonglycosylated peptide had no effect (Dzhambazov *et al.* 2006). It has also been reported that the galactosylation of Hyl1265 in the α1 chain of type IV collagen causes a dramatic reduction in adhesion and spreading of melanoma cells suggesting that even subtle changes in collagen carbohydrate content may have significant biological consequences (Lauer-Fields *et al.* 2003).

Glycosylated hydroxylysines have also been found in other proteins with collagenous sequences, like in the C1q subcomponent (for a review, see Kivirikko *et al.* 1992). The serum mannan-binding lectin has glycosylated hydroxylysines which are critical for the assembly of the triple helix and the secretion of the protein (Heise *et al.* 2000). Similarly, hydroxylation and glycosylation of the lysines in the collagenous domain of adiponectin regulate its multimerization and secretion and consequently contribute to its activity as an insulin sensitizer (Richards *et al.* 2006, Wang *et al.* 2006).

2.3.5 Collagen cross-links

Covalent cross-links are formed within and between collagen molecules and they provide the collagen fibrils with tensile strength and mechanical stability. The formation of cross-links starts with oxidative deamination of specific telopeptide lysyl and hydroxylysyl residues generating the reactive aldehydes, allysine and hydroxyallysine. This reaction is catalyzed by lysyl oxidase, and after that the formation and further maturation of cross-links occurs spontaneously (for a review, see Kielty & Grant 2002).

The formation of cross-links is illustrated in Figure 2. Briefly, allysine or hydroxyallysine reacts with helical lysine or hydroxylysine to form intermediate, aldimine or keto-imine cross-links. These reducible di-functional cross-links are then converted into nonreducible tri-functional, mature cross-links to provide additional stability during maturation of the tissue. Tri-functional cross-links can also be formed by the reaction of two di-functional cross-links. These cross-links have been characterized in fibril-forming collagens, and other collagen types probably contain different cross-links (for reviews, see Eyre *et al.* 1984, Knott & Bailey 1998, Robins 1999, Kielty & Grant 2002, Robins & Brady 2002).

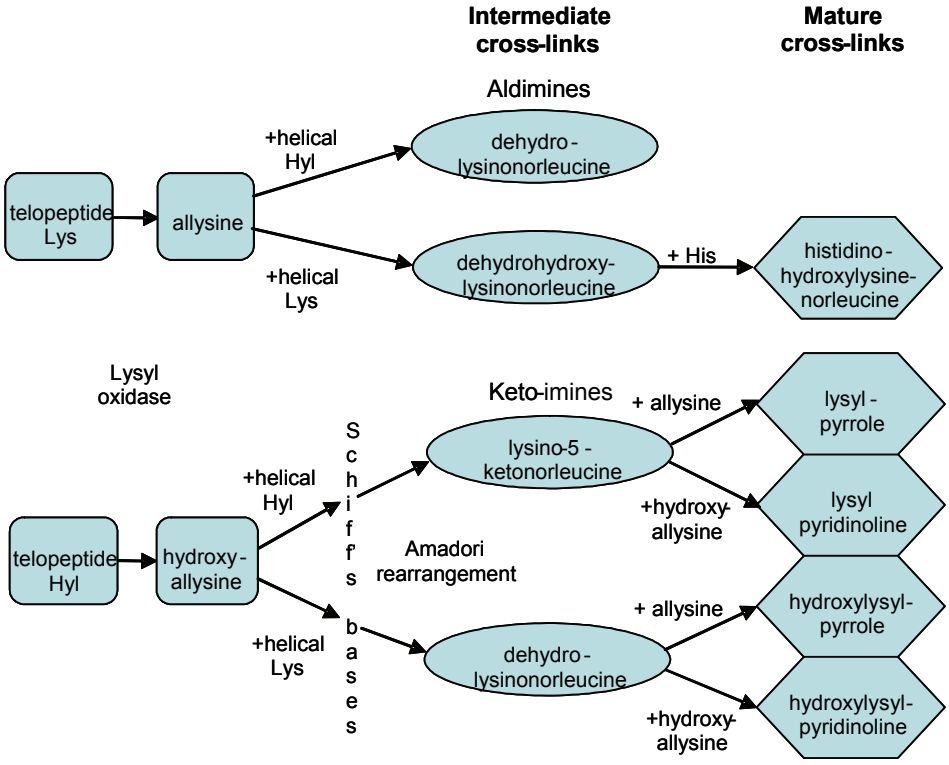

Fig. 2. The formation of collagen cross-links through different pathways.

Cross-links derived from a hydroxyallysine are more stable than those derived from allysine. Adult skin, cornea and sclera contain lysine aldehyde derived cross-links, whereas hydroxylysine aldehyde derived cross-links predominate in bone, cartilage, ligament, most tendons, embryonic skin and most major intestinal tissues (for reviews, see Robins 1999, Kielty & Grant 2002). Glycosylated hydroxylysines can also be involved in cross-linking. Type I, II and III collagens have four cross-linking sites, one in both telopeptides and two in the helix, and the N-telopeptide-derived cross-links are usually linked to the C-terminal part of the helix, whereas the C-telopeptide-derived cross-links are adjacent to the N-terminal end of the helix (for reviews, see Knott & Bailey 1998, Robins & Brady 2002).

2.4 Lysyl hydroxylase

Lysyl hydroxylase (LH, EC 1.14.11.4) was first purified as a homogenous protein from chick embryos (Turpeenniemi-Hujanen *et al.* 1980) and later from human placenta (Turpeenniemi-Hujanen *et al.* 1981). The human lysyl hydroxylase is a dimer (α_2) with a molecular weight of 190,000, which consists of only one type of subunit. The molecular weight of the monomer is about 80,000-85,000 depending on the glycosylation (Turpeenniemi-Hujanen *et al.* 1981, Myllylä *et al.* 1988). Lysyl hydroxylase contains asparagine-linked carbohydrate units which are required for maximal lysyl hydroxylase activity (Myllylä *et al.* 1988, Pirskanen *et al.* 1996).

Lysyl hydroxylase was first cloned from chicken and it was shown to be a polypeptide consisting of 710 amino acids and a signal peptide of 20 amino acids. Although the catalytic properties of lysyl hydroxylase are very similar to prolyl-4-hydroxylase, there is no significant homology between the sequences. Chick lysyl hydroxylase has four potential attachment sites for asparagine-linked oligosaccharides and 9 cysteine residues, one of which is probably involved in the binding of a Fe^{2+} atom to a catalytic site (Myllylä *et al.* 1991). The cloning of human lysyl hydroxylase revealed a polypeptide with 709 amino acids and a signal peptide of 18 amino acids. The sequence of human lysyl hydroxylase is well conserved with the chick sequence, especially in the C-terminus with over 90% homology suggesting that the C-terminus probably contains functionally significant regions (Hautala *et al.* 1992a).

2.4.1 Reaction catalyzed by lysyl hydroxylase

Lysyl hydroxylase belongs to the group of 2-oxoglutarate dioxygenases, which all have a similar reaction mechanism. The reaction requires Fe^{2+}, 2-oxoglutarate, O_2 and ascorbate and produces succinate and CO_2. During the hydroxylation reaction 2-oxoglutarate is stoichiometrically decarboxylated and one oxygen atom is incorporated into succinate while the other is incorporated into the hydroxyl group formed (for reviews, see Kivirikko *et al.* 1992, Kivirikko & Pihlajaniemi 1998).

The reaction mechanism catalyzed by lysyl hydroxylase involves an ordered binding of Fe^{2+}, 2-oxoglutarate, O_2 and the peptide substrate in this order, and an ordered release

of the hydroxylated peptide, CO_2, succinate and Fe^{2+}. The Fe^{2+} need not leave the enzyme during each catalytic cycle and the order of release of the hydroxylated peptide and CO_2 is uncertain (Puistola *et al.* 1980b). LH can also catalyze the uncoupled decarboxylation of 2-oxoglutarate without subsequent hydroxylation of the peptide substrate (Puistola *et al.* 1980a). The uncoupled decarboxylation occurs even in the presence of saturating conditions of the peptide substrate and some non-hydroxylatable peptides increase the rate of the reaction (for a review, see Kivirikko & Pihlajaniemi 1998).

2.4.1.1 Peptide substrate

The lysine residue must be in a peptide linkage in order to be hydroxylated. The minimum sequence requirement for lysyl hydroxylase reaction is an –X-Lys-Gly- triplet (Kivirikko *et al.* 1972), but lysines are also hydroxylated in –X-Lys-Ser-, -X-Lys-Ala and –X-Lys-Thr-sequences of arginine-rich histones (Ryhänen 1975). The amino acid sequence around the lysyl residue and the peptide chain length influence the interaction of the substrate with the enzyme (Kivirikko *et al.* 1972).

The conformation of the peptide substrate is also an important determinant of the reaction. A triple-helical conformation of the collagenous peptide prevents lysine hydroxylation (Kivirikko *et al.* 1973, Ryhänen & Kivirikko 1974). It has been suggested that a folded β-turn in the peptide substrate would be necessary for the hydroxylation at the catalytic site. The peptide may also need an extended polyproline-II structure for the interaction at the binding site of the lysyl hydroxylase (Jiang & Ananthanarayanan 1991, Ananthanarayanan *et al.* 1992).

However, binding of the collagenous peptides to recombinant LH isoforms in an aqueous environment is not affected by structural motifs. Furthermore, the analysis of the binding and hydroxylation of collagenous peptides *in vitro* demonstrated that specific amino acids surrounding the lysyl residue together with the net charge of the peptide promote its binding to all LH isoforms. The peptides with a positive net charge and stronger preference for an aqueous environment bound all LH isoforms more often. These data suggest that the LH binding site is not a deep hydrophopic pocket, but rather that it is open, and hydrophilic and acidic amino acids affect the binding (Risteli *et al.* 2004).

2.4.1.2 Co-substrates, activators and inhibitors

The reaction catalyzed by lysyl hydroxylase requires Fe^{2+}, 2-oxoglutarate, O_2 and ascorbate (for a review, see Kivirikko & Pihlajaniemi 1998). The Fe^{2+} is bound to the lysyl hydroxylase by three side chains, His-656, His-708 and Asp-658, the mutation of any of these residues inactivate the enzyme completely (Pirskanen *et al.* 1996).

The 2-oxoglutarate is an absolute and highly specific requirement for the hydroxylation reaction (for a review, see Kivirikko *et al.* 1992). The 2-oxoglutarate binding site of collagen hydroxylases can be divided into two main subsites (Hanauske-Abel & Günzler 1982, Majamaa *et al.* 1984). However, the K_m of 2-oxoglutarate for lysyl hydroxylase is higher than for other hydroxylases suggesting that different collagen

hydroxylases have slightly different structure in the 2-oxoglutarate binding site (for a review, see Kivirikko & Pihlajaniemi 1998). Subsite I in human LH1 is Arginine-700 which ionically binds the C-5 carboxyl group (Passoja *et al.* 1998a) while subsite II consists of two *cis*-positioned coordination sites of the enzyme bound Fe^{2+} and it is chelated by the C1-C2 moiety of 2-oxoglutarate (Hanauske-Abel & Günzler 1982, Majamaa *et al.* 1984).

The molecular oxygen required in the hydroxylation reaction comes from the atmosphere. The oxygen atoms are incorporated into an enzyme-bound intermediate and they are exchangeable with water (Kikuchi *et al.* 1983). Ascorbate is a specific requirement for the reaction (Puistola *et al.* 1980a). It is required to reactivate the enzyme by reducing the enzyme-bound Fe^{3+} after the uncoupled decarboxylation reaction (Myllylä *et al.* 1984).

In addition to co-substrates, purified lysyl hydroxylase needs dithiothreitol, bovine serum albumin and catalase for maximal activity. The dithiothreitol probably keeps the essential free thiol groups at the catalytic site reduced, and the action of bovine serum albumin is also in part due to the presence of free thiol groups. Bovine serum albumin and catalase act partly by a non-specific protein effect, and catalase also destroys peroxide (for reviews, see Kivirikko & Myllylä 1982, Kivirikko *et al.* 1992).

Lysyl hydroxylase can be inhibited competitively with respect to Fe^{2+}, 2-oxoglutarate and O_2 by many divalent cations, structural analogues of the 2-oxoglutarate and citric acid cycle intermediates and superoxide dismutase, respectively (Ryhänen 1976, Myllylä *et al.* 1979, Puistola *et al.* 1980b, Majamaa *et al.* 1985). Furthermore, the organophosphates malathion and malaoxon inhibit lysyl hydroxylase by competing with collagen for the peptide substrate binding site (Samimi & Last 2001). In addition, the amount of LH protein can be downregulated by minoxidil, an antihypertensive drug, which specifically decreases LH mRNA (Hautala *et al.* 1992b, Yeowell *et al.* 1992, Zuurmond *et al.* 2005).

2.5 Lysyl hydroxylase isoforms

The presence of lysyl hydroxylase isoforms was first proposed to explain the differences in the level of lysyl hydroxylation between different tissues and collagen types. The variation in the extent of hydroxylation was especially seen in patients with Ehlers-Danlos syndrome type VI (EDS VI) that showed markedly decreased hydroxylysine content in skin, reduced levels in bone but normal amounts in cartilage. In addition, some collagen types had very low levels of hydroxylysine, while the level was normal in other types, suggesting the existence of tissue- or collagen type-specific LH isoenzymes (Pinnell *et al.* 1972, Risteli *et al.* 1980, Ihme *et al.* 1984).

The existence of LH isoforms was debated over the years, until the development of DNA techniques enabled cloning of a lysyl hydroxylase cDNA sequence and the search for other gene products with sequence homology. Two novel lysyl hydroxylase isoforms, named lysyl hydroxylase 2 (LH2) and lysyl hydroxylase 3 (LH3) were first characterized from human (Valtavaara et al. 1997, Passoja et al. 1998b, Valtavaara et al. 1998) and later from mouse, rat and zebrafish (Ruotsalainen et al. 1999, Mercer et al. 2003, Schneider &

Granato 2007). Furthermore, the gene coding for LH2 can be alternatively spliced resulting in two different forms, LH2a and LH2b (Valtavaara 1999, Yeowell & Walker 1999b). After the cloning of the new lysyl hydroxylase isoforms, the previously known isoform was named LH1. Phylogenetic analysis suggests that the lysyl hydroxylase isoforms are derived from an ancestral gene by two gene duplication events. LH3 is the oldest LH isoform whereas LH1 and LH2 are derived by a more recent gene duplication (Ruotsalainen et al. 1999).

2.5.1 Characteristics of lysyl hydroxylase isoforms

The lengths of the human LH1, the shorter LH2 form, LH2a and LH3 polypeptides without the putative signal peptides are 709, 712 and 714 and the lengths of the corresponding mouse isoforms are 710, 710 and 714, respectively (Hautala *et al.* 1992a, Valtavaara *et al.* 1997, Valtavaara *et al.* 1998, Ruotsalainen *et al.* 1999).

The amino acid sequences are well conserved between different species, the similarity between the human, mouse and rat isoforms is over 90%. The amino acid sequences of different isoforms show about 60% similarity among the same species, and the C-terminus, which is important for the lysyl hydroxylase activity, is the most conserved part of the molecule. All four critical residues in the catalytic sites, two histidines and one aspartate that bind the Fe^{2+} atom (Pirskanen *et al.* 1996) and the arginine involved in the binding of 2-oxoglutarate (Passoja *et al.* 1998a) are conserved in all lysyl hydroxylases (Hautala *et al.* 1992a, Armstrong & Last 1995, Valtavaara *et al.* 1997, Passoja *et al.* 1998b, Valtavaara *et al.* 1998, Ruotsalainen *et al.* 1999, Mercer *et al.* 2003).

Furthermore, all human and mouse LH isoforms contain nine conserved cysteine residues and several potential attachment sites for asparagine-linked oligosaccharides, which are not conserved between isoforms (Hautala *et al.* 1992a, Valtavaara *et al.* 1997, Passoja *et al.* 1998b, Valtavaara *et al.* 1998, Ruotsalainen *et al.* 1999). Although glycosylation of one of the asparagines may be required for full activity of LH1 (Pirskanen *et al.* 1996), this particular site is not conserved in LH3, suggesting that different isoforms may have different glycosylation requirements for catalytic activity (Passoja *et al.* 1998b).

The recombinant LH isoforms are present as homodimers at protein level, and they have two main protease sensitive regions that divide the polypeptides into three fragments (Rautavuoma *et al.* 2002). The K_m values for Fe^{2+}, 2-oxoglutarate, ascorbate and a peptide substrate have been determined for recombinant human lysyl hydroxylases. The catalytic properties of LH1 and LH3 were essentially identical (Passoja *et al.* 1998b), while the kinetic constants of LH2 were different from the other isoforms (Valtavaara 1999).

2.5.2 Genes for lysyl hydroxylase isoforms

The gene structure has been determined for human LH1, mouse LH2 and both human and mouse LH3 (Heikkinen *et al.* 1994, Rautavuoma *et al.* 2000, Ruotsalainen *et al.* 2001).

The genes have been named *PLOD1*, *Plod2*, *PLOD3* and *Plod3*, respectively. *PLOD1, PLOD3* and *Plod3* have 19 exons, whereas *Plod2* has 20 exons, one of which is alternatively spliced. The exons of the different LH genes are quite similar in size, but the sizes of the introns vary significantly between isoforms. *Plod2* is the largest gene among the lysyl hydroxylases, being about 50 kb, the size of *PLOD1* is 40 kb, whereas the *PLOD3* and *Plod3* are much smaller, 11.6 and 10 kb. Both *PLOD1* and *PLOD3* have several repetitive Alu-sequences, which can create potential recombination sites in the genes, and many repetitive retroposon-like sequences have also been found in *Plod3*. None of the genes have a TATAA box in the promoter area and they all have several transcription start sites. However, the 5'-flanking regions of the LH genes are different suggesting differences in the regulation of gene expression (Ruotsalainen *et al.* 2001).

The genes for human lysyl hydroxylases have been mapped to different chromosomes, *PLOD1* to chromosome 1p36.2-1p36.3 (Hautala *et al.* 1992a), *PLOD2* to 3q23-q24 (Szpirer *et al.* 1997) and *PLOD3* to 7q22 (Valtavaara *et al.* 2000). Similarly, rat lysyl hydroxylase genes reside in different chromosomes, the gene for LH1 on the chromosome 5q36 (NCBI), the gene for LH2 on 8q31and the gene for LH3 on 12q12 (Mercer *et al.* 2003).

2.5.3 Subcellular localization of lysyl hydroxylase isoforms

Lysyl hydroxylase 1 has been localized to the cisternae of the rough endoplasmic reticulum (Guzman *et al.* 1976, Peterkofsky & Assad 1979) as a peripherally associated membrane protein which binds to membranes via weak electrostatic interactions (Kellokumpu *et al.* 1994). However, LH1 does not contain either of the previously known ER-retention motifs, KDEL or double lysine (Myllylä *et al.* 1991, Hautala *et al.* 1992a). When the ER retention of LH1 was studied in more detail, a 40-amino acid peptide in the C-terminus of LH1 was found to contain all the information necessary for the correct localization in the ER (Suokas *et al.* 2000). Further analysis of this peptide showed that it localizes LH1 to the ER permanently, without recycling between the Golgi and ER. The critical localization motif consists of 17 amino acids forming an extended loop within the iron-binding domain of LH. The loop is exposed and readily accessible for binding (Suokas *et al.* 2003). The 40-amino acid peptide is almost identical in the different LH isoforms, suggesting that all the LH isoforms use the same mechanism for their localization in the ER (Suokas *et al.* 2000, Suokas *et al.* 2003).

LH3 has been localized to the ER in cultured cells (Heikkinen *et al.* 2000) and in mouse kidney (Salo *et al.* 2006). Surprisingly, LH3 has also been detected in the extracellular space in the mouse liver and in the serum. In addition, LH3 can be found inside cultured cells, but also on the cell surface and secreted into the medium. The LH3 secreted into the medium and in serum is more heavily glycosylated than the intracellular enzyme (Salo *et al.* 2006).

2.5.4 Specificity of lysyl hydroxylase isoforms

Despite the variation of lysyl hydroxylation in different tissues (Pinnell *et al.* 1972), all LH isoforms are expressed in several tissues (Table 3). The human LH1 shows constitutive expression in several tissues (Heikkinen *et al.* 1994, Yeowell *et al.* 1994), whereas the distribution of LH2 and LH3 is more restricted to specific tissues (Valtavaara *et al.* 1997, Passoja *et al.* 1998b, Valtavaara *et al.* 1998). The mouse lysyl hydroxylases show distinct tissue distribution (Ruotsalainen *et al.* 1999), while the rat LH isoforms show widespread expression in several tissues and cell types (Armstrong & Last 1995, Mercer *et al.* 2003).

Table 3. The tissue distributions of human and mouse lysyl hydroxylases

Isoform	Species	Tissues with high expression	Tissues with lower expression	Reference
LH1	human	liver, spleen, skeletal muscle, cartilage, brain, lung, placenta, vein, aorta, artery, gall bladder		(Heikkinen *et al.* 1994, Yeowell *et al.* 1994)
LH1	mouse	heart, liver, lung, skeletal muscle, kidney		(Ruotsalainen *et al.* 1999)
LH2	human	heart, placenta, pancreas	brain, lung, liver, kidney	(Valtavaara *et al.* 1997)
LH2	mouse	heart, lung, kidney	skeletal muscle, testis	(Ruotsalainen *et al.* 1999)
LH3	human	placenta, pancreas, spinal cord	brain, lung, liver, skeletal muscle, kidney	(Passoja *et al.* 1998b, Valtavaara *et al.* 1998)
LH3	mouse	heart, lung, liver and testis	brain, skeletal muscle, kidney	(Ruotsalainen *et al.* 1999)

The presence of collagen type specific LH isoforms has been suggested to explain the variable hydroxylation of lysines detected in EDS VI patients. Normal fibroblasts preferentially hydroxylate type I collagen, but cells from EDS VI patients that had reduced LH activity, hydroxylated type IV collagen more effectively than type I collagen (Risteli *et al.* 1980). Furthermore, type I and III collagens extracted from different tissues of EDS VI patients showed low hydroxylysine content, whereas that in type in II, IV and V collagens was normal (Ihme *et al.* 1984).

However, when the mRNA expression of LH1, LH2 and LH3 and collagen types I, III, IV and V were studied in different cell lines, there was no correlation between different LH isoforms and different collagen types, suggesting a lack of clear collagen type specificity (Wang *et al.* 2000). The data obtained from *in vitro* binding of collagenous peptides to different LH isoforms indicate that there is no strict sequence specificity for the LH isoforms either. However, there is a clear preference for some sequences to be bound and hydroxylated by a specific isoform, and a tendency for the isoforms to favor some collagen types (Risteli *et al.* 2004).

2.5.5 Lysyl hydroxylase 1

Lysyl hydroxylase 1 was the first lysyl hydroxylase to be cloned (Myllylä *et al.* 1991, Hautala *et al.* 1992a). Due to its constitutive expression (Heikkinen *et al.* 1994, Yeowell *et al.* 1994), it is the most studied isoform and its importance *in vivo* is clearly demonstrated in patients with type VI Ehlers-Danlos syndrome.

2.5.5.1 Mutations in the gene for LH1cause type VI Ehlers-Danlos syndrome

EDS VI, the kyphoscoliotic type of Ehlers-Danlos syndrome is an autosomal recessively inherited connective tissue disorder. The patients show severe muscular hypotonia at birth, generalized joint laxity, progressive kyphoscoliosis from birth, skin fragility, scleral fragility and rupture of the ocular globe (for reviews, see Beighton *et al.* 1998, Steinmann 2002). EDS VI was the first collagen disorder with a known biochemical and genetic basis; the patients showed decreased hydroxylysine content of collagen in skin and other organs and a reduced activity of lysyl hydroxylase in skin fibroblasts (Krane *et al.* 1972, Pinnell *et al.* 1972, Sussman *et al.* 1974).

Due to decreased LH activity the EDS VI patients also show an abnormal cross-linking pattern: the skin lacks the mature cross-links containing histidine (Eyre & Glimcher 1972) and the lysyl pyridinoline:hydroxylysyl pyridinoline ratio is altered in bone (Steinmann *et al.* 1995). However, the LH activity and dermal hydroxylysine content correlate poorly with the severity of the phenotype (for a review, see Steinmann 2002). Patients with a phenotype resembling EDS VI but with normal lysyl hydroxylase activity have been reported (Judisch *et al.* 1976, Royce *et al.* 1989), and this form of the syndrome has been classified as EDS VIB (for a review, see Steinmann 2002).

At least 20 different mutations contributing to EDS VI have been identified in the LH1 gene (for a review, see Yeowell & Walker 2000). The most common of these mutations is a large, 8.9 kb duplication of seven exons caused by a homologous recombination of Alu sequences in introns 9 and 16, and the frequency of this mutation is almost 25% among EDS VI families (Hautala *et al.* 1993, Pousi *et al.* 1994, Heikkinen *et al.* 1997, for a review, see Yeowell & Walker 2000). Three other mutations, a 15-bp deletion in exon 11 and single base substitutions in exons 10 and 14 generating premature termination codons, have been found in unrelated patients (Yeowell & Walker 1997, Walker *et al.* 1999, Pousi *et al.* 2000, Yeowell *et al.* 2000a, Yeowell *et al.* 2000b). Other mutations characterized in *PLOD1* include four splicing defects, two deletions and insertions, seven point mutations causing premature stop codons or nonsense codons and a combination of point mutations and a deletion (Hyland *et al.* 1992, Ha *et al.* 1994, Heikkinen *et al.* 1997, Yeowell & Walker 1997, Brinckmann *et al.* 1998, Pajunen *et al.* 1998, Pousi *et al.* 1998, Heikkinen *et al.* 1999, Yeowell *et al.* 2000a, Yeowell *et al.* 2000b).

The genetic basis for EDS VIB is unknown. In some patients the amount of LH2 or LH3 mRNA have been decreased, but the LH activity and cross-linking patterns were normal and no mutations have been found in the *PLOD2* gene (Walker *et al.* 2004a, Walker *et al.* 2004b).

2.5.5.2 LH1 hydroxylates lysines in the triple-helical domain

Studies on EDS VI have given new information about the substrate specificity of LH1. The amount of cross-links, which require hydroxylation of lysines in the helical part of collagen, is decreased in the EDS VI patients (Eyre & Glimcher 1972, Steinmann *et al.* 1995). Furthermore, in an EDS VI patient type I collagen of bone and type II collagen of cartilage showed a loss or dramatic reduction of hydroxylysyl pyridinoline cross-links indicating that the helical cross-linking sites are more markedly underhydroxylated by *PLOD1* deficiency than other sites of the triple helix (Eyre *et al.* 2002). Moreover, when the mRNA level of LH1 was decreased in fibroblasts by minoxidil treatment, the hydroxylation of helical lysine residues was reduced to 50% and the amount of hydroxylysyl pyridinoline cross-links was decreased non-proportionally (Zuurmond *et al.* 2005). Hence, the data indicates that LH1 has a preference for helical lysines as a substrate, and it preferentially hydroxylates lysines in the triple-helical region at the cross-link positions.

2.5.6 Lysyl hydroxylase 2

The LH2 is expressed as two alternatively spliced forms, LH2a and LH2b. LH2b contains one extra exon of 63 bp which introduces a 21 amino acid proline-rich sequence into the previously cloned LH2a. The alternatively spliced exon has been named 13A, and it is located between exons 13 and 14 in the *PLOD2* gene (Valtavaara 1999, Yeowell & Walker 1999b). The amino acid sequence coded by exon 13A has been conserved between human, mouse and rat (Mercer *et al.* 2003). Furthermore, the intron sequences surrounding exon 13A are also highly homologous between human and mouse, and they contain short identical sequences, which may contribute to the alternative splicing (Ruotsalainen *et al.* 2001).

Although both LH2 forms have lysyl hydroxylase activity *in vitro*, the K_m values for ascorbate and especially for the peptide substrate are different between LH2a and LH2b. These differences suggest that the alternative splicing modifies the active center of LH2 (Valtavaara 1999). Furthermore, an *in vitro* binding assay with collagenous peptides indicated that the alternative splicing of LH2 changes the peptide binding properties of LH2. The data suggest that the alternatively spliced exon participates in the peptide binding to the active site, or it changes the three-dimensional structure of the active site thus altering the binding properties (Risteli *et al.* 2004).

2.5.6.1 Tissue distributions of LH2a and LH2b

RT-PCR analysis of the expression of the human LH2a and LH2b indicates that the alternative splicing of LH2 may be tissue specific. LH2b was expressed in all the tissues studied and was the major isoform in most of the tissues. Particularly, LH2b was the only transcript in skin, lung, aorta and dura while LH2a predominated in kidney and spleen

(Yeowell & Walker 1999a). The amount of human LH2b mRNA is high in heart and skeletal muscle (Valtavaara 1999).

The distribution of rat LH2a and LH2b is also tissue specific. The rat LH2a was never expressed in the absence of LH2b; the expression of LH2a was higher in xiphoid cartilage and kidney whereas LH2b was the predominant form in heart and bone (Mercer *et al.* 2003). The fetal skin at different developmental stages showed expression of only LH2b suggesting that alternative splicing is not developmentally regulated (Yeowell & Walker 1999b).

The alternative splicing in cell culture conditions is regulated by cell density. In skin fibroblasts, where LH2b is normally the only transcript, and in kidney cells, where both forms are equally expressed, low cell density and cycloheximide treatment increased the expression level of LH2a (Walker *et al.* 2005).

2.5.6.2 LH2 hydroxylates lysines in the telopeptides

The pattern of lysyl hydroxylation in the non triple-helical telopeptide domains of collagen determines the tissue-specific cross-linking patterns and consequently the biomechanical properties of the tissues (Uzawa *et al.* 1999). The presence of a lysyl hydroxylase specific for the telopeptide lysines was first suggested by Barnes and co-workers (1974) who showed that the extent and age-related changes in hydroxylation of N-terminal telopeptide lysines were unrelated to the corresponding values of the whole collagen chain. Furthermore, highly purified LH hydroxylated lysyl residues only in the triple-helical domain of type I collagen, but not in the telopeptides (Royce & Barnes 1985). The existence of telopeptidyl LH was later supported by data obtained from chick hypertrophic tendon type I collagen, showing increased hydroxylation of telopeptide lysines while the hydroxylation of lysines in triple-helical domain was unaffected (Gerriets *et al.* 1993).

In vitro studies on differentiating osteoblasts suggested that LH2 has a role in the hydroxylation of lysines in the telopeptides of type I collagen (Uzawa *et al.* 1999). Up-regulation of LH2 expression coincided with a higher lysyl hydroxylation level in telopeptides and an increased amount of telopeptide hydroxylysyl aldehyde derived cross-links, whereas the expression levels of LH1 and LH3 did not change.

The function of LH2 as a telopeptide lysyl hydroxylase was further supported by studies of the Bruck syndrome showing incomplete hydroxylation of lysines in telopeptides of bone type I collagen. This deficient hydroxylation of telopeptide lysines results in aberrant collagen cross-linking of bone causing osteoporosis, joint contractures, fragile bones and short stature (Bank *et al.* 1999). Three missense mutations in exon 17 of *PLOD2* have been detected from three Bruck syndrome families indicating that LH2 is the putative telopeptide lysyl hydroxylase (van der Slot *et al.* 2003, Ha-Vinh *et al.* 2004). However, Bruck syndrome has also been linked to chromosome 17q12 in one family indicating a heterogeneous genetic basis for the syndrome (Bank *et al.* 1999).

2.5.6.3 LH2b, the telopeptide lysyl hydroxylase is involved in the formation of fibrosis

Fibrosis is a disorder characterized by an excessive accumulation of collagen (for a review, see Trojanowska *et al.* 1998). In addition, lysyl hydroxylase activity and lysyl hydroxylation are increased in induced fibrosis (Reiser & Last 1986, Last *et al.* 1990, Gerriets *et al.* 1996) and consequently hydroxylysine aldehyde derived cross-links are increased in fibrotic conditions (Reiser *et al.* 1986, Brinckmann *et al.* 1999, 2001, van der Slot *et al.* 2003).

Fibrotic skin revealed an elevated LH2b mRNA level while the mRNA levels of the other isoforms were normal. The data thus indicate that LH2b is responsible for the overhydroxylation of lysines in collagen telopeptides leading to increased formation of hydroxylysine aldehyde derived pyridinoline cross-links (van der Slot *et al.* 2004). Up-regulation of LH2b leading to an increase in the pyridinoline cross-links has been detected in many fibrotic disorders, and many profibrotic cytokines were shown to increase the LH2b mRNA level and consequently the amount of pyridinoline cross-links (van der Slot *et al.* 2004, van der Slot *et al.* 2005).

Similarly, up-regulation of LH2 mRNA leading to an increase of hydroxylysyl-aldehyde derived cross-links have been detected in skin in systemic scleroderma (SSc), an autoimmune disease characterized by excess deposition of collagen in skin and/or internal organs. Furthermore, the up-regulation of LH2 leading to altered cross-linking can be induced by exposing fibroblasts to interleukin 4 and prolonged hypoxia (Brinckmann *et al.* 2005).

In addition to increasing the amount of hydroxylysine aldehyde derived cross-links, overexpression of LH2b leads to elevated collagen synthesis. Therefore, LH2b has a bifunctional role in the pathogenesis of fibrosis and it may be a good target for an antifibrotic therapy (Wu *et al.* 2006). Overexpression of LH2b also leads to defective collagen fibrillogenesis and impaired matrix mineralization (Pornprasertsuk *et al.* 2005). The role of LH2b in the hydroxylation of telopeptidyl hydroxylysines and consequently in directing collagen cross-linking pathways was further confirmed by increased production of pyridinoline cross-links in CHO-K1 and MC3T3-E1 cells transfected with LH2b (Mercer *et al.* 2003, Pornprasertsuk *et al.* 2004). LH2a has no role in hydroxylating lysines in telopeptide regions (Mercer *et al.* 2003, van der Slot *et al.* 2004), whereas LH2b is probably not capable of hydroxylating lysyl residues in the helical part of collagens (Wu *et al.* 2006).

2.5.7 Lysyl hydroxylase 3

LH3 is exceptional among LH isoforms because of its multifunctionality. LH3 has, in addition to lysyl hydroxylase activity (Passoja *et al.* 1998b, Valtavaara *et al.* 1998), hydroxylysyl galactosyltransferase (GT) and galactosylhydroxylysyl glucosyltransferase (GGT) activities *in vitro*, whereas LH1, LH2a and LH2b have only lysyl hydroxylase activity (Heikkinen *et al.* 2000, Wang *et al.* 2002a). Moreover, the only lysyl hydroxylase ortholog of *C. elegans* (Norman & Moerman 2000) has all these three activities (Wang *et*

al. 2002a, Wang *et al.* 2002b) and the zebrafish LH3 (*diwanka*) is also multifunctional (Schneider & Granato 2006). When these activities were studied *in vitro* with recombinant protein, LH3 worked more effectively as glucosyltransferase than as galactosyltransferase or lysyl hydroxylase (Wang *et al.* 2002a).

The different enzyme activities have been linked to different parts of the LH3 molecule. The amino acids critical for LH activity reside in the C-terminal part of the molecule (Pirskanen *et al.* 1996, Heikkinen *et al.* 2000, Wang *et al.* 2002b), whereas the amino acids important for GT and GGT activity localize near the N- terminus (Heikkinen *et al.* 2000, Rautavuoma *et al.* 2002, Wang *et al.* 2002a).

In vitro mutagenesis studies indicate that Cys-144 and Leu-208 in human LH3 and their corresponding amino acids in *C. elegans* LH are important for GGT activity (Wang *et al.* 2002b). Cys-144 is also important for GT activity and it is required for the functional integrity of GT and GGT. Cys-144 is not disulfide-linked and probably does not participate directly in the enzyme catalysis (Wang *et al.* 2002a, Wang *et al.* 2002b). The LH3 sequence also contains a DXD-like motif (Wang *et al.* 2002b), which is characteristic of many glycosyltransferases and is thought to play a role in Mn2+ binding and catalysis (Ünligil & Rini 2000). Mutations of aspartates in this sequence of LH3 eliminated GGT and GT activities suggesting that this motif is important for the catalytic activity of both glycosyltransferases (Wang *et al.* 2002a, Wang *et al.* 2002b).

2.6 Collagen glycosyltransferases

The galactosylation of hydroxylysine and the glucosylation of galactosylhydroxylysine in collagens are catalyzed by hydroxylysyl galactosyltransferase (EC 2.4.1.50) and galactosylhydroxylysyl glucosyltransferase (EC 2.4.1.66), respectively. So far, LH3 is the only protein having collagen glycosyltransferase activities whose gene has been cloned (Heikkinen *et al.* 2000, Wang *et al.* 2002a). Previously collagen glycosyltransferases have been studied on the protein level (for a review, see Kivirikko & Myllylä 1979).

Both enzymes have been purified from chick embryo homogenate, galactosyltransferase at about 1000-fold purification and glucosyltransferase as a homogenous protein. When analyzed by gel filtration, the activity of the partially purified galactosyltransferase appears in two major fractions with molecular weights of 450,000 and 200,000 and one minor fraction with a molecular weight of 50,000 (Risteli *et al.* 1976). The molecular weight of chick glucosyltransferase is about 72,000-78,000 and it consists of only one polypeptide (Myllylä *et al.* 1977). Both enzymes are glycoproteins and they require the presence of free sulfhydryl groups, probably in the catalytic site, for the activity (for a review, see Kivirikko & Myllylä 1979).

Both collagen glycosyltransferases have been localized to the rough endoplasmic reticulum and their distribution within microsomes was similar to that of lysyl hydroxylase (Blumenkrantz *et al.* 1984). The collagen glucosyltransferase activity has also been detected in serum and in platelets (for a review, see Kivirikko & Myllylä 1979).

Collagen glycosyltransferase activities vary between different tissues and in different physiological and pathological states. High activity has been measured in the pregnant uterus and generally the activities are high during embryonic development (Spiro & Spiro

1971) and decrease with increasing age. Usually the glycosyltransferase activities correlate with collagen synthesis activity. However, the activities of collagen glycosyltransferases are not always regulated in parallel with with prolyl-4-hydroxylase and lysyl hydroxylase indicating specific regulation of glycosyltransferases (for a review, see Kivirikko & Myllylä 1979).

2.6.1 Collagen glycosyltransferase reactions

The free ε-amino group of the hydroxylysine residue is an absolute requirement for both galactosyl and glucosyltransferase reactions. Free hydroxylysine does not act as the sugar acceptor for the galactosyltransferase, whereas free galactosylhydroxylysine does act as a substrate for the glucosyltransferase. Interaction with both enzymes is further influenced by the amino acid sequence and the chain length of the peptide and the triple-helical conformation of native collagens prevents both glycosylations. The preferential carbohydrate donor of both glycosyltransferases is the corresponding UDP-glycoside and both enzymes require a bivalent cation, preferably Mn^{2+}. The reaction can be inhibited by several bivalent cations, the reaction product UDP and some other nucleotides (for reviews, see Kivirikko & Myllylä 1979, Kivirikko 1995).

The reaction mechanism of chick glucosyltransferase involves binding of Mn^{2+}, UDP-glucose and collagen in this order, and the binding site of UDP-glucose is separate from those of Mn^{2+} and the substrate. The products are probably released in the order glucosylated collagen, UPD and Mn^{2+}, and Mn^{2+} need not leave the enzyme during each catalytic cycle (Myllylä 1976).

2.6.2 LH3 as a collagen glycosyltransferase

The characteristics of recombinant human LH3 are similar to those of the purified chick enzymes. The molecular weight of recombinant human LH3 is about 80,000-85,000 (Passoja *et al.* 1998b, Heikkinen *et al.* 2000, Wang *et al.* 2002a). Since the chick LH3 has not been cloned, it is not known if the apparent size difference is due to the different species or to differences in the glycosylation of the recombinant protein when compared to protein isolated from tissues (Heikkinen *et al.* 2000). LH3 is also a glycoprotein (Passoja *et al.* 1998b, Valtavaara *et al.* 1998), and a cysteine residue is critical for its glycosyltransferase activities (Wang *et al.* 2002b). LH3 has been localized to the ER (Heikkinen *et al.* 2000, Salo *et al.* 2006), but it has also been found in serum (Salo *et al.* 2006). Unlike the other LH isoforms, LH3 seems to be regulated independently from total collagen synthesis (Wang *et al.* 2000).

Moreover, the GGT activity was shown to correlate with the amount of LH3 protein in mouse tissues and serum (Salo *et al.* 2006). The levels of galactosyltransferase and glucosyltransferase activities of LH3 correspond to the enzyme activities purified from chick embryos. The Km values of the chick enzymes for UDP-galactose and UDP-glucose, being 20-30 and 5-30, respectively, also correspond to 35 and 17 determined for LH3 (Wang *et al.* 2002a).

3 Aims of the present work

Lysyl hydroxylase catalyzes the formation of hydroxylysyl residues in collagens and other proteins with collagenous domains. Some of the hydroxylysyl residues are further glycosylated and the extent of hydroxylation and glycosylation shows wide variation between different tissues and collagen types. At the time this work was started, two novel lysyl hydroxylase isoforms, LH2 and LH3, had recently been cloned from human and mouse. The importance of the previously characterized lysyl hydroxylase, LH1 *in vivo* had already been demonstrated by a hereditary disorder, type VI Ehlers Danlos syndrome, but differences between the different lysyl hydroxylases and roles of the newly discovered LH2 and LH3 were not understood. This thesis study was carried out in order to differentiate the lysyl hydroxylase isoforms to better understand their functions. The specific aims of the present work were to:

1. Map the genes of different mouse lysyl hydroxylases to mouse chromosomes.
2. Characterize the expression of mouse lysyl hydroxylases during embryonic development and to compare the distribution of LH2 and LH3 in adult mouse tissues.

During this work LH3 was found to be a multifunctional enzyme having lysyl hydroxylase, galactosyltransferase and glucosyltransferase activities *in vitro*. Therefore an additional goal was set to:

3. Characterize the *in vivo* functions of the different enzymatic activities of LH3 by generating mouse lines with mutations of LH3 including null (knockout) mutations as well as domain-specific mutations.

4 Materials and methods

The materials and methods are described in more detail in the original articles (I-IV).

4.1 Chromosomal localization (I)

The mouse LH genes were localized into chromosomes by comparing the pattern of segregation of LH isoform specific polymorphisms with the patterns of segregation of several other molecular markers in pre-typed DNA panels. Three different panels were used: a panel of 10 samples prepared from a set of partially consomic strains, each carrying a different assortment of chromosomal segments of *Mus spretus* origin in a C57BL/6 background, a subset of samples from a EUCIB (Breen *et al.* 1994) panel for more accurate localization and the mouse/hamster radiation hybrids (RH) panel (T31) from Research Genetics for refining the localization.

For the localization of *Plod1*, DNA samples from the consomic set and the EUCIB subset were analyzed by Southern analysis (Ausubel *et al.* 1989) using *Hind*III digestion, separation on a 0.8% agarose gel, transfer to a nylon membrane and hybridization with radioactively labeled LH1 cDNA (Ruotsalainen *et al.* 1999) in ExpressHyb (Clontech) solution. *Plod2* was localized with PCR amplification of intron 9 of *Plod2* from the consomic set and EUCIB DNA samples followed by digestion with *Hae*III and separation on a 4% agarose gel or sequencing. The localization was refined with PCR amplification of the intron 9 from the T31 RH panel. *Plod3* was mapped by PCR amplification of intron 5 of *Plod3* only from the T31 RH panel.

The pattern of segregation of polymorphisms for the *Plod* genes were compared with the established pattern for the EUCIB resource with GENE-LINK software (Montagutelli 1990) and the data collected with the T31 RH panel was processed at: http://www-genome.wi.mit.edu.

4.2 Production of polyclonal antibodies against mouse LH2 and LH3 (II)

For the production of polyclonal antibodies against mouse LH2 and LH3 the 5' moiety of mouse LH2 corresponding to amino acids 28-227 or the mouse LH3 cDNA (Salo *et al.* 2006) were cloned into a pQE30-vector (Qiagen) with a $(His)_6$-tag at the N-terminus. The recombinant proteins were produced in *E.coli* XL-1 cells, purified first with an Ni-NTA agarose column (Qiagen) under denaturing conditions and further with 15 % SDS-PAGE. The proteins were eluted from the gel, dialyzed (Prussak *et al.* 1989), and used as antigen to produce antibodies in rabbits by Davids Biotechnologie (Regensburg, Germany). The antibodies were purified by Protein A-Sepharose or by antigen coupled to CNBr activated Sepharose 4B.

4.3 Whole mount *in situ* hybridization (II)

The expression of LH1, LH2 and LH3 in mouse embryos was studied by whole mount *in situ* hybridization. The sense and anti-sense probes were prepared by cloning cDNA fragments of mouse LH1, LH2 and LH3 into pBluescript and digoxigenin probes were synthesized with a Digoxigenin RNA labeling kit (Boehringer Mannheim) using T7 and T3 RNA polymerases. The whole mount *in situ* hybridization was performed based on standard procedures (Wilkinson 1992), and the embryos were stained with alkaline phosphatase using Anti-Digoxigenin-alkaline phosphatase-Fab fragments and BM-Purple substrate (Boehringer Mannheim).

4.4 RNA isolation (II, III), RT-PCR (II) and Northern blot analysis (II, III)

To analyze the LH expression levels in normal mouse embryos and tissues (II) or genetically modified mouse embryos (III), total RNA was extracted with the Trizol Reagent (Invitrogen) and mRNA was further purified with Dynabeads oligo d(T) (Dynal). Alternatively, mRNA was directly extracted with an Oligotex Direct mRNA mini kit (Qiagen) (III).

For studying the expression of LH2a and LH2b by RT-PCR (II), the Oligo-d(T) primed cDNA was prepared (Hautala *et al.* 1993), exon 13A was amplified with oligonucleotides from exon 9 and exon 14 of the mouse gene for LH2 (Ruotsalainen *et al.* 2001), and the products were analyzed on a 1% agarose gel.

To analyze the LH expression levels in normal mouse embryos (II), mouse embryo multiple tissue Northern blots (Clontech) were used. For Northern blot analysis of the genetically modified embryos (III), the mRNA was separated by electroforesis in a 0.8% agarose gel containing 0.22 M formaldehyde and transferred to a nylon membrane (Ausubel *et al.* 1989). The blots were hybridized with radioactively labeled cDNA

fragments of mouse LH1, LH2 and LH3 (Ruotsalainen *et al.* 1999) (II, III), and with a cDNA clone of zinc finger HIT-1 (BC026751) (III). Actin cDNA was used as a control probe to standardize the quantities of mRNA. Quantification of mRNA levels (III) was performed with ImageQuant 5.2 software (Molecular Dynamics).

4.5 Protein isolation and Immunoblotting (II, III, IV)

To analyze the LH2 and LH3 protein in normal mouse tissues (II) and in genetically modified mice and embryos (III), tissues or pooled embryos were homogenized and the soluble proteins were bound to Concanavalin A-Sepharose. To analyze the expression of collagens in LH3 manipulated cell lines (IV), the cells were homogenized to obtain soluble proteins. The proteins were then separated under reducing conditions by 6 % or 7.5 % SDS-PAGE, transferred to a PVDF-membrane, which was incubated with Protein A-Sepharose purified anti-mouse LH2 and LH3 antibodies (II, III), rabbit anti-mouse collagen type I and VI (Rockland) and a rat monoclonal anti-mouse collagen type IV α2 NC1 domain (Sado *et al.* 1995) (IV). 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 (III) was performed with ImageQuant 5.2 software (Molecular Dynamics).

4.6 Immunohistochemistry (II, III) and Immuno-Electron microscopy (II, IV)

For studying the LH2 and LH3 expression in normal mice (II), the tissues were fixed in 4% paraformaldehyde in PBS and embedded in paraffin. Thick sections were stained with affinity purified anti-LH2 and LH3 antibodies and with biotinylated goat anti-rabbit IgG (DAKO) and a Vectastain ABC kit (Vector laboratories), and the antibody complex was detected with DAB. For immunohistological staining of the embryos and tissues of LH3 manipulated mice (III), the samples 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), and rabbit anti-mouse laminin (Sigma). The primary antibody was detected with a fluorescent-labeled secondary antibody, AlexaFluor 594 conjugated goat anti-rabbit IgG (Molecular Probes).

Immunostaining of LH2 and LH3 in normal mice (II) and of collagens in genetically modified mice (IV) for electron microscopy (EM) was performed by fixing the samples in 4% paraformaldehyde in a 0.1 M phosphate buffer with 2.5% sucrose followed by immersion in 2.3 M sucrose and freezing in liquid nitrogen. For immunolabeling, thin cryosections were incubated with primary antibody, affinity purified anti-mouse LH2 and LH3 antibodies (II), or rabbit anti-mouse collagen type I (Rockland), type IV (Chemicon), type VI (Rockland) (IV), rabbit anti-mouse decorin (Fisher *et al.* 1995) and

then with a protein A-gold complex. Sections were examined in a Philips CM100 transmission electron microscope with a CCD-camera.

4.7 Generation of genetically modified LH3 mice (III)

The targeting vectors were constructed by cloning genomic fragments of the *Plod3* gene digested from the BAC clone together with a selection marker into modified pBluescript. The constructs were linearized with *Not*I, electroporated into R1 ES cells and the G418-resistant clones were screened for homologous recombination by Southern blot hybridization with an external probe after *EcoR*V or *Hind*III digestion (Fässler & Meyer 1995). ES cells carrying the disrupted allele were microinjected into C57BL/B6 blastocysts, which were transferred into pseudopregnant females to generate chimeras, which were subsequently mated with C57BL/B6 mice.

Two different constructs were generated to create a LH3 knockout mouse line, a hypomorphic mouse line with a reduced mRNA level of LH3 with mutated LH activity and an LH mutant mouse line with mutated LH activity (Fig. 1A, III). The targeting vector for the LH3 knockout 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 to interrupt the *Plod3* gene. The mice were genotyped by PCR with primers from exon 6, exon 7, and from the neomycin (*neo*) gene.

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 (Heikkinen *et al.* 2000). The targeting construct 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. The point mutation was confirmed by amplifying and sequencing exon 18 of the *Plod3* gene. To establish the mouse strain lacking the *neo* cassette (LH mutant), $LH^{+/-}$, $neo^{+/-}$ mice were intercrossed with a transgenic Cre-deletor mouse line (Sakai & Miyazaki 1997). The offspring were genotyped by PCR with primers from intron 18 and the removal of the *cre* locus was verified by PCR with primers from the *cre* gene (Sakai & Miyazaki 1997).

4.8 Histology and transmission electron microscopy (III, IV)

The embryos and tissues of genetically modified mice were fixed in 4% paraformaldehyde in PBS or in 10% neutral formalin overnight and embedded in paraffin. The 5 μm thick sections were stained with hematoxylin and eosin (HE) and observed under a light microscope.

For ultrastructural analysis, the tissues or embryos were fixed in 1% glutaraldehyde and 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 by observer, who did not know the genotype of the samples. The thickness of the epidermal BM and lamina densa of LH mutated mice (III)

were measured from the images captured with a CCD-camera equipped with EM-Menu version 3 assisted by Tcl scripts from Tietz Video and Image Processing Systems GmbH. Statistical analysis was performed using the Student's t-test.

4.9 Enzyme activity measurements (III)

The effect of the D669A mutation was verified by measuring the LH activity in LH mutant and wild type mouse embryos. The embryos were homogenized and LH3 was separated from other LHs with UDP-hexanolamine beads (Sigma). The partially purified LH3 was used in the lysyl hydroxylase activity assay based on decarboxylation of 2-oxo[1-14C]glutarate (Kivirikko & Myllylä 1982), using the synthetic peptide IKGIKGIKG as a substrate.

The galactosyltransferase and glucosyltransferase activities were measured in the genetically modified mice. The embryos or adult mouse tissues were homogenized and the supernatant was used for the enzyme assay based on the transfer of [H3]-labeled sugar from UDP-galactose or UDP-glucose to hydroxylysyl or galactosyl hydroxylysyl residues in a calfskin gelatine substrate (Myllylä *et al.* 1975, Kivirikko & Myllylä 1982).

4.10 Analysis of hydroxylysyl residues and hydroxylysyl cross-links (III)

Collagenous proteins were extracted from LH mutant and control 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 & Rhodes 1982), which were hydrolyzed by acid hydrolysis. The free phenylthiocarbamyl amino acid derivates were separated on a reversed phase column and analyzed with an amino acid analyzer.

The cross-link measurements were carried out in skin and bone of the LH mutant and wild type mice. The samples were reduced with sodium borohydride. After acid hydrolysis the collagen content was measured (hydroxyproline assay), and hydroxylysyl aldehyde derived cross-links (hydroxylysyl pyridinoline and lysyl pyridinoline) were determined by reverse phase chromatography (Mercer *et al.* 2003).

4.11 Mouse embryonic fibroblast and newborn skin fibroblast cell culture (IV)

Fibroblast cell lines were established from E8.5 LH3 knockout embryos and LH mutant newborn skin and from corresponding controls for more detailed analysis of collagen secretion. Embryos or skin samples were dissected, sliced, washed with PBS and placed in Dulbecco's MEM (Gibco) supplemented with 20% fetal calf serum (Promocell),

penicillin-streptomycin (Sigma) and 50 µg/ml ascorbic acid. The cells were grown to confluence and trypsinized and the culture was continued in the medium containing 10% fetal calf serum.

In order to study the secretion of collagen in these cells, protein synthesis was inhibited by cycloheximide. The cells were treated with 10 µg/ml cycloheximide for 0, 1, 1.5, 2, 2.5, 3, 3.5, 4 or 6 hours. Cells and medium were collected after each time point and used for immunoblot analysis and immunofluorescence stainings.

4.12 Immunofluorescence stainings (IV)

The localization of collagens and structure of cell organelles in genetically modified mouse fibroblasts was studied with immunofluoresence stainings. For collagen stainings the cells were fixed with 95 % ethanol/5% acetic acid and for other stainings with 4% paraformaldehyde. The cells were blocked with 1% BSA, 0.05% saponin in PBS, stained with rabbit anti-mouse collagen type I (Rockland), type IV (Chemicon), type VI (Rockland), rabbit anti-calnexin (Stressgen) and mouse anti-GM130 (Transduction Laboratories) and with Alexa Fluor 488 -conjugated anti-rabbit or anti-mouse IgG (Molecular Probes).

5 Results

5.1 Mapping of the mouse lysyl hydroxylase genes to chromosomes (I)

The chromosomal localization of mouse lysyl hydroxylases was determined by matching the pattern of segregation of LH isoform specific polymorphisms to the patterns of segregation of several other molecular markers in pre-typed DNA panels.

The lysyl hydroxylase 1 gene (*Plod1*) was localized into chromosome 4 within a segment flanked by the microsatellite markers at 69.5 cM and 77 cM. This localization combined with the previously published localization in chromosome 4 between 76.3 cM and 77.6 cM (McClive & Morahan 1994) maps *Plod1* to chromosome 4 at 77 cM (Table 1, I). In addition, based on this localization and the conservation of synteny between human, mouse and rat, the rat gene for LH1 was predicted to map to chromosome 5.

The mouse lysyl hydroxylase 2 gene (*Plod2*) was localized to chromosome 9 at 51 cM, very close to the breakpoint of synteny conservation with human chromosomes 3 and 15 and the lysyl hydroxylase 3 gene (*Plod3*) was mapped to chromosome 5 at 80 cM, very close to the breakpoint of synteny conservation with human chromosomes 7 and 13 (Table 1, I). Both of these localizations are thus in chromosomal segments which are very close to the breaks in the conservation of linear gene order between human and mouse chromosomes.

5.2 Characterization of the expression of mouse LH isoforms (II)

5.2.1 Widespread expression of LH1, LH2 and LH3 during embryonic development

The expression of mouse LH1, LH2 and LH3 was studied during mouse embryonic development. Northern blot analysis (Fig.1, II) indicated that the expression of all lysyl hydroxylases starts at E7 and continues throughout embryonic development, but the

amounts of the mRNAs of the different isoforms showed variation. LH1 was highly expressed at all stages, while the expression of LH2 and LH3 mRNAs varied. Both LH2 and LH3 were highly expressed at E7, but the LH2 mRNA was decreased at later stages while LH3 was downregulated at E11 and upregulated at E15 and E17. The RT-PCR analysis also revealed variation in the alternative splicing of LH2 during embryonic development (Fig.3.C, II). The results indicate that LH2a is the major form during early development (at E7.5 and E9.5), while LH2b predominates at later stages (at E13.5), and at E11.5 both forms are equally expressed.

A more detailed analysis of LH1, LH2 and LH3 expression by whole mount *in situ* hybridization revealed similar expression patterns for all LH isoforms during early development and organogenesis (Fig.2, II). The embryonic mesoderm of E7.5 embryos showed expression of all LH isoforms. However, LH3 was expressed more broadly during gastrulation and it was also expressed in extraembryonic tissues, especially in the differentiating trophoblast, where no significant expression of LH1 or LH2 was detected. In E9.5 and E11.5 embryos, all LHs show similar expression; at E9.5 and E11.5 in tail bud, somites, dorsal aorta, branchial arches, hindbrain and forebrain, and at E11.5, expression was also detected in limb buds.

5.2.2 Different distributions of LH2 and LH3 in adult mouse tissues

The distribution of mouse LH2 in different mouse tissues was studied using a polyclonal LH2 antibody. The antibody recognized an LH2 protein of 89 kDa in heart, spleen, lung and muscle, whereas a protein of 86 kDa was observed in kidney and testis (Fig.3.A, II). Since the LH2 antibody showed no cross-reactivity with LH1 and LH3, the bands of different sizes represented the splice variants of the gene for LH2, LH2a and LH2b. RT-PCR analysis confirmed that heart, spleen, lung and muscle contained mostly LH2b whereas kidney and testis expressed only LH2a (Fig.3.B, II). Moreover, LH2a is the prominent form in brain, which showed expression of both forms.

Since immunoblot analysis revealed some differences between the expression of LH2 and the previously characterized LH3 (Salo *et al.* 2006), the tissue distributions of these isoforms was studied in more detail by immunohistochemistry (Fig.4, II). In some tissues LH2 and LH3 were produced in a cell-specific manner whereas in other tissues they were distributed in same cells, but predominated in different cell types. As LH3 has been observed both intracellularly and extracellularly in some tissues (Salo *et al.* 2006), the more detailed tissue distribution of LH2 and LH3 was analyzed by immuno-EM. The results revealed more differences between the expression patterns of LH2 and LH3; LH2 was observed only intracellularly in the ER (Fig.5, II) whereas LH3 showed intracellular or extracellular localization or both. Interestingly, the distributions of both LH2 and LH3 were different in embryonic (E13.5 and E15.5.) and adult liver. LH2 showed faint expression in embryonic stages while no expression was detected in the adult. Furthermore, the intracellular distribution of LH3 detected in embryonic livers changed to extracellular in adult (Fig.6, II). The tissue distributions of LH2 and LH3 are summarized in Table 4.

Table 4. Distributions of LH2 and LH3 in different mouse tissues

Tissue	LH2	LH3
Lung	Smooth muscle bundles of bronchioles	Type II pneumocytes
Spleen	Reticular cells in white and red bulb border, in ER	Reticular cells in red bulb area, in ER, also in extracellular space
Brain	Purkinje cells, fainter in pyramidal cells	Pyramidal cells, fainter in Purkinje cells
Muscle	In the Z-band area, near t-tubes, in ER	In the Z-band area, near t-tubes, in ER, also in extracellular space
Pancreas	Exocrine cells	In extracellular space between exocrine cells
Kidney	Epithelial cells of the areas of distal and collecting tubules, in ER	Distal part of renal tubules, in ER and in extracellular space[1]
Liver	No expression in adult, faint in embryos	Sinusoids in adult, only in extracellular space[1], hepatocytes in embryos

[1](Salo *et al.* 2006)

5.3 Generation and analysis of LH3 manipulated mice (III, IV)

To characterize the different enzymatic activities of LH3 *in vivo*, three different genetically manipulated mouse lines were generated by two targeting constructs (Fig.1, III). In the LH3 knockout the *Plod3* gene was inactivated by inserting an *IRES-β-gal-polyA-neo* cassette into exon 2. In the hypomorphic mouse line, LH activity was specifically disrupted by an D669A mutation, which leaves the GT and GGT activity intact (Heikkinen *et al.* 2000), and the *neo* cassette was inserted into intron 18. ES clones with targeted mutations were obtained with both constructs and they were used to produce chimeras with germline transmission. Heterozygous mice of both mouse lines were viable and fertile but produced no homozygous offspring indicating an embryonic lethal phenotype. The third mouse line, the LH mutant was generated by removing the *neo* cassette from the hypomorphic line. Homozygous LH mutant mice containing the D669A mutation were born with a normal mendelian ratio and they were viable and fertile (Table 1, III). The different manipulations of the *Plod3* gene and their consequences in different mouse lines are summarized in Table 5.

Table 5. Characteristics of the different LH3 manipulated mouse lines

Mouse line	Manipulation of *Plod3*	LH3 mRNA/protein	Enzyme activities % of control		Homozygous phenotype
			GGT	LH	
Knockout	Inactivation by insertion of gene cassette	Not detectable	2-4	Not analyzed	Lethal at E9.5
Hypomorph	D669A mutation *neo* cassette	Reduced/10% wild type	11-20	Not analyzed	Lethal at E9.5-14.5
LH mutant	D669A mutation	normal	55-100	< 9	Viable

5.3.1 Lack of GGT activity of LH3 leads to embryonic lethality (III)

Characterization of the phenotype of the homozygous LH3 knockout embryos revealed fragility, growth retardation, dilated blood vessels mainly in the region of the sinus venosus, and death at E9.5 (Fig.2, III). Interestingly, the hypomorphic embryos showed variation in the phenotype and in the time of death. Some of the homozygous hypomorphic embryos showed abnormalities similar to the LH3 knockout leading to death before E10.5, while some embryos characterized by slightly retarded growth and intracranial hemorrhage at E12.5-13.5 died before E14.5 (Fig.2, III). The LH mutant mice showed no anatomical or obvious behavioral abnormalities when followed for up to 2 years.

Since LH3 is a multifunctional protein having LH, GT and GGT activities, the three LH3 manipulated mouse lines were further characterized by analyzing the LH3 mRNA and protein levels (Fig.1, III) and measuring the GGT activity (Table1, III). In LH3 knockout embryos, LH3 mRNA was undetectable in homozygotes and reduced in heterozygotes. Similarly the GGT activity was nearly absent (2-4%) in the homozygotes and decreased (50% in embryos, 60-70% in adult tissues) in the heterozygotes.

The correlation between the amount of LH3 protein and GGT activity was further confirmed by analysis of the hypomorphic mouse line. Hypomorphic homozygous embryos had dramatically reduced amounts of LH3 mRNA and protein (10% of the wild type). This reduction of the LH3 protein is due to the inserted *neo* cassette; a similar effect has been described in different genes (Nagy 2000). Consequently, the GGT activity of hypomorphic embryos was also decreased, being 11-20% of the controls. In addition, older embryos had higher GGT activity indicating that the survival of the embryos correlated with increasing GGT activity.

The LH mutant showed a normal amount of LH3 mRNA and protein. Similarly, the GGT activity was normal or slightly reduced (80% of the control) in LH mutant embryos and most of the adult tissues. The GGT activity was clearly decreased (55% of the control) only in lung. The GT activity in both LH3 knockout and hypomorphic embryos was also slightly reduced. The GT activity of E9.5 LH3 knockout and E10.5 hypomorphic embryos are presented in Table 6.

Table 6. Galactosyltransferase activities of LH3 knockout and hypomorphic embryos.

Mouse line	Phenotype	Specific GT activity (dpm/μg)	% of wild type
LH3 knockout[1]	+/+	21.2	
	+/-	20.2	95
	-/-	15.5	73
Hypomorph[2]	+/+	43.9	
	+/-	39.5	90
	-/-	26.4	60

[1]average of two measurements, [2]average of three measurements

To reveal possible compensation of LH3 by LH1 and LH2, the amounts of LH1 and LH2 mRNA were analyzed (Fig.1, III). The LH3 knockout showed a slight increase in the LH1 (1.6-fold) and LH2 (1.95-fold) mRNA when compared to the wild-type, whereas in the

hypomorphic and LH mutant mice the mRNA levels of LH1 and LH2 were comparable with the wild type. In addition, the manipulations of *Plod3* did not affect the expression of the putative zinc-finger gene (*Znhit1*) located head to head with *Plod3*.

5.3.2 Disrupted basement membranes in non-viable embryos (III)

The embryos of LH3 manipulated mouse lines were analyzed in more detail by immunohistochemistry (Fig.3, III) and electron microscopy. The homozygous embryos of both lethal mouse lines showed abnormal type IV collagen staining in the embryo and in Reichert's membrane. In the LH3 knockout, type IV collagen was located mostly inside the cells, not in the BM zone. The hypomorphic embryos showed type IV collagen staining inside the cells, but fragmented staining was also detected in the BM zone. Furthermore, in the LH mutant embryos, type IV collagen was normal and localized to the BM. The staining for laminin was normal in all mouse lines.

The effect of defective GGT activity on the altered BM structure was confirmed by ultrastructural analysis. Transmission EM analysis of the neural tube (Fig.3C, III) revealed that the LH3 knockout embryos had no BM, and the hypomorphic embryos had discontinuous or fragmented BMs, while the LH mutant had normal BM structure. Similarly, the LH3 knockout embryos lacked the endothelial basement membranes and the hypomorphic embryos showed some amorphous material in the BM zone while the LH mutant and wild type embryos had stretches of BM underlining the endothelial cells (Fig.3D, III). Due to the lack of BM, ruptures of the endothelial cell layer were also detected in knockout and hypomorphic embryos (Fig.3D, III). In addition, the knockout and hypomorphic embryos had dilated ER (Fig.3D, III) showing accumulation of proteins. In hypomorphic embryos decreasing GGT activity correlated with increasing protein accumulation and more abnormal BM structure. The LH3 knockout also showed increased apoptosis and shortening of Golgi stacks (Fig.1, IV).

5.3.3 Abnormal secretion of type IV collagen in the LH3 knockout (IV)

The distribution of type IV collagen in embryos was further studied by immuno-EM (Fig.1, IV). In the wild type embryos, type IV collagen was localized in the continuous BM while the LH3 knockout embryos had no BM or type IV collagen aggregates in the extracellular space. In the LH3 knockout embryos, type IV collagen was mostly retained inside the cells, in the dilated ER, or as individual particles scattered in the cytoplasm or in aggregated material in intracellular vesicles near the plasma membrane.

The collagens produced by the LH3 knockout were analyzed in more detail in cultured fibroblasts isolated from LH3 knockout embryos. Immunoblot analysis of type I and IV collagens produced by these cells revealed a decrease in the amount of both collagens in the LH3 knockout when compared with control cells (Fig. 3A and B, IV). The collagens produced by the LH3 knockout also migrated faster than the controls. The size difference between collagens produced by LH3 knockout and by control cells was determined and it corresponds to the calculated loss of 17 and 10 hydroxylysyl-linked disaccharides in type

I collagen chains and loss of 40 hydroxylysyl-linked disaccharides in type IV collagen. The immunoblot data thus demonstrate that the smaller sizes of type I and IV collagens are due to defective glycosylation of hydroxylysines in the LH3 knockout cells.

The inhibition of protein synthesis in the fibroblasts by cycloheximide resulted in delayed secretion of type IV collagen in the LH3 knockout cells (Fig.2, IV). Immunofluorescence studies indicated that the type IV and VI collagens were mostly retained inside the cells in the LH3 knockout, whereas type I collagen was detected both intracellularly and in the extracellular space, but with different staining intensity.

5.3.4 Altered collagens in the LH mutant (III, IV)

In the LH mutant mice, *Plod3* contained the D669A mutation, which abolishes the LH activity *in vitro*, but leaves the glycosyltransferase activities intact (Heikkinen *et al.* 2000). The effect of the mutation *in vivo* was confirmed by measurement of the LH activity of LH3 separated from the other LH isoforms. The LH activity of the partially affinity purified LH3, measured with the 2-oxoglutarate decarboxylase assay was less than 9% of controls, which probably represents the residual activities of the other 2-oxoglutarate dioxygenases.

The effect of abolishment of LH activity was further studied by determining the number of hydroxylysine residues in collagen fractions extracted from kidneys and lungs of LH mutant mice. The fraction containing type IV and V collagens had a 30% reduction of the hydroxylysine residues when compared with the wild type, whereas no change in hydroxylysine amount was observed in the fraction containing type I, II and III collagens (Table 2A, III). Moreover, cross-link analysis of the skin and bone of the LH mutant mice revealed a reduction in the hydroxylysine aldehyde derived cross-links (hydroxylysyl pyridinoline and lysyl pyridinoline) in the skin of homozygotes, but the amount was normal in bone (Table 2B, III). The amount of 4-hydroxyproline was normal in these tissues indicating no changes in the amount of collagens.

Collagens produced by the LH mutant were studied in more detail in cultured fibroblasts isolated from the skin of LH mutant newborn mice. Immunoblot analysis revealed that type IV and VI collagens and maybe also type I collagen produced by LH mutant fibroblasts migrated slightly faster than the collagens produced by control cells (Fig.3C, IV). The size difference cannot be due to lack of only hydroxylysines suggesting that disruption of LH activity leads to lack of some glycosylated hydroxylysines in LH mutant cells. However, the size difference between the collagens produced by the LH mutant and control cells was smaller than between the LH3 knockout and control fibroblasts (Fig.3C, IV) suggesting that the LH mutant lacks only some of the hydroxylysine-linked carbohydrates.

5.3.5 Ultrastructural changes in the LH mutant mice (III, IV)

The histological analysis of the tissues of the LH mutant mice revealed no obvious abnormalities in kidney, heart, liver, spleen, testis, skin and lung structure. However, mild

alterations in the structure of muscle were observed; some of the muscle fibers were less organized in the LH mutants than in wild types. In addition, the immunohistochemical staining of type I, III, IV and VI collagens and laminin were normal in lung and skin of LH mutant mice.

However, the ultrastructure of the skin and lung of the newborn LH mutant homozygotes showed some alterations (Fig.4, III), which were more evident in the newborn than in the adult mice. The lamina densa of the epidermal BM was significantly ($p<0.0001$) thinner in the homozygotes (23.1 ± 6.4) than in the controls (31.2 ± 7.1). Furthermore, the collagen fibrils in skin and lung of the homozygotes were covered by diffuse material not detected in the control, and they were more disorganized and loosely packed in collagen bundles than in the wild type.

Further analysis of the LH mutant mice revealed alterations in the skeletal muscle that were not detected in the wild type muscles (Fig.5, IV). In particular, the sarcoplasmic reticulum showed dilatations and vacuolization and the mitochondria were swollen in the LH mutant. Moreover, IEM analysis revealed that in LH mutants, the BM was not tightly bound to the sarcolemma of the muscle fibers as in the control. Instead, it was more distant from the muscle fibers and detached in several places.

5.3.6 Abnormal distribution of type IV and VI collagens in the LH mutant mice (IV)

The distribution of collagens and other molecules affecting the organization of collagen fibrils was studied in LH mutant with immuno-EM (Fig.4, IV). The distribution of type I collagen and decorin were similar in the LH mutant and control skin, but the distribution of type VI collagen was abnormal in the LH mutants. Type VI collagen was detected on the surface of the collagen fibrils both in the LH mutant and control skin, but in the LH mutants it was less evenly distributed. Strikingly, the LH mutant skin showed abnormal aggregation of the type VI collagen in the extracelullar space and occasionally some individual gold particles or aggregates of type VI collagen were seen inside the cells. No aggregates or intracellular accumulation of type VI collagen was seen in the controls.

Since the LH mutant mice showed thinning of the epidermal lamina densa, the distribution of type IV collagen in skin was also studied. Type IV collagen was detected in the epidermal BM both in the LH mutant and wild type newborn mice. The distribution of type IV collagen was slightly more uneven in the LH mutant than in the wild type, but no prominent aggregation of type IV collagen was detected in the mutant or in the wild type skin.

The distribution of collagens was also studied in the muscle of the adult LH mutant mice. In agreement with the skin results, the distribution of type I collagen was similar in the wild type and in the LH mutant muscle, whereas the distributions of type IV and VI collagens were altered in the LH mutant (Fig.5, IV). Both type IV and VI collagens showed uneven distribution in the LH mutant when compared with the wild type. In addition, in the LH mutant muscle the type VI collagen formed extracellular aggregates similar to those detected in the skin.

6 Discussion

6.1 The genes for mouse LH isoforms map to different chromosomes

Three lysyl hydroxylase isoforms, LH1, LH2 and LH3, encoded by three different genes have been characterized from mouse (Ruotsalainen *et al.* 1999, Ruotsalainen *et al.* 2001). In this thesis these genes, *Plod1*, *Plod2* and *Plod3,* were assigned to mouse chromosomes.

Plod1 was mapped to mouse chromosome 4 at 77.0 cM thus confirming the previously suggested localization (McClive & Morahan 1994). In addition, the localization of *Plod1* together with conservation of synteny between human, mouse and rat chromosomes was used to predict the localization of the rat gene for LH1 to chromosome 5, which has been later mapped to 5q36 (NCBI). Moreover, *Plod2* was mapped to chromosome 9 at 51.0 cM and *Plod3* was mapped to chromosome 5 at 80.0 cM. These localizations are in good agreement with the synteny of conservation between the human and mouse chromosomes. The mapping of *Plod1*, *Plod2* and *Plod3* have been later confirmed by the mouse genome project to chromosomes 4 at 76.5 cM, 9 at 52.0 cM and 5 at 80.0 cM, respectively (NCBI).

The mouse LH genes thus reside each on a different chromosome as has been shown with the human and rat genes (Hautala *et al.* 1992a, Valtavaara *et al.* 1997, Valtavaara *et al.* 2000, Mercer *et al.* 2003). The structures of the LH genes are well conserved between different isoforms and between human and mouse. Phylogenetic analysis suggests that the LH genes have evolved as a result of two gene duplications from an ancestral gene. LH3 is the oldest isoform and evolution of the LH1 and LH2 genes is associated with lengthening of the introns (Ruotsalainen *et al.* 1999, 2001).

6.2 Lysyl hydroxylases are highly expressed during embryogenesis

The tissue distributions of different LH isoforms have been previously studied in human and mouse in order to understand their functions. There are slight differences between the expression patterns of the LH isoforms between different species, but in general, LH1 is

expressed in several tissues while the expression of LH2 and LH3 is more restricted (Heikkinen *et al.* 1994, Yeowell *et al.* 1994, Valtavaara *et al.* 1997, Passoja *et al.* 1998b, Valtavaara *et al.* 1998, Ruotsalainen *et al.* 1999).

The characterization of the expression of the LH isoforms during mouse embryonic development presented in this study suggests important developmental roles for lysyl hydroxylases. All LH isoforms probably have significant roles during development because they show widespread developmental expression, which starts earlier than the expression of most collagen types, including the quantitatively major fibrillar collagens (see Table 1. in this thesis). The importance of lysyl hydroxylases for early development is supported by the recently characterized zebrafish LH1 and LH2 which are also expressed in mesoderm already during gastrulation (Schneider & Granato 2007), thus resembling the mouse lysyl hydroxylases.

Despite the similar widespread distribution, the amount of each LH isoform seems to be independently regulated suggesting an isoform specific developmental regulation for the mouse lysyl hydroxylases, similar to that indicated for the zebrafish LH1 and LH2 (Schneider & Granato 2007). The widespread distribution of lysyl hydroxylases in developing embryos also correlates with the expression of several collagen types in the same tissues (see Table 1. in this thesis). In particular, the strong LH1 expression is in agreement with its role in hydroxylating the lysyl residues in the triple-helical domain of the fibrillar collagens (Steinmann *et al.* 1995, Eyre *et al.* 2002) and expression patterns of the multifunctional LH3 (Heikkinen *et al.* 2000) resemble those of the highly glycosylated collagens IV and VI.

6.3 Alternative splicing of LH2 shows developmental and tissue-specific regulation

LH2 seems to be the most regulated isoform during development and in adult tissues. More regulation can be seen in the alternative splicing of LH2 which results in two different forms; LH2a and LH2b (Valtavaara 1999, Yeowell & Walker 1999b). In whole embryos, alternative splicing is developmentally regulated, although in an earlier study only LH2b was expressed in cultured cells derived from embryonic skin (Yeowell & Walker 1999b). The zebrafish LH2a and LH2b also show different expression levels during development (Schneider & Granato 2007), thus supporting the developmental regulation of the alternative splicing.

The alternative splicing is also regulated in adult tissues. The distribution of mouse LH2a and LH2b are similar to those previously published for human and rat showing LH2b as the prominent form in most of the tissues whereas LH2a is the major form in kidney (Yeowell & Walker 1999b, Mercer et al. 2003). The developmental regulation of LH2b and its distribution in adult tissues are in good agreement with its recently characterized role as a telopeptide lysyl hydroxylase, which is responsible for the formation of the more stable pyridinoline cross-links (Mercer *et al.* 2003, van der Slot *et al.* 2004).

LH2b probably cannot hydroxylate lysines in the triple-helical domain (Wu *et al.* 2006), while LH2a does not hydroxylate telopeptide lysines in cultured cells, and its role

is not known (Mercer *et al.* 2003, van der Slot *et al.* 2004). However, due to its high expression during early development and conserved specific expression in kidney, LH2a probably has an important role in embryonic development and in some tissues. Since LH2b directs collagen cross-linking pathways by hydroxylating telopeptidyl hydroxylysines (Mercer *et al.* 2003, Pornprasertsuk *et al.* 2004), the regulation of the alternative splicing could be used to regulate the biomechanical properties of the tissues.

6.4 The expression of mouse LH2 and LH3 differ in adult tissues

The widespread expression of both LH2 and LH3 during embryonic development becomes more restricted in adult tissues. It is therefore probable that the LH isoforms have different functions and substrates during extensive tissue formation in the developing embryo and in adult tissues. In addition, the mRNA expression of some collagens, like types II and III is more restricted in adult tissues than in embryos (Cheah *et al.* 1991, Niederreither *et al.* 1995).

In some adult mouse tissues LH2 and LH3 are produced in a cell-specific manner while in other tissues they co-localize. Previously cell-specific expression of lysyl hydroxylases has been suggested by Mercer and co-workers (2003) reporting differential expression of rat LH2 and LH3 in different bone marrow cell types. The distributions of LH2 and LH3 also diverge at the cellular level. Supporting the earlier finding (Salo *et al.* 2006), LH3 is localized in the ER, in the extracellular space, or in both, while LH2 is located in the ER. LH2 and LH3 are localized to the ER despite a lack of KDEL signals. The mechanism of ER retention for LH2 and LH3 is not known, but due to their high homology to LH1 (Ruotsalainen *et al.* 1999), they probably associate with the ER membrane using the same mechanism as described for LH1 (Suokas *et al.* 2000, Suokas *et al.* 2003). The secretion mechanism of LH3 is not known. The extracellular LH3 has a higher molecular weight than the intracellular enzyme suggesting that the secretion is not regulated by a cleavage of a retention motif (Salo *et al.* 2006).

The specific patterns of distribution suggest that LH2 and LH3 probably have different substrates. Although both isoforms are involved in collagen biosynthesis in the ER, they may prefer different collagen types as substrates. In addition, the multifunctional LH3 possibly has other substrates in the extracellular space.

6.5 GGT activity of LH3 is indispensable for embryonic development

LH3 is a multifunctional enzyme, which has LH, GT and GGT activities *in vitro* (Heikkinen *et al.* 2000). The significance of these activities *in vivo* was studied by generating three different mouse lines with different manipulations of the gene for LH3. The LH3 knockout with an embryonic lethal phenotype lacks the LH3 protein and consequently all activities catalyzed by LH3. The two other mouse lines have LH activity specifically destroyed by a point mutation, which leaves GT and GGT activities intact (Heikkinen *et al.* 2000). The embryonic lethal hypomorphic mouse line also has a

decreased LH3 mRNA level due to the insertion of a *neo* cassette in the *Plod3* gene while the viable LH mutant has a normal LH3 level.

LH3 knockout embryos die earlier than any collagen knockout (see Table 2. in this thesis). LH3 thus seems to be necessary for normal mouse development as could be predicted on the basis of its early developmental expression. Similarly, in zebrafish (Schneider & Granato 2006) and *C. elegans* (Norman & Moerman 2000) the multifunctional LH3 (Wang *et al.* 2002b) is critical for normal development. The roles of glycosyltransferase activities and LH activity of LH3 were distinguished by specifically destroying the LH activity. The mouse lines with this mutation demonstrated that the LH activity of LH3 is not necessary for embryonic development, whereas the viability of the embryos is dependent on GGT activity. Furthermore, in the LH3 knockout the GGT activity was reduced in proportion to the reduction in LH3 level. The LH3 manipulated mice thus confirm the earlier observed correlation between GGT activity and the amount of LH3 protein (Salo *et al.* 2006). Together the data indicate that LH3 is the main collagen glucosyltransferase *in vivo*.

Evidently, the GGT activity of LH3 has an indispensable role in mouse development. Therefore, LH1 and LH2, which lack GGT activity (Heikkinen *et al.* 2000), could not rescue the lethal phenotype of the LH3 knockout, although their expression during development is similar to that of LH3. Comparison of the mouse lines also indicates that the GGT activity of LH3 is more important *in vivo* than its LH activity as previously suggested by *in vitro* analysis (Wang *et al.* 2002a).

However, it is not yet known whether the inactivation of GT, the third enzymatic activity of LH3, causes abnormalities in the LH3 knockout, or whether they are due solely to inactivation of LH and GGT activities. The LH3 knockout produces collagens that seem to lack all the hydroxylysine-linked disaccharides. However, the GT activity of LH3 deficient embryos is only slightly decreased. Mammalian cells contain many different galactosyltransferases, which can glycosylate multiple unrelated proteins (for reviews, see Amado *et al.* 1999, Ohtsubo & Marth 2006). It is therefore possible that the GT activity of LH3 has a role in galactosylation of collagen hydroxylysyl residues, but it can be compensated for by some other galactosyltransferase when LH3 is not present.

6.6 The lethality in LH3 knockout mice is due to disruption of BM

The defect leading to embryonic death in LH3 manipulated mice is disruption of basement membranes due to an intracellular accumulation of type IV collagen. The knockout of several BM components and their receptors leads to embryonic death demonstrating that disruption of BM structure is a common cause of embryonic lethality (for a review, see Yurchenco *et al.* 2004). A lack of laminin-γ1 and the laminin receptor β1-integrin causes lethality at E5.5 (Fässler & Meyer 1995, Smyth *et al.* 1999) while type IV collagen knockout and collagen chaperone Hsp47 knockout mice that produce misfolded type IV collagen show embryonic death between E10.5-E11.5 (Nagai *et al.* 2000, Marutani *et al.* 2004, Pöschl *et al.* 2004). The data suggest that laminin is essential for the early formation of the BM whereas type IV collagen is essential for maintaining the structural integrity of the BM later when more mechanical stress is induced. This is

clearly demonstrated by the LH3 knockout embryos that show dilatation of blood vessels and rupture of the endothelial cell layer due to the lack of endothelial BMs.

Like many other knockout mice with BM abnormalities (for a review, see Yurchenco *et al.* 2004), the LH3 knockout shows abnormalities in the extra-embryonic Reichert's membrane suggesting that the lethality is associated with an insufficient barrier between maternal and embryonic environments. However, disruption of BMs can lead to different defects that contribute to early lethality in the LH3 knockout. The *fukutin* null mice lacking O-mannosyl sugar moieties of the laminin receptor α-dystroglycan show an embryonic lethal phenotype similar to the LH3 knockout. As in the LH3 knockout, the *fukutin* null embryos also exhibit increased apoptosis, which is most likely due to the inability of the BM to transmit survival signals to differentiating cells (Kurahashi *et al.* 2005).

The importance of LH3 to the formation of BMs has been confirmed by BM abnormalities and the embryonic lethality of another LH3 knockout producing low amounts of a truncated LH3 (Rautavuoma *et al*. 2004). However, comparison of different LH3 manipulated mice demonstrates that the BM abnormality is in particular due to deficient glycosylation of hydroxylysyl residues. The importance of correct post-translational modifications of type IV collagen has been further demonstrated by a type I prolyl-4-hydroxylase knockout characterized by an embryonic lethal phenotype at E10.5 due to abnormal type IV collagen localization and disruption of basement membranes (Holster *et al.* 2006). Interestingly, the type IV collagen and Hsp47 null embryos (Nagai *et al.* 2000, Marutani *et al.* 2004, Pöschl *et al.* 2004) lived longer than LH3 knockout embryos. It can thus be concluded that defective glycosylation of hydroxylysines in type IV collagen is more detrimental than its total loss or abnormal folding.

The early lethality of the LH3 knockout may be in part due to the intracellular accumulation of type IV collagen. In the Hsp47 knockout embryos, abnormally folded type IV collagen accumulates in the ER causing ER stress, which induces apoptosis (Marutani *et al.* 2004). However, LH3 dependent glycosylations are probably also important for other collagen types. Type V collagen null mice die at E10.5 due to vasculature defects (Wenstrup *et al.* 2004) suggesting a role for abnormal type V collagen in the dilated blood vessels of LH3 deficient embryos. Type XVIII collagen shows widespread BM expression in the mouse embryo (Miosge *et al.* 2003) and its importance for the BM structure has been established in type XVIII knockout mice (Utriainen *et al.* 2004). Since studies on zebrafish revealed that type XVIII collagen is a substrate for LH3 (Schneider & Granato 2006), defective glycosylation of type XVIII collagen may contribute to the disruption of basement membranes in the LH3 knockout.

6.7 Defective glycosylation of hydroxylysines disturbs the secretion of type IV collagen

The lack of LH3-dependent glycosylations disrupts the secretion of type IV collagen causing its intracellular accumulation and abnormalities in the ER and Golgi structures in the LH3 knockout. However, in the LH mutant type IV collagen lacks some glycosylated hydroxylysines but is still secreted into the BM. The data suggest that the secretion of

type IV collagen is dependent on glycosylation of hydroxylysyl residues, but only some glycosylations are necessary for secretion.

Previously, the *C. elegans* mutant lacking the multifunctional (Wang *et al.* 2002b) LH showed intracellular ER accumulation of type IV collagen (Norman & Moerman 2000). Similarly, LH3-dependent glycosylations are also important for normal secretion of type II collagen in zebrafish (Schneider & Granato 2006). In addition, glycosylated hydroxylysines have also been shown to be important for normal multimerization and secretion of adiponectin (Richards *et al.* 2006, Wang *et al.* 2006) and serum mannan-binding lectin (Heise *et al.* 2000).

It has been established earlier that folding and secretion of other secreted proteins are dependent on the correct N-linked glycosylation (for reviews, see Helenius 1994, Parodi 2000). Moreover, abnormally folded type IV collagen produced by Hsp47 null cells is secreted fourfold slower than normal (Matsuoka *et al.* 2004). In LH3 knockout cells the secretion of type IV collagen is delayed suggesting that deficient glycosylation of hydroxylysyl residues could also affect the folding of type IV collagen. Since most of the misfolded collagens are degraded intracellularly (Lamande *et al.* 1995, Fitzgerald *et al.* 1999), degradation of unfolded collagens could explain the reduced amount of type IV collagen in LH3 knockout fibroblasts.

6.8 Underglycosylation of hydroxylysines leads to abnormal distribution of type VI collagen

Abolishing the LH activity of LH3 does not cause dramatic changes in development or tissue structure. However, the loss of LH activity of LH3 reduces the number of glycosylated hydroxylysines, which causes an abnormal distribution of some collagen types. The effect is best seen in the highly glycosylated collagen types; especially type VI collagen that shows abnormal aggregation, and type IV collagen that has a slightly altered distribution.

Type VI collagen contributes to the formation of collagen fibrils by affecting the arrangement of fibronectin (Sabatelli *et al.* 2001), which is needed for fibrillogenesis (Canty & Kadler 2005), and by binding to type I collagen (Minamitani *et al.* 2004). It is therefore likely that the disorganization of collagen fibrils in the skin of LH mutant mice is mainly due to the abnormal distribution of type VI collagen. In addition, an uneven distribution of the incompletely glycosylated type VI collagen causes defects in the ultrastructure of skeletal muscle of LH mutant mice similar to those previously detected in type VI collagen knockout mice (Irwin *et al.* 2003).

The underglycosylation of hydroxylysyl residues also seems to alter the distribution of type IV collagen, which probably causes abnormalities in BM ultrastructure in skin and muscle. However, the BM of skin also contains, in addition to type IV collagen, type VII and XVII collagens (for a review, see McMillan *et al.* 2003), which may also be different in the LH mutant. In skeletal muscle, collagen VI associates closely with type IV collagen in BMs (Kuo *et al.* 1997) suggesting that alterations seen in the BMs of the LH mutant muscles are due to an abnormal distribution of both these collagen types.

6.9 Deficient glycosylation of hydroxylysines may alter the interactions and assembly of collagens

Glycosylations in general affect the intermolecular binding and intramolecular conformation of proteins (for a review, see Ohtsubo & Marth 2006). A role for the O-linked carbohydrates of collagens in cell-extracellular matrix interactions has been previously suggested by studies which show that T-cell recognition of type II collagen derived peptides (Corthay *et al.* 1998, Myers *et al.* 2004, Van den Steen *et al.* 2004) and the binding of type IV collagen to melanoma cell receptors (Lauer-Fields *et al.* 2003) depend on O-linked glycosylation of collagens. Therefore, loss of some glycosylated hydroxylysines can also change the binding properties of collagens in LH mutant mice. Since in the type VI collagen knockout the changes in muscle ultrastructure were suggested to be due to abnormal engagement of integrins (Irwin *et al.* 2003), similar changes in the LH mutant could be due to disturbed interactions between underglycosylated type VI collagen and integrins. Moreover, abnormal binding of underglycosylated type VI collagen to fibronectin and type I collagen might lead to disorganization of collagen fibrils in skin.

Underglycosylation of type IV collagen may also affect its assembly into basement membranes and thus cause the abnormalities of BM ultrastructure in the LH mutants and the lack of type IV collagen in BM zones in the LH3 knockout. The absence of type IV collagen in the BM has also been reported in the embryos producing a truncated LH3 despite the presence of extracellular type IV collagen aggregates (Rautavuoma *et al.* 2004). The data suggest that the underglycosylated type IV collagen is not assembled normally in the basement membrane. This hypothesis is supported by the finding that hydroxylysine-linked carbohydrates affect the assembly of the 7S tetramer of type IV collagen, which is involved in its alignment in the BM (Langeveld *et al.* 1991). Moreover, the lack of O-linked oligosaccharides in α-dystroglycan abolishes its binding to laminins and other BM components thus disrupting the basement membrane (Michele *et al.* 2002).

It has also been suggested that hydroxylysine-linked carbohydrates might regulate the packing of collagen molecules into supramolecular assemblies (for a review, see Kivirikko 1995). The *in vitro* assembly of type VI collagen tetramers is dependent on the correct glycosylation of hydroxylysines (Kuo *et al.* 1995). Since the tetramerization of type VI collagen occurs already inside the cells (Kielty & Grant 2002), it can be hypothesized that abnormal tetramerization of underglycosylated type VI collagen could cause its abnormal assembly and intracellular and extracellular aggregation in the LH mutant.

6.10 LH activity of LH3 *in vivo*

The alterations detected in the LH mutant mice and a decrease in the amount of hydroxylysines in the collagen fraction enriched with type IV and V collagens demonstrate that the lysyl hydroxylase activity of LH3 has physiological significance.

The lack of LH activity of LH3 is not totally compensated for by other lysyl hydroxylases even in tissues like muscle that show a co-distribution of LH2 and LH3. Since LH3 knockout cells produce collagens that lack more sugar moieties than collagens of LH mutant cells, it can be concluded that LH1 and LH2 can partially compensate for the LH3 by hydroxylating part of the lysyl residues that will also be glycosylated. However, some of the lysyl residues are specific substrates for LH3 and these residues are not glycosylated in the LH mutant.

The effect of the LH activity of LH3 differs in different collagen types; type IV and VI collagens showed altered distributions in the LH mutant while type I collagen was distributed normally. The results thus indicate that the highly glycosylated collagen types, such as type IV and VI collagens, are the main substrates for LH3 *in vivo* as suggested earlier by the *in vitro* studies showing a preference of LH3 to bind and hydroxylate type IV and VI collagens (Risteli *et al.* 2004). However, also type I collagens produced by the LH mutant seem to be underglycosylated suggesting that LH3 may hydroxylate mainly the lysyl residues that are further glycosylated in all collagen types.

Different LH isoforms seem to be specific for certain lysyl residues. LH1 hydroxylates the lysines in the helical parts of collagens (Eyre *et al.* 2002, Zuurmond *et al.* 2005) while LH2b is responsible for hydroxylating the telopeptidyl lysines (Uzawa *et al.* 1999, Mercer *et al.* 2003, van der Slot *et al.* 2004) involved in cross-linking. Therefore, the LH mutant bone showed a normal amount of hydroxylysine derived cross-links. However, LH mutant skin showed a decrease in the amount of hydroxylysine derived cross-links. Since collagen fibrils are also disorganized in skin of lysyl oxidase knockout mice (Mäki *et al.* 2005), the altered cross-linking pattern may contribute to abnormal collagen fibrils in LH mutant skin.

6.11 Future prospects

This thesis has provided new information about the LH3 catalyzed lysyl hydroxylase and glucosyltransferase activities *in vivo*. However, physiological role of galactosyltransferase activity remains to be elucidated. In addition, the physiological significance of extracellular LH3 still needs to be studied. Further analysis of different LH3 manipulated mice and cells derived from these mice could be used to study these activities and also to define whether LH3 also has other substrates in addition to collagens.

The results presented in this thesis also propose important roles for hydroxylysine linked carbohydrates. However, many questions remain to be answered. For instance, glycosylated hydroxylysines are important for the secretion of type IV collagen, but the mechanism leading to the intracellular accumulation of deficiently glycosylated collagen remains unclear. A loss of glycosylation probably affects collagen folding, but it is not known if the loss of glycosylated hydroxylysines delays or even prevents the formation of the triple helix. Furthermore, although hydroxylysine glycosylation is important for normal assembly of type IV and VI collagen, the effect of hydroxylysyl glycosylation on the formation of supramolecular assemblies is not known. In addition, the function of these glycosylations in other collagen types including fibrillar collagens is not yet

understood. The LH3 knockout and mutant fibroblasts provide tools to address these questions and a model for studying the interactions between underglycosylated collagens and their affinity for other matrix macromolecules.

7 Conclusions

In this thesis study the genes for the mouse lysyl hydroxylases were mapped to chromosomes, and the expression of the LH isoforms was studied in mouse. The lysyl hydroxylase genes were shown to localize to three different mouse chromosomes. The characterization of the expression of different LH isoforms indicated that all lysyl hydroxylases are widely expressed but independently regulated during embryonic development. In many adult mouse tissues LH2 and LH3 were produced or regulated in a cell-specific manner suggesting that the LH isoforms have more specialized functions in the adult. Both LH2 and LH3 were shown to be versatile enzymes; the functions of LH2 can be developmentally and tissue-specifically regulated by alternative splicing, and the multifunctional LH3 can localize either to the ER together with LH2, or it can appear in the extracellular space.

The different enzyme activities of LH3 were studied by generating three different mouse lines with different manipulations in the gene for LH3. The LH3 knockout lacking the LH3 protein and the hypomorphic mouse line with a decrease in LH3 mRNA and mutated LH activity were embryonically lethal, whereas the LH mutant with a normal amount of LH3, but with mutated LH activity, were viable but showed ultrastructural alterations. The results therefore indicate that LH3 has GGT and LH activities *in vivo* and in particular the glycosyltransferase activity of LH3 is essential for embryonic development. The deficient LH activity of LH3 caused abnormalities in the highly glycosylated collagen types IV and VI suggesting that LH3 preferentially hydroxylates lysyl residues that will also be glycosylated.

Study of the LH3 manipulated mice provides new insights into the roles of glycosylated hydroxylysines. A lack of glycosylation of hydroxylysines caused intracellular accumulation and deficient secretion of type IV collagen leading to disruption of basement membranes, while reduced hydroxylation and glycosylation of lysyl residues caused abnormal assembly and distribution of type IV and VI collagens leading to disorganization of collagen fibrils and abnormalities in the ultrastructure of skeletal muscle and BM. The glycosylation of hydroxylysines is thus essential for normal secretion of type IV collagen and its assembly into basement membrane. In addition, glycosylated hydroxylysines are important for the correct assembly of type IV, and especially VI collagen and probably for collagen-collagen interactions.

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