Anne Tuomisto

THE ROLE OF COLLAGEN XIII IN B-CELL LYMPHOMA DEVELOPMENT, AND CHARACTERIZATION OF ITS BIOSYNTHESIS AND TISSUE DISTRIBUTION
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Academic dissertation to be presented, with the assent of the Faculty of Medicine of the University of Oulu, for public defence in Auditorium 101 A of the Faculty of Medicine (Aapistie 5 A), on December 5th, 2008, at 10 a.m.

OULUN YLIOPISTO, OULU 2008
Tuomisto, Anne, The role of collagen XIII in B-cell lymphoma development, and characterization of its biosynthesis and tissue distribution
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Abstract

Collagen XIII belongs to the subgroup of collagenous transmembrane proteins. It has a wide tissue distribution and has been localized to many sites of cell-matrix and cell-cell interaction in tissues.

Biochemical and in silico analyses of collagen XIII and other collagenous transmembrane proteins revealed that the biosynthesis of this structurally varied group is characterized by a coiled-coil motif following the transmembrane domain, and these trimerization domains appear to be associated with each of the collagenous domains. The collagen XIII trimer was shown to have an interchain disulfide bond at the junction of the NC1 and COL1 domains, and several other collagenous transmembrane proteins have a pair of cysteines in the same location. Furthermore, furin cleavage at the NC1 domain can be expected in most of the proteins.

Mice heterozygous for the Col13a1del transgene, encoding a mutant collagen XIII, developed clonal mature B-cell lineage lymphomas originating in the mesenteric lymph node (MLN). The incidence of disease in conventionally reared mice was 2-fold higher than for mice raised in a specific pathogen-free facility. The lymphomas often associated with large populations of macrophages and T cells. Lymphomas expressed little if any collagen XIII, suggesting that the effect of the mutation was B-cell extrinsic and likely to be associated with collagen XIII-positive tissues drained by the MLN. Studies of the small intestines of transgenic mice showed highly abnormal subepithelial basement membranes (BM), associated with heightened expression of genes involved in immune responses. These findings suggest that collagen XIII-dependent maintenance of the intestinal BM is a critical determinant of cancer susceptibility.

Collagen XIII exhibited a wide tissue distribution at the protein level, and the most intense expression was found in lung. Tissues contained 1-4 collagen XIII polypeptides, their size ranging between 78 and 102 kDa. Collagen XIII staining was detected in a restricted set of blood vessels in the liver, pancreas, adrenal gland, epididymis and brain. Moreover, Col13a1del transgene expression in the absence of endogenous collagen XIII proved to be deleterious for mouse embryonal development, leading to early fetal mortality.

Keywords: collagen, Collagen XIII/biosynthesis, collagenous transmembrane proteins, extracellular matrix, immunity, lymphoma, transgenic mice
Acknowledgements

This study was carried out at the Oulu Center for Cell-Matrix Research, at the Department of Medical Biochemistry and Molecular Biology, University of Oulu.

I wish to express my deepest gratitude to my supervisor, Professor Taina Pihlajaniemi, for providing excellent opportunities for research. I wish to thank Professors Johanna Myllyharju, Leena Ala-Kokko and Seppo Vainio, Docents Peppi Kerppinen and Minna Männikkö, Research Professors Emeritus Kari Kivirikko and Ilmo Hassinen for creating excellent research facilities and an inspiring scientific atmosphere.

I am grateful to Associate Professor Marion Gordon and Docent Jarmo Käpylä for their valuable comments on the manuscript. Sandra Hänninen is acknowledged for her careful revision of the language.

I want to thank my co-authors Anne Snellman, Ph.D., Anu Koski, M.D., Malin Sund, M.D., Ph.D., Jenni Tahkola, M.D., Professor Eeva-Riitta Savolainen, Docent Helena Autio-Harmainen, Annikki Liakka, M.D., Ph.D., Docent Raija Sormunen, Jussi Vuoristo, Ph.D., Anne West, Ph.D., and Professor Riitta Lahesmaa for their essential contribution in preparing the original articles. Especially Herbert C. Morse III, M.D., is acknowledged for sharing his mouse pathology skills as well as his deep knowledge of immunology. Anne Latvanlehto, Ph.D., is acknowledged for both her excellent scientific skills and friendship. Docent Sinikka Eskelinen is acknowledged for her experienced guidance in cell biology.

I would like to thank all members of Taina’s group and other personnel in the department for creating such a pleasant working environment. Many of you have become important friends to me during these years. I am grateful to Aila White for her outstanding technical assistance. The skillful staff of the department, Pertti Vuokila, Auli Kinnunen, Marja Leena Karjalainen, Seppo Lähdesmäki and Risto Helminen, are acknowledged for their valuable help in everyday life. I wish to thank the personnel of the Laboratory Animal Center and the animal facilities at the Department of Biochemistry for good care of mice. Jenni Tahkola is warmly acknowledged for smooth preparations for the dissertation and party.

I want to thank my friends Anne Rajaniemi, Sanna Ruusunen, Sari ja Jari Kallio for their priceless friendship over decades. My dear friends Joanna and Vesa Ilvesaro are acknowledged for ‘deep-sharing’ our lives. My father and late mother are sincerely acknowledged for establishing the excellent basis for my life. My brother Ari’s family is acknowledged for their kind hospitality during the
Sievi visits. My younger brother Marko and sister-in-law Ulla deserve my deepest gratitude for all their help and support. My aunts Marketta Silvola and Lea Silvola are acknowledged for their extreme helpfulness in various matters, as well as taking care of my daughter. Raili Tuomisto is acknowledged for numerous days of childcare during Tiia’s holidays and illnesses. Nelli Tuomisto is acknowledged for her joyous character. My most loving thanks belong to my precious daughter Tiia. You are my sunshine.

This work was supported by grants from the Health Sciences Council of the Academy of Finland (115237), the Finnish Cancer Foundation and the Sigrid Jusélius Foundation, by the sixth EU Framework Programme (Integrated Project ‘Angiotargeting’; contract no 504743), Cancer Society of Northern Finland, and Tauno Tönning foundation.

Oulu, November 2008

Anne Tuomisto
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>Aβ</td>
<td>amyloid β peptide</td>
</tr>
<tr>
<td>ADAM</td>
<td>a disintegrin and metalloproteinase</td>
</tr>
<tr>
<td>AVVR</td>
<td>atrioventricular valve regurgitation</td>
</tr>
<tr>
<td>BM</td>
<td>basement membrane</td>
</tr>
<tr>
<td>C-</td>
<td>carboxy-</td>
</tr>
<tr>
<td>Clq</td>
<td>complement component, q subcomponent</td>
</tr>
<tr>
<td>CAC</td>
<td>colitis-associated cancer</td>
</tr>
<tr>
<td>CARD</td>
<td>caspase activation and recruitment domain</td>
</tr>
<tr>
<td>CLAC</td>
<td>collagen-like Alzheimer amyloid plaque component</td>
</tr>
<tr>
<td>CLAC-P</td>
<td>precursor of the collagen-like Alzheimer amyloid plaque component</td>
</tr>
<tr>
<td>CL-P1</td>
<td>collectin placenta 1</td>
</tr>
<tr>
<td>COL</td>
<td>collagenous domain</td>
</tr>
<tr>
<td>CRD</td>
<td>carbohydrate recognition domain</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DLBCL</td>
<td>diffuse large B-cell lymphoma</td>
</tr>
<tr>
<td>E</td>
<td>embryonal day</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDA</td>
<td>ectodysplasin</td>
</tr>
<tr>
<td>Edar</td>
<td>Eda receptor</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FACIT</td>
<td>fibril-associated collagens with interrupted triple helices</td>
</tr>
<tr>
<td>FACS</td>
<td>flow cytometry</td>
</tr>
<tr>
<td>FAE</td>
<td>follicle-associated epithelium</td>
</tr>
<tr>
<td>FELASA</td>
<td>federation of European laboratory animal science association</td>
</tr>
<tr>
<td>GAG</td>
<td>glycosaminoglycan</td>
</tr>
<tr>
<td>GALT</td>
<td>gut-associated lymphoid tissue</td>
</tr>
<tr>
<td>GC</td>
<td>germinal center</td>
</tr>
<tr>
<td>HED</td>
<td>hypohidrotic ectodermal dysplasia</td>
</tr>
<tr>
<td>HSPG</td>
<td>heparin-sulphate proteoglycan</td>
</tr>
<tr>
<td>IKK-β</td>
<td>inhibitor of NF-κB kinase-β</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LAIR-1</td>
<td>leukocyte-associated immunoglobulin-like receptor-1</td>
</tr>
</tbody>
</table>
LDL: low-density lipoprotein
MALT: mucosa-associated lymphoid tissue
MARCO: macrophage scavenger receptor with collagenous structure
MASP: MBL-associated serine proteases
MBL: mannose-binding lectin
MLN: mesenteric lymph node
MMP: matrix metalloproteinase
m.o.i.: multiplicity of infection
MSR1: macrophage scavenger receptor 1
N- amino
NC: noncollagenous domain
NF-κB: nuclear factor-κB
NK: natural killer
NMJ: neuromuscular junction
Nod: nucleotide-binding oligomerization domain
NrCam: neuronal cell adhesion molecule
OLF: olfactomedin
P: proline
PALS: periarteriolar lymphoid sheath
PAMPs: pathogen-associated molecular patterns
PBS: phosphate-buffered saline
PRR: pattern recognition receptors
RT-PCR: reverse transcription-polymerase chain reaction
SDS: sodium dodecyl sulphate
SPA: surfactant protein A
SPD: surfactant protein D
SPF: specific pathogen-free
SRA: scavenger receptor class A
SRCL: scavenger receptor with C-type lectin
TACE: TNF-α converting enzyme
TAM: tumor-associated macrophages
TCRβ: T cell receptor β chain
TLR: Toll-like receptor
TNF: tumor necrosis factor
UGRP1: uteroglobin-related protein 1
Xedar: X-linked Eda-A2 receptor
XLHED: X-linked hypohidrotic ectodermal dysplasia
List of original articles

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


Contents

Abstract
Acknowledgements 5
Abbreviations 7
List of original articles 9
Contents 11
1 Introduction 15
2 Review of the literature 17
   2.1 Collagens ......................................................... 17
   2.2 Collagenous transmembrane proteins ...................... 19
      2.2.1 Collagen XVII ............................................... 21
      2.2.2 Collagen XXIII .............................................. 22
      2.2.3 Collagen XXV .............................................. 22
      2.2.4 Ectodysplasin .............................................. 23
      2.2.5 The class A scavenger receptors ....................... 24
      2.2.6 The MARCO receptor ..................................... 25
      2.2.7 Macrophage scavenger receptor (MSR1) ............ 25
      2.2.8 Scavenger receptor with C-type lectin (SRCL) ...... 26
      2.2.9 Colmedins ................................................. 27
   2.3 Collagen XIII .................................................... 27
      2.3.1 Biosynthesis of the collagen XIII ..................... 29
      2.3.2 Shedding of collagen XIII ............................ 30
      2.3.3 Expression of collagen XIII in cancer tissue ...... 30
   2.4 Mouse models for studying collagen XIII function .......... 31
      2.4.1 N-terminally altered collagen XIII mice (Col13a1N/N) .......... 31
      2.4.2 Mice overexpressing collagen XIII (Col13a1oe) ...... 32
      2.4.3 Mice overexpressing truncated collagen XIII (Col13a1del) ................................................. 32
   2.5 Defense collagens .................................................. 34
      2.5.1 Function of the collagenous domain .................. 35
      2.5.2 Complement activation by C1q, MBL and ficolins .... 36
   2.6 Basement membranes ............................................. 36
   2.7 Immunity ............................................................ 37
      2.7.1 Innate and adaptive immunity .......................... 37
      2.7.2 Intestinal immunity ....................................... 38
      2.7.3 Intestinal epithelial cells in intestinal immunity ...... 38
2.7.4 Regulation of the immune response by the LAIR-1 receptor implicates cell-matrix interactions ................................. 40
2.7.5 Microbial infection, inflammation and cancer.............................. 42
2.8 Collagens in autoimmune diseases.......................................................... 43
2.9 Diffuse large B-cell lymphoma............................................................... 43

3 Outlines of the present study 45

4 Materials and methods 47

4.1 Construction of recombinant baculoviruses (I) ....................................... 47
4.2 Analysis of recombinant proteins by Western blotting (I) ...................... 48
4.3 Amino acid sequence analysis and secondary structure prediction (I)............................................................................................................. 48
4.4 Generation of Col13a1del and Col13a1del;Col13a1− transgenic mice, their health monitoring and pregnancy terminations (II, III)......... 49
4.5 Tissue preparation, histological and immunofluorescence analysis (II, III) ....................................................................................... 49
4.6 Flow cytometry (FACS) (II).................................................................... 50
4.7 Molecular analysis (II)............................................................................. 50
4.8 Electron microscopy (II) ......................................................................... 50
4.9 Microarray analysis (II)........................................................................... 51
4.10 Western blotting of tissues (III)............................................................... 51

5 Results 53

5.1 The two cysteines at the end of the first noncollagenous domain (NC1) are responsible for linking the three α1(XIII) chains together by interchain disulfide bonds (I) .................................................. 53
5.2 The NC3 coiled-coil domain is important for correct folding and stability of collagen XIII in vitro (I)................................................................. 54
5.3 In silico analysis of the novel collagenous transmembrane proteins (I)............................................................................................... 55
5.4 Occurrence of lymphomas in Col13a1del mice (II) .................................. 56
5.5 Analysis of the lymphomas and preneoplastic changes (II)............... 56
5.6 Expression and localization of collagen XIII in lymphomas, lymphoid tissues and intestine (II) .......................................................... 57
5.7 Electron microscopy of the small intestine (II)......................................... 58
5.8 Gene expression profiles from wild-type and Col13a1del mice differ for small intestinal tissues (II) ........................................................ 58
5.9 Expression of collagen XIII protein in mouse tissues analyzed by Western blotting (III).......................................................... 59
1 Introduction

In multicellular organisms, the extracellular matrix (ECM) between the cells provides physical support to tissues and organs. The ECM is present in every tissue, but is most highly enriched in connective tissues and basement membranes (BM). Cells in tissues synthetize and maintain it, but the matrix reciprocally influences cellular functions. Cell-matrix interactions have a major effect on cell attachment and migration, but also regulate cellular differentiation and gene expression profiles. For example, collagens have been shown to regulate immune responses by interacting with leukocyte-associated immunoglobulin-like receptor-1 (LAIR-1).

Collagen XIII belongs to the group of collagenous transmembrane proteins together with collagens XVII, XXIII and XXV, as well as ectodysplasin, the class A macrophage scavenger receptors and colmedin proteins. Collagen XIII is a large molecule spanning from the interior of the cell to the extracellular matrix, and the ectodomain mostly collagenous is a 150 nm long rod with flexible hinges. Collagen XIII is involved in both cell-matrix and cell-cell adhesion in a wide range of tissues including muscle, skin, bone and other connective tissues. In the present work, we analyzed and, with the help of in silico approaches, characterized features of the biosynthesis of collagenous transmembrane proteins. In transgenic Col13a1del mice we found a propensity to lymphoma development in mesenteric lymph nodes (MLN). Subepithelial BMs in jejunum tissues drained by MLN were highly abnormal, and these tissues were associated with heightened expression of immune response genes. In humans, 15–20% of all cancers are estimated to be linked to underlying infections and inflammatory responses. Studies in Col13a1del mice define the ECM functioning as a critical determinant of cancer susceptibility.

We characterized further the tissue distribution of collagen XIII at the protein level, and found it in a range of tissues in association with blood vessels. Moreover, its polypeptide pattern showed tissue-specific distribution.
2 Review of the literature

2.1 Collagens

Collagens are the most abundant proteins in the human body, representing approximately 30% of all proteins. They are found in the interstitial tissue of virtually all parenchymal organs, contributing to the stability of tissues and organs and maintaining their structural integrity. Their importance for tissue function has been highlighted by the wide spectrum of human diseases caused by mutations in collagen genes. The size, function and tissue distribution of different collagen types vary remarkably. At the moment, vertebrates are known to have at least 29 collagen types consisting of 44 distinct α-chains, and more than 20 additional proteins have collagen-like domains. Binding of the collagens to their cellular receptors such as integrins, discoidin domain receptors, glycoprotein VI and proteoglycan receptors modulates cell adhesion, differentiation, growth, cellular reactivity and survival. Collagens have a great impact on organ development, wound healing and tissue repair. (O'Reilly et al. 1997, Davis et al. 2000, Ortega & Werb 2002, Gelse et al. 2003, Myllyharju & Kivirikko 2004, Söderhall et al. 2007.)

Based on their structure and supramolecular organization, collagens can be grouped into subfamilies of fibril-forming collagens, fibril-associated and related collagens (FACIT), collagens forming hexagonal networks, type IV collagens, type VI collagens forming beaded filaments, type VII collagens forming anchoring fibrils, transmembrane collagens and endostatin-containing collagens XV and XVIII. In addition, a heterogenous group within the collagen superfamily is composed of proteins with collagenous domains but has not been defined as collagens. (Myllyharju & Kivirikko 2004.)

The characteristic feature of all members of the collagen family of proteins is the triple helix composed of three α-chains. The triple helix can be formed by identical chains as in collagen types II, III, VII and X, or by two or more different chains as in collagen types I, IV, V, VI, IX, XI and possibly type VIII. (Myllyharju & Kivirikko 2004.) Each of the three α-chains in a trimeric collagen molecule forms an extended left-handed helix in which 18 amino acids are needed per turn (Hofmann et al. 1978). The three chains are supercoiled around a central axis in a right-handed manner to form the triple helix (Fraser et al. 1979). A requirement for triple helix formation is that every third amino acid is glycine,
the smallest amino acid existing. As a result a Gly-X-Y repeat, the characteristic structure of collagens, is formed. In the α-chain assembly around the central axis, glycine residues are located in the center of the triple helix, while larger side chains of other amino acids occupy the outer positions. The X and Y positions are often occupied by proline and hydroxyproline. (Bornstein 1974, Miller 1976, Gelse et al. 2003, Myllyharju & Kivirikko 2004.)

Depending on the collagen type, specific proline and lysine residues are modified by posttranslational enzymatic hydroxylation. The presence of 4-hydroxyproline is essential for the formation of intramolecular hydrogen bonds and contributes to the stability of the triple-helical conformation. Some of the hydroxylysines are further modified by glycosylation. (Gelse et al. 2003.) The extent of lysine hydroxylation and hydroxylysine glycosylation may regulate collagen fibril formation and affect the diameter of the formed collagen fibril (Notbohm et al. 1999).

Although the collagen triple helix represents the major part of the collagen molecule, especially in the group of fibril-forming collagens, noncollagenous domains flanking the central helical part are also important structural components. In the case of collagen I, the C-propeptide, or more specifically its C-terminal globular domain, plays a fundamental role in the initiation of triple helix formation and heterotrimer selectivity (Malone et al. 2005). A shed C-terminal propeptide has been shown to be chemotactic for endothelial cells and to stimulate migration and production of metalloproteinases by mammary carcinoma cell lines (Palmieri et al. 2000, Palmieri et al. 2003). C-propeptide fragments have also been reported to stimulate extracellular matrix production (Katayama et al. 1993). The functions of the N-terminal propeptides are less understood. Mice lacking the majority of the non-triple-helical sequence in the N-propeptide of collagen I had normal procollagen synthesis, secretion and proteolytic processing (Bornstein et al. 2002). However, transfection of the mutant cDNA into COS-7 cells led to reduced collagen synthesis and impaired cell adhesion to the fibronectin matrix, suggesting that the N-propeptide plays an important role in feedback regulation (Oganesian et al. 2006). The short non-helical telopeptides of the processed collagen monomers are involved in the covalent cross-linking of the collagen molecules as well as in the linking to other molecular structures of the surrounding matrix (Rossert & de Crombrugghe 2002).
2.2 Collagenous transmembrane proteins

Collagenous transmembrane proteins are type II transmembrane proteins which contain at least one stretch of Gly-X-Y repeats. In addition to collagen types XIII, XVII, XXIII and XXV, current members of this group include ectodysplasin, the class A macrophage scavenger receptors and colmedin proteins containing an olfactomedin domain (OLF) in addition to collagenous domains (Figure 1). (Franzke et al. 2005.) The group of class A macrophage scavenger receptors contains three members: macrophage scavenger receptor 1 (MSR1), macrophage scavenger receptor with collagenous structure (MARCO) and scavenger receptor with C-type lectin (SRCL) (Murphy et al. 2005). Collagens XIII and XVII participate in cell adhesion, and also collagen XXIII and ectodysplasin may have functions in cell adhesion. The ectodomain of collagen XXV has been identified as a component of Alzheimer amyloid plaques (Hashimoto et al. 2002). The class A macrophage scavenger receptors are involved in host defense and the colmedins in neuromuscular signaling. (Franzke et al. 2005.)

The transmembrane collagens XIII, XVII, XXIII and XXV have dual functions both as cell surface receptors as well as matrix components, when the ectodomain is shed from the cell surface (Hirako et al. 1998, Schacke et al. 1998, Snellman et al. 2000b, Hashimoto et al. 2002, Banyard et al. 2003). The soluble ectodomains have been shown to interact with a number of ECM proteins in vitro, for example, the ectodomain of collagen XIII with fibronectin, nidogen-2 and perlecan, the collagen XXIII ectodomain with heparin, and the collagen XXV ectodomain with the fibrillar β-amyloid peptide (Tu et al. 2002, Hashimoto et al. 2002, Banyard et al. 2003).

Collagens XIII, XXIII and XXV have a similar structural organization, containing three collagenous domains (COL1-COL3) interrupted and flanked by four noncollagenous domains (NC1-NC4) (Hägg et al. 1998, Hashimoto et al. 2002, Banyard et al. 2003). Collagenous transmembrane proteins are discussed here in detail, while collagen XIII is reviewed thoroughly in section 2.3.
Fig. 1. Schematic structures of the human collagenous transmembrane proteins. Reprinted from the Journal of Biological Chemistry, 282, Snellman et al. (2007), with permission from American Society for Biochemistry and Molecular Biology. The noncollagenous domains are shown as black boxes, the transmembrane domain as a bricked box, the collagenous domains as white boxes, SRCR domains as netted boxes, C-type CRD as squares in a box, a tumor necrosis factor motif as a spotted box, and an OLF domain as a striped box. Potential coiled-coil regions are indicated with bars and potential furin cleavage sites with arrows above the corresponding polypeptides. NH₂, N terminus; COOH, C terminus; C, cysteine.
2.2.1 Collagen XVII

Collagen XVII, initially identified as the 180-kDa bullous pemphigoid antigen (BP180), is a structural hemidesmosomal transmembrane protein with a non-triple-helical cytoplasmic domain and a large interrupted collagenous extracellular domain (Giudice et al. 1992, Nishizawa et al. 1993). Collagen XVII has a critical role in maintaining the linkage between the intracellular and the extracellular structural elements involved in epidermal adhesion. The intracellular ligands of collagen XVII include β4-integrin, plectin, and BP230 in the hemidesmosomal plaque (Koster et al. 2004), and the extracellular ligands α6-integrin and laminin-5 in the anchoring filaments (Hopkinson et al. 1995, Tasanen et al. 2004). Thus, collagen XVII contributes to stable adhesion of epithelial cells by multiple protein-protein interactions. Mutations in the collagen XVII gene, \textit{COL17A1}, lead to junctional epidermolysis bullosa, a human blistering skin disorder (McGrath et al. 1995). In bullous autoimmune skin diseases, autoantibodies to collagen XVII perturb cell adhesion and lead to epidermal-dermal separation and skin blistering (Schumann et al. 2000). In addition, serum levels of collagen XVII-recognizing autoantibodies correlate with the disease activity in these patients (Feng et al. 2008).

Collagen XVII contains three 180-kDa α-chains that fold to form a homotrimer with collagenous triple helical segments. The ectodomain contains 15 collagenous subdomains, intervened and flanked by 16 short noncollagenous sequences. (Giudice et al. 1992.) The ectodomain of collagen XVII is constitutively shed from the cell surface (Hirako et al. 1998, Schacke et al. 1998). Metalloproteases of the ADAM (A Disintegrin and Metalloproteinase) family catalyze this process, with ADAM17/TACE (TNF-α Converting Enzyme) appearing to be the major sheddase of collagen XVII (Franzke et al. 2002). The transmembrane enzyme TACE is involved in the shedding of a variety of type I and II transmembrane proteins. Substrate recognition by TACE is thought to depend on structural motifs rather than a common consensus sequence. (Black 2002.) The ectodomain shedding of collagen XVII plays a role in the regulation of epithelial cell adhesion, detachment and motility during development, differentiation and regeneration. Cleavage of the ectodomain facilitates release of the cell from its binding partners in the microenvironment. (Franzke et al. 2005.) The mechanisms regulating the shedding are slowly unraveling. Zimina et al. (2005) showed that plasma membrane lipid organization regulates collagen XVII shedding. Collagen XVII is localized inside lipid rafts, the dynamic cholesterol-
and sphingolipid-enriched membrane microdomains, where it is less accessible to its sheddase, which is outside the rafts. A small decrease in cell-surface cholesterol increased collagen XVII shedding. In another study, Zimina et al. (2007) showed that extracellular phosphorylation of collagen XVII regulates the shedding of the ectodomain. Extracellular phosphorylation is one of the mechanisms in the regulation of many biological processes, such as cell adhesion and proliferation, as well as mechanisms of immune responses (Redegeld et al. 1999).

### 2.2.2 Collagen XXIII

Collagen XXIII has been found in rat prostate carcinoma cells. It is a cell surface protein which can be cleaved by furin protease (Banyard et al. 2003, Koch et al. 2006, Veit et al. 2007). At the mRNA level its expression is highest in lung, and it is clearly expressed also in cornea, skin, tendon and amnion. Minor amounts can be found in kidney and placenta. At the protein level collagen XXIII expression is restricted to the epithelial layer of the epidermis, small intestine, oropharynx and the dorsum of the tongue. Collagen XXIII likely interacts with the BM, but it is not a component deposited into it. (Koch et al. 2006.)

Collagen XXIII is expressed in tissues both in full-length and shed forms. It appears to be in full-length form in mouse skin and kidney and mostly full-length in lung. In brain, collagen XXIII is expressed as two processed forms, the size of one of them being the expected size of the shed form of collagen XXIII and the lower molecular weight polypeptide likely representing additional processing of collagen XXIII. (Koch et al. 2006.)

Recently, Banyard et al. (2007) showed increased expression of collagen XXIII in epithelial cells in prostate cancer tissues. Collagen XXIII was also shown to be a predictor of prostate cancer recurrence, rendering it a potential biomarker for assessing prostate cancer progression and metastasis.

### 2.2.3 Collagen XXV

Collagen XXV, also called CLAC-P (precursor of the collagen-like Alzheimer amyloid plaque component), is expressed in neurons and at low levels also in heart, testis and eye. At least four splice isoforms of collagen XXV exist. Collagen XXV ectodomain is cleaved from the plasma membrane by furin, and the newly formed N-terminal end is stabilized by pyroglutamate modification. A
secreted ectodomain of collagen XXV is localized to senile plaques in Alzheimer’s disease brains, where it is an integral component of amyloid deposits. (Hashimoto et al. 2002.)

Both the full-length and secreted forms of collagen XXV bind to the fibrillar, but not monomeric form of amyloid β peptide (Aβ) in vitro (Hashimoto et al. 2002). When Söderberg et al. (2005a) analyzed the effect of CLAC on fibrillar Aβ, they detected further aggregation of Aβ-fibrils. Moreover, CLAC assembled Aβ-fibrils into protease-resistant aggregates, which may be relevant in the pathogenesis of Alzheimer’s disease. The interaction of collagen XXV with Aβ-fibrils is mediated by the COL1 of CLAC, and the triple-helical structure of CLAC is required. (Osada et al. 2005.) On the other hand, the soluble form of collagen XXV restricts the fibrillization of monomeric Aβ in vitro, suggesting an inhibitory role of collagen XXV in β-amyloid formation in Alzheimer’s disease brains (Osada et al. 2005). NC2 domain of CLAC is needed for the interaction with monomeric Aβ (Söderberg et al. 2005b).

2.2.4 Ectodysplasin

Ectodysplasin (EDA) is a member of the tumor necrosis factor (TNF) superfamily, the only member of this family containing a collagenous domain (Kere et al. 1996, Copley 1999, Ezer et al. 1999, Mikkola et al. 1999). Mutations in the components of the TNF signaling pathway result in a malformation syndrome called hypohidrotic (anhidrotic) ectodermal dysplasia (HED). The most frequent form of congenital ectodermal dysplasia, the X-linked form of HED, is due to mutations in EDA (Pinheiro & Freire-Maia 1994, Kere et al. 1996). Eda signaling has an important function in embryonic development, especially in the development of ectodermally derived organs (Mikkola & Thesleff 2003, Mustonen et al. 2003).

The Eda transcript is known to undergo complicated alternative splicing yielding numerous ectodysplasin transcripts. EDA-A1 (391 aa) and EDA-A2 (389 aa), the functional forms of the molecule, differ only by an insertion of two amino acids in the TNF domain due to the usage of alternative splice donor sites (Bayes et al. 1998). Despite the minor difference between EDA-A1 and EDA-A2, these molecules were found to engage two different receptors: EDAR is the specific receptor for EDA-A1, whereas the X-linked EDA-A2 receptor (XEDAR), another related but distinct TNF receptor, binds EDA-A2 but not EDA-A1 (Yan et al. 2000).
The EDA-A1 and EDA-A2 isoforms have a short N-terminal intracellular domain and a fairly large C-terminal extracellular domain containing 19 (Gly-X-Y) collagen-like repeats with one interruption followed by the TNF homology domain (Ezer et al. 1999, Mikkola et al. 1999). The collagenous domain of EDA appears to play a role in the multimerization of EDA trimers (Schneider et al. 2001). However, mutation studies of the human EDA gene have suggested additional functions for the collagenous domain, since a number of families with the X-linked form of HED (XLHED) have in-frame deletions in the collagenous domain. However, the multimerization of this mutated protein is unaffected when it is expressed in vitro (Ezer et al. 1999, Schneider et al. 2001).

Eda signaling is not limited to mammals; both the sequence and function of Edar have been conserved in all vertebrates. Edar has been shown to regulate feather development in chicken and scale development in teleost Medaka fish as well as the formation of skeletal and dental structures in adult zebrafish. (Kondo et al. 2001, Houghton et al. 2005, Harris et al. 2008.) The binding of Eda-A1 to its receptor Edar activates the nuclear factor-κB (NF-κB) both in vitro and in vivo (Mikkola & Thesleff 2003).

2.2.5 The class A scavenger receptors

Scavenger receptors are a family of transmembrane proteins involved in tissue macrophage functions such as endocytosis, adhesion, phagocytosis and intracellular signaling. They are capable of binding a wide variety of ligands including modified or oxidized low-density lipoproteins (LDL), apoptotic cells and pathogens. These receptors are divided into eight classes (A–H). A common feature for class A scavenger receptors is a collagen-like domain. (Murphy et al. 2005.) The class A scavenger receptors derive from three related genes: macrophage scavenger receptor (MSR1) [also called scavenger receptor class A, member 1 (SRA)], macrophage receptor with collagenous structure (MARCO) and scavenger receptor with C-type lectin (SRCL) (Figure 1) (Freeman et al. 1990, Emi et al. 1993, Elomaa et al. 1995, Nakamura et al. 2001). MSR1 and SRCL mRNAs can be alternatively spliced to generate at least three isoforms in the case of MSR1 and two isoforms for SRCL (Freeman et al. 1990, Gough et al. 1998, Nakamura et al. 2001).
2.2.6 The MARCO receptor

The MARCO receptor is constitutively expressed in a subpopulation of macrophages in the marginal zone of the spleen and in the medullary cord of the lymph nodes. These are regions of active removal of pathogens and other substances from the blood and lymph fluid by macrophages. (Elomaa et al. 1995.) The MARCO receptor is also expressed by splenic dendritic cells (Granucci et al. 2003). MARCO can be induced by a variety of infectious (van der Laan et al. 1997, van der Laan et al. 1999) and non-infectious (Sakaguchi et al. 1998, Seta et al. 2001) inflammatory stimuli in most tissue macrophages both in vivo and in vitro. Pro- and anti-inflammatory cytokines have no effect on MARCO induction (van der Laan et al. 1999). Knockout mice lacking MARCO display reduced bacterial clearance from lung tissue and increased bacterial infection (Arredouani et al. 2004).

MARCO can bind acetylated LDL and Gram-positive and Gram-negative bacteria but not yeast (Elomaa et al. 1995, Elomaa et al. 1998). In addition to microbial ligands MARCO also has other ligands. First of all, MARCO is the main receptor for large environmental particles in the lung alveolar macrophages (Palecanda et al. 1999). The endogenous ligand for MARCO in lung is uteroglobin-related protein 1 (UGRP1). The UGRP1-MARCO-ligand-receptor pair is probably involved in inflammation and pathogen clearance in the lung. (Bin et al. 2003.) In the spleen, the MARCO receptor on the surface of the marginal zone macrophages interacts directly with marginal zone B cells enabling the retention of the marginal zone B cells in this location (Karlsson et al. 2003).

At the cellular level, MARCO can modulate the actin cytoskeleton dynamics, rendering it a possible cell surface sensor. Its ectopic expression in several cell lines induces the formation of large lamellipodia-like structures and long dendritic processes. (Pikkarainen et al. 1999.) During dendritic cell maturation its expression correlates with changes in actin cytoskeleton organization. As such the expression of MARCO is sufficient to induce the cytoskeleton modifications in dendritic cells. (Granucci et al. 2003.)

2.2.7 Macrophage scavenger receptor 1 (MSR1)

MSR isoforms are expressed by almost all macrophage populations, but they can also be detected in endothelial and smooth muscle tissues (Naito et al. 1991, Li et al. 1995). Two isoforms of MSR1 can both bind modified LDL, polynucleic
acids, bacterial components and some carbohydrate ligands (Dhaliwal & Steinbrecher 1999). A third MSR1 isoform is retained within the mammalian endoplasmic reticulum (ER) and thus cannot bind extracellular ligands (Gough et al. 1998).

MSR1 can mediate the phagocytosis of Gram-positive and Gram-negative bacteria and endotoxin clearance (Hampton et al. 1991, Peiser et al. 2000, Thomas et al. 2000). Interestingly, the MSR1 proteins are also implicated in regulating cell adhesion by binding to proteoglycans in the extracellular matrix (Santiago-Garcia et al. 2003).

Knockout mice lacking the MSR1 receptor are resistant to the development of atherosclerosis, suggesting that the receptor contributes to the uptake of modified lipoproteins and to cholesterol ester accumulation in macrophages in vivo, which leads to the appearance of foam cells (Suzuki et al. 1997). These macrophage-derived foam cells make up the fatty streak lesions that precede more advanced atherosclerotic lesions, which may ultimately progress to more adverse vascular and cardiovascular events, such as thrombosis and myocardial infarction (Ross 1993).

2.2.8 Scavenger receptor with C-type lectin (SRCL)

SRCL (also known as collectin placenta 1; CL-P1) shows features of both the type A scavenger receptors for modified LDL and the collectins, C-type lectins involved in pathogen recognition (Peiser et al. 2002). Collectins are a group of molecules that have collagen-like sequences and carbohydrate recognition domains (CRD) (Drickamer 1988). They are involved in host defense through their ability to bind to the carbohydrate antigens on microorganisms. Collectins are the innate immune system molecules present in plasma and on mucosal surfaces. Collectins are more widely discussed in section 2.3. SRCL is the only membrane-bound member of the collectin family. (Ohtani et al. 2001.)

SRCL is widely expressed in human tissues in vascular endothelial cells, with the highest levels in placenta, heart and lung. SRCL can bind and phagocytose Gram-positive and Gram-negative bacteria as well as yeast, and it also reacts with oxidized LDL. Due to its membrane-bound nature, SRCL may have important roles in host defense that are different from those of soluble collectins. SRCL is also unique when compared to the other class A scavenger receptors, MSR1 and MARCO, since it is expressed on endothelial cells but not macrophages,
suggesting distinct functions compared with the MSR1 and MARCO proteins. (Ohtani et al. 2001.)

2.2.9 Colmedins

The OLF domain has been discovered in more than 100 proteins in species ranging from *Caenorhabditis elegans* to *Homo sapiens*, with essential roles in various physiological processes (Zeng et al. 2005). The colmedins (collagen repeat plus olfactomedin domain), as the name suggests, are transmembrane proteins containing collagenous and olfactomedin-like domains (Loria et al. 2004). *Rattus norvegicus* gliomedin, CG6867, cof-2 (colmedin family member 2) and unc-122 (uncoordinated movement) are characterized members of this subfamily (Franzke et al. 2005). Gliomedin has been identified in all sequenced vertebrate genomes. In invertebrates several orthologous genes have been identified, with one copy of the gene found in *Drosophila melanogaster* and two copies, unc-122 and cof-2, identified in *Caenorhabditis elegans*. Of all traditional collagen types, only collagens IV and XVIII are conserved throughout the animal kingdom (Myllyharju & Kivirikko 2004). Thus, conservation of gliomedin-like genes in such a broad range of species suggests functions fundamental to life also in the case of these collagenous proteins. In *Caenorhabditis elegans*, the protein unc-122 is located at neuromuscular junctions (NMJ) and is proposed to be involved in maintaining a structural microenvironment that allows efficient NMJ signaling. Another *Caenorhabditis elegans* colmedin, cof-2, has a similar structure to unc-122, but its function is still unknown. (Loria et al. 2004.) In contrast, gliomedin is a glial ligand for the axonal adhesion molecules neurofascin and NrCam (neuronal cell adhesion molecule) at the nodes of Ranvier in the peripheral nervous system (Eshed et al. 2005). Unique features of *Drosophila melanogaster* CG6867 are its two immunoglobulin domains not found in any other OLF domain protein (Loria et al. 2004).

2.3 Collagen XIII

Collagen XIII is widely expressed in many tissues such as bone, cartilage, heart, muscle, lung, intestine and skin throughout development and adult life (Sandberg et al. 1989, Sund et al. 2001a). It is located in the focal adhesions of cultured fibroblasts and other cells, and in the adhesive structures of tissues, such as the myotendinous and neuromuscular junctions in muscle, intercalated discs in the

Collagen XIII consists of three collagenous domains (COL1 to COL3) (Figure 1) separated and flanked by four noncollagenous domains (NC1 to NC4) (Pihlajaniemi & Tamminen 1990, Hägg et al. 1998). Full-length human α(XIII) chains have four pairs of cysteine residues located in the NC1, COL1, NC2 and NC4 domains (Hägg et al. 1998). Interchain disulfide bonds are formed by cysteines in the NC1 and possibly in the COL1 and NC2 domains, whereas the cysteine residues in the NC4 domain form intrachain bonds (Snellman et al. 2000a).

Based on rotary shadowing electron microscopy, the length of the collagen XIII ectodomain is 150 nm and it consists of three collagenous rods separated by two flexible hinges, most likely representing noncollagenous domains NC2 and NC3. Collagen XIII binds with high affinity to the extracellular matrix protein fibronectin, the BM proteins nidogen-2 and perlecan, and to heparin. This last observation suggests that collagen XIII may interact with matrix- or membrane-bound heparan sulphate proteoglycans. (Tu et al. 2002.) Collagen XIII has been shown to interact with the I-domains of α1 (Nykvist et al. 2000) and α11 integrins (Tu 2004). In in vitro conditions the collagen XIII ectodomain associates with the fibrillar fibronectin matrix. The association occurs between the conserved C-terminal end of collagen XIII and the N-terminal part of fibronectin containing the collagen/gelatin-binding domain. The collagen XIII ectodomain can also interfere with the assembly of the fibronectin matrix, suggesting that the biological activities of the collagen XIII ectodomain may include the ability to remodel the structure of extracellular matrix. (Väisänen et al. 2006.) The features of collagen XIII are listed at table 1.

### Table 1. Features of collagen XIII.

<table>
<thead>
<tr>
<th>Level</th>
<th>Specific properties</th>
<th>Outcome</th>
<th>Biological relevance</th>
</tr>
</thead>
<tbody>
<tr>
<td>mRNA</td>
<td>Extensive alternative splicing</td>
<td>17 different transcripts</td>
<td>COL1, NC2, COL3 and NC4 domains affected</td>
</tr>
<tr>
<td>Protein</td>
<td>Coiled-coil domains</td>
<td>Triple-helix formation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>N- to C-terminus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Proteolytic shedding</td>
<td>Membrane-bound and shed forms</td>
<td>Influence on cell behaviour</td>
</tr>
<tr>
<td>Molecular interaction</td>
<td>Several binding partners</td>
<td>Interferes the assembly of fibronectin matrix</td>
<td>Remodelling of the ECM</td>
</tr>
</tbody>
</table>

28
2.3.1 Biosynthesis of the collagen XIII

The collagen XIII mRNA undergoes extensive alternative splicing, producing at least 17 different transcripts (Peltonen et al. 1997). In both mouse and human, a total of 10 exons encoding either collagenous or noncollagenous sequences can be alternatively spliced, affecting the sizes of the COL1, NC2, COL3 and NC4 domains (Pihlajaniemi & Tamminen 1990, Tikka et al. 1991, Juvonen & Pihlajaniemi 1992, Juvonen et al. 1992).

In the multimerization of collagen XIII α-chains, sequences important for chain recognition and the association of three individual α-chains reside in the N-terminal region of the molecule (Snellman et al. 2000b) rather than in the C-terminal part as in the case with the fibril-forming collagens (Doege & Fessler 1986, Lees et al. 1997). Hence, the triple helix formation proceeds from the N- to the C-terminus, in the opposite orientation known to occur for the fibrillar collagens (Bachinger et al. 1981, Bruckner et al. 1981). The association domain seems to be conserved among several other transmembrane collagens, suggesting common feature in their chain association (Snellman et al. 2000b). In further studies, the association domain sequence revealed a high probability of forming a coiled-coil structure that determines the association state and chain composition of forming multimeric protein complexes (Beck & Brodsky 1998, Engel & Kammerer 2000, Burkhard et al. 2001, Latvanlehto et al. 2003). As such, collagenous α-chains are characterized by a slow folding speed and poor selection properties (Beck & Brodsky 1998). Later it was shown by McAlinden et al. (2003) that most members of the collagen superfamily contain coiled-coil domains, suggesting a general role in triple-helical assembly. Coiled-coil domains can be located along collagen polypeptide chains either before, after or between triple helical regions.

The predicted N-terminal coiled-coil structure of collagen XIII extends also within the transmembrane domain albeit with lower probability. Moreover, another coiled-coil structure is predicted to be located in the NC3 domain of collagen XIII. It has been proposed that the NC1 coiled-coil domain is important for association of the N-terminal part of the collagen XIII α-chains, while the NC3 coiled-coil domain accounts for the association of the C-terminal part of the molecule. Both the NC1 and NC3 coiled-coil structures are also predicted for the corresponding domains in the related collagen types XXIII and XXV. It seems that these two widely separated coiled-coil domains in collagen XIII and in the
related collagens function as independent oligomerization domains participating in the folding of distinct areas of the collagen molecule (Latvanlehto et al. 2003.)

A coiled-coil domain is not always sufficient for trimerization, as found for the biosynthesis of collagen IX of the FACIT subgroup (Jäälinoja et al. 2008). The collagen IX molecule consists of three collagenous domains, COL1–COL3, separated and flanked by four noncollagenous domains numbered from the C-terminus (Ninomiya et al. 1985). The most C-terminal collagenous (COL1) and noncollagenous (NC1) domains are not essential for trimerization of this collagen, but these domains contribute to the specificity of chain association (Jäälinoja et al. 2008). Despite the coiled-coil structure in the NC2 region (McAlinden et al. 2003), NC2 is not able to trimerize a short variant containing only the COL2 and NC2 domains and the trimerization has been found to also require the N-terminal NC3 and COL3 domains (Jäälinoja et al. 2008).

2.3.2 Shedding of collagen XIII

Subtilisin-like proprotein convertases are responsible for the shedding of the collagen XIII ectodomain (Snellman et al. 2000b, Väisänen et al. 2004). Cleavage occurs both in the trans-Golgi network and in the plasma membrane. The released ectodomain reduces cell adhesion, migration and proliferation. Hence, the collagen XIII ectodomain appears to be a biologically active molecule which influences cell behavior. (Väisänen et al. 2004.) Collagen XIII binds to heparin with high affinity, rendering collagen XIII capable of binding matrix- or membrane-bound heparan sulphate proteoglycans. Heparin inhibits the shedding of the collagen XIII ectodomain by an unknown mechanism in insect cell cultures. (Tu et al. 2002.) In this regard, heparin molecules and heparin-containing proteoglycans might have a role in regulating the cleavage of collagen XIII.

2.3.3 Expression of collagen XIII in cancer tissue

Recently, collagen XIII expression was shown to be increased in the reactive stroma cells in epithelial tumors and throughout mesenchymal tumors. In a cell culture model, tumor cells led to upregulated collagen XIII, vimentin and α-smooth muscle actin expression in a paracrine manner in the primary fibroblasts. Together with the phenotypic changes of the cells, the changes are characteristic to myofibroblast transdifferentiation. On the other hand, increased
amounts of the soluble collagen XIII ectodomain altered the growth milieu of the
tumor cells by decreasing cell adhesion and spreading. Hence, it is conceivable
that tumor cells induce the expression of collagen XIII in the tumor stroma, and
this may contribute to tumor progression and altered behavior. (Väisänen et al.
2005.)

2.4 Mouse models for studying collagen XIII function

In order to study the function of collagen XIII in vivo, several genetically
modified mouse lines have been generated (Table 2) (Kvist et al. 2001, Sund et
al. 2001b, Ylönen et al. 2005). Due to the high similarity of the human and mouse
genomes (Mouse Genome Sequencing Consortium et al. 2002) and the possibility
to specifically modify the mouse genes, mice are widely used as models to study
gene function and disease processes. These similarities with humans are reflected
in the mouse genome; where almost every gene in the human has a counterpart in
the mouse. Moreover, mice are small, easy to maintain in the laboratory and have
a short breeding cycle (about 2 months).

Table 2. Collagen XIII mouse models.

<table>
<thead>
<tr>
<th>Mouse model</th>
<th>Mutation</th>
<th>Outcome</th>
<th>Main phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col13a1NN</td>
<td>targeted</td>
<td>Lack transmembrane collagen XIII</td>
<td>progressive myopathy</td>
</tr>
<tr>
<td>Col13a1neo</td>
<td>transgenic</td>
<td>overexpress collagen XIII</td>
<td>massive bone overgrowth</td>
</tr>
<tr>
<td>Col13a1he</td>
<td>transgenic</td>
<td>overexpress shortened collagen XIII</td>
<td>embryonal lethality as homozygote</td>
</tr>
</tbody>
</table>

2.4.1 N-terminally altered collagen XIII mice (Col13a1NN)

Mice expressing N-terminally altered collagen XIII molecules, lacking the short
cytosolic and transmembrane domains (Col13a1NN), were generated by
homologous recombination. These mice were deficient in transmembrane form of
collagen XIII but retained the secreted molecules with altered N-terminus and
intact collagenous ectodomain. These mice were viable and fertile. In cultured
skin fibroblasts from Col13a1NN mice, the secreted collagen XIII was localized to
focal adhesions in the same way as normal collagen XIII in control mouse
fibroblasts. However, decreased adhesion of the Col13a1NN-derived embryonal
fibroblasts was detected on uncoated wells and even more dramatically on wells
coated with collagen IV. In skeletal muscle of the mice, the N-terminally altered
collagen XIII chains were expressed at comparable levels to intact molecules, but
there were disturbances in the muscle integrity. Ultrastructural characterization showed that the BM of the skeletal muscle was infirm and fuzzy. Also, vacuolization and disorganization of myofilaments and z-bands were observed in the mutant mice. Collagen XIII was located in close contact with the plasma membrane in the skeletal muscle of wild-type mice when studied by immunoelectron microscopy. In contrast, N-terminally mutated collagen XIII molecules were located mostly in the extracellular space. Moreover, the mutant muscles contained areas where the BM was detached from the sarcolemma. Col13a1^{N/N} mice subjected to forced running were more sensitive to exercise-induced muscle damage than control mice. According to these findings, collagen XIII participates in the linkage between muscle fibers and the BM. (Kvist et al. 2001.)

2.4.2 Mice overexpressing collagen XIII (Col13a1^{oe})

Transgenic mice overexpressing collagen XIII (Col13a1^{oe}) have a striking phenotype in the bones. In these mice the collagen XIII expression level is similar to control mice, except that in skin, cartilage and skeletal tissues elevated collagen XIII expression is detected, the highest level of expression being in skeletal tissues. At birth no phenotypical differences between the transgenic mice and their wild-type littermates could be detected. However, signs of enhanced bone formation were observed at the age of 3–4 weeks onwards, and by the age of 2 months, severe skeletal abnormalities were found. Histological analysis of the tibias and the femurs showed a massive increase in cortical bone. Endogenous collagen XIII is expressed in periosteal and endosteal osteoblasts. In the age-matched transgenic mice a thickening of periosteum was detected, with several layers of osteoblasts when only one cell layer exists in the controls. Bone formation and mineralization occurred at a rate several times higher in the overexpression mice than in their wild-type littermates. These findings suggest that collagen XIII has an important role in bone modeling, and it may have a function in coupling the regulation of bone mass to mechanical use. (Ylönen et al. 2005.)

2.4.3 Mice overexpressing truncated collagen XIII (Col13a1^{del})

The Col13a1^{del} mice express endogenous collagen XIII as well as mutant α1(XIII) chains which have an 83-amino acid deletion in their second collagenous
domain (COL2). They also lack the terminal 36 amino acids including the whole NC4 domain. The expression pattern of the transgene closely resembled that of the endogenous collagen XIII gene, however with lower levels in most tissues studied. Since heterozygous \textit{Col13a1} \textit{del} mice did not show any overt phenotype, heterozygous matings were performed. A percentage of embryos were spontaneously aborted between embryonal days of development (E) 10.5 and 13.5. In the fetuses of early lethal phenotype, fusion of the chorionic and amniotic membranes failed preventing the formation of functional placenta. Histological analysis showed the absence of fetal portion of the placenta, and no fetal blood cells could be detected. (Sund \textit{et al.} 2001b.)

The late phenotype fetuses, aborted by E13.5, were pale and had an irregular and weak heartbeat, suggesting a cardiovascular defect. The endocardium, myocardium and epicardium were developed, but the myocardial layer appeared thinner than in the control fetuses, and the trabeculation of the ventricles was reduced. Adherens junctions of the myocardium were less electron dense with no adhesion of myofilaments to these structures in mutant embryos. The adherence junction marker cadherin displayed a disorganized staining pattern and reduced signal intensities in mutant heart. Nonetheless, desmosomes had developed normally in the mutant heart. (Sund \textit{et al.} 2001b.)

In the late phenotype fetuses vascular defects were also detected in the cranial region, more specifically in the developing central nervous system and the ganglia, whereas the development of the microvessels in other parts, such as the cephalic mesenchyme and the upper limb of the same fetuses, was normal. Collagen XIII was not expressed in the capillary endothelia either in mutants or controls, while the surrounding tissue stained strongly for collagen XIII. A defect in placental blood vessel formation was observed in the mutant embryos, when the vessels showed less spreading in the labyrinth layer of mutant placentas than in the control placentas. The observed defect in angiogenesis might be due to altered binding or signal transduction between the endothelial cells and the surrounding matrix. Studies of \textit{Col13a1} \textit{del} embryos confirmed the role of collagen XIII in adhesive interactions and also in embryonal development. (Sund \textit{et al.} 2001b.)

Tahkola \textit{et al.} (2008) further analyzed the cardiovascular disorders of \textit{Col13a1} \textit{del} embryos of heterozygous matings at E12.5. Doppler ultrasonography revealed atrioventricular valve regurgitation (AVVR) in 9 transgene-positive fetuses. Altogether 33 fetuses were analyzed, 23 of which were transgene-positive and 10 transgene-negative (wild-type) The fetuses with AVVR had a lower heart
rate and outflow than those without AVVR, and also a greater isovolumetric relaxation time in the cardiac cycle. Ductus venosus pulsatility indices for veins and the umbilical artery pulsatility indices were increased. Histological analysis showed reduced trabeculation of the ventricles and thinned myocardium in the fetuses with AVVR. Therefore, in mid-gestation Col13a1del transgene expression results in cardiac dysfunction in mouse fetuses.

2.5 Defense collagens

Defense collagens contain a collagen-like sequence continuous with noncollagenous sequences, which recognize microbe-specific structures called pathogen-associated molecular patterns (PAMPs) shared by broad classes of microorganisms (Tenner 1999). The interaction of the host’s innate immune system with PAMPs initiates the host protective response. Defense collagens include membrane-bound class A macrophage scavenger receptors MSR1, MARCO and SRCL, and soluble members, which are a component of the complement system C1q and the collectin family of proteins (Figures 1 and 2). (Bohlson et al. 2007.)

The collectin family of collagen-like region containing mammalian lectins (carbohydrate-recognizing proteins) includes eight members defined so far, of which mannose-binding lectins (MBL), surfactant protein A (SP-A) and surfactant protein D (SP-D) are the best characterized. In the case of the ficolins members of the collectin family, the lectin domain responsible for microorganism recognition is a fibrinogen-like domain. (Tenner 1999.) Collectins are the innate immune system molecules present in plasma and on mucosal surfaces. All collectins share a domain structure consisting of four regions; a cysteine-rich N-terminal domain, a collagen-like region, an α-helical neck domain and a C-terminal carbohydrate recognition domain. Collectins represent the first line of host defense. (Gupta & Surolia 2007.) Upon recognition of the infectious agents, collectins put into action effector mechanisms like direct opsonization (van Iwaarden et al. 1992), neutralization (Wakamiya et al. 1992), agglutination (Kuan et al. 1992), complement activation (Kawasaki et al. 1989) and phagocytosis (Pikaa et al. 1995) to curb microbial growth. In addition, they also modulate inflammatory and allergic responses and apoptotic cell clearance (Stuart et al. 2005). These functions limit infection and subsequently modulate the adaptive immune responses (Gupta & Surolia 2007).
Adiponectin (adipocyte complement related protein of 30 kDa) has a similar structure as the other soluble defense collagens (Suzuki et al. 2007). In addition to its role in glucose and lipid metabolism, it has also been reported to have antisuppressive properties due to its ability to induce the production of anti-inflammatory cytokines and inhibit the production of proinflammatory cytokines (Wolf et al. 2004, Wulster-Radcliffe et al. 2004).

Fig. 2. Schematic illustration of defence collagens. Collagenous domains are denoted by black lines and globular domains by grey circles. Modified from Bohlson et al. 2007, reprinted with permission of Elsevier.

2.5.1 Function of the collagenous domain

The collagenous domain of the defense collagens is responsible for trimerization of the polypeptide chains, but it also has other functions such as linking the invading organism to the complement or phagocyte effector mechanisms of the immune system (Bohlson et al. 2007). In MBL and SP-A the collagenous domain is involved in recognition of the C1q receptor (Geertsma et al. 1994, Arora et al. 2001). MBL activates the complement pathway via MBL-associated serine proteases (MASPs) involving their collagen-like domain (Thiel et al. 1997). The large collagen-like segment in SP-D functions as a platform to crosslink carbohydrates on microbial surfaces to enhance their aggregation, uptake and neutralization (Hartshorn et al. 2002). SRCL has a positively charged collagen-like domain, which binds microbes and oxidized LDL particles in the vascular space (Ohtani et al. 2001).
2.5.2 Complement activation by C1q, MBL and ficolins

The C1q molecule initiates the classical pathway of the complement cascade by binding to the IgG of immune complexes (Loos 1983, Duncan & Winter 1988). The complement system is one of the major means by which the body recognizes foreign antigens and pathogens. Activation of the complement system promotes the opsonization of pathogens, chemotaxis and activation of leukocytes, and direct killing of pathogens. Moreover, the complement system enhances and directs the adaptive immune response and also functions to dispose of apoptotic cells. (Fujita et al. 2004.)

The C1q molecule is composed of three different polypeptide chains, A, B and C. Six copies of each chain are found per C1q molecule (Reid & Porter 1976, Kishore & Reid 2000). Overall, the molecule looks like six tulips, each tulip consisting of an A, B and C chain (Figure 2) (Tenner 1999). The stalk of each tulip consists of a collagenous sequence of ~81 aa, in which the A, B, and C chains form a heterotrimeric collagenous triple helix (Reid 1976). Kaul and Loos have shown the C1q molecule to have, in addition to the serum form, a membrane-bound form (mC1q) on tissue macrophages in a type II orientation (Kaul & Loos 1993, Kaul & Loos 1995).

In addition to the classical IgG-dependent C1q utilizing pathway, the complement system can be activated by alternative or lectin pathways (Endo et al. 2006). The alternative pathway does not involve specific recognition molecules, whereas the lectin pathway utilizes MBL and ficolins to bind PAMPs on the surface of microbes. These carbohydrate-binding proteins contain a collagenous region attached to the lectin domain. However, they contain a different lectin domain: a carbohydrate recognition domain (CRD) in the case of MBL and a fibrinogen-like domain in ficolin. (Ikeda et al. 1987, Matsushita et al. 2000, Endo et al. 2006.) The structure of MBL and ficolins, as well as adiponectin, is very similar to that of C1q (Figure 2) (Holmskov et al. 2003, Suzuki et al. 2007).

2.6 Basement membranes

The basement membrane (BM), a specialized extracellular matrix in contact with cells, is a dense, sheet-like structure 50–100 nm in thickness. BMs provide structural support, divide tissues into compartments, regulate cell behavior and also serve as a semipermeable selective barrier in mammalian kidneys. BMs are
usually found basolateral to the epithelium and the endothelium, and around peripheral nerve axons, fat cells and muscle cells.

At least 50 proteins are now known to be associated with BMs. The main components of BMs are collagen IV, laminin, perlecan and nidogen/entactin. Collagen IV and laminin self-assemble into networks which are interconnected by nidogen/entactin. Among others, agrin, SPARC/BM-40/osteopontin, fibulins, and collagen types XV and XVIII are additional components of the BMs.

Variation of the relative amounts of BM components, as well as the subtypes involved, permit tissue specific properties of BMs. BM components, several of which are proteoglycans, also have isoforms that are differentially expressed in different organs. Laminin heterotrimers are encoded by 11 genes (α1-5, β1-3, γ1-3), and at least 15 possible combinations of these gene products exist. Collagen IV is encoded by six distinct α-chains. Collagen IV and laminin heterogeneity creates the major molecular basis for tissue-specific BM compositions. These isoforms differ in their cell-adhesion properties, proteolytic susceptibility and ability to interact with other BM constituents. Other mechanisms contributing to BM composition include variations in splice variants, tissue-specific gene regulation and posttranslational modifications. Alternative splicing occurs for collagen IV, laminin, agrin, fibulin and fibronectin. Proteolytic cleavage of laminins is an example of posttranslational modification, as well as glycosaminoglycan (GAG) chain differences on perlecan. The GAG chains can differ in length and in sulfation. (Kalluri 2003, Aszodi et al. 2006, LeBleu et al. 2007.)

2.7 Immunity

2.7.1 Innate and adaptive immunity

Immunity is divided into innate and adaptive immunity. The elements of innate immunity – neutrophils, monocytes, macrophages, complement, cytokines, and acute phase proteins – provide the immediate host defense. The innate immune response is highly conserved as it is seen even in the simplest animals, which confirms its importance to survival. Higher animals utilize also the adaptive immunity where the reaction is antigen-specific through T lymphocytes and B lymphocytes. The innate immunity is rapid but sometimes nonspecific leading to damages in normal tissues. The pathogen-specific reaction in the adaptive
immune system takes several days or weeks to develop. However, the adaptive
system has a memory to antigens it has encountered, and the subsequent
exposure to these antigens lead to more vigorous and rapid response, yet more
slowly than the innate system. (Parkin & Cohen 2001.)

2.7.2 Intestinal immunity

The intestinal immune system is the largest and most complex part of the immune
system. It encounters more antigens than any other part of the body, and it also
discriminates clearly between invasive organisms and harmless antigens, such as
food proteins and commensal bacteria (Mowat 2003). The abundant and highly
active gut immune system is tightly regulated to prevent excessive immune
responses to food and gut bacteria (MacDonald 2003, Brandtzaeg & Pabst 2004).

The gut-associated lymphoid tissue (GALT) can be divided into effector sites
consisting of lymphocytes scattered throughout the epithelium and lamina propria
of the mucosa, and organized tissues responsible for the induction phase of the
immune response. These are the Peyer’s patches and mesenteric lymph nodes
(MLNs), as well as smaller, isolated lymphoid follicles, which have the
appearance of microscopic Peyer’s patches and are distributed throughout the
walls of the small and large intestines. (Hamada et al. 2002.) The MLNs are the
largest lymph nodes in the body. Their development is distinct from that of both
Peyer’s patches and peripheral lymph nodes (Debard et al. 1999, Cuff et al. 1999,
Alcamo et al. 2002, Scheu et al. 2002) and the accumulation of lymphocytes in
the MLNs requires both L-selectin and α4β7 integrin adhesion molecules, which
normally direct lymphocytes to enter peripheral and mucosal tissues (Wagner et
al. 1998). Hence, MLNs might be the crossroads between the peripheral and
mucosal recirculation pathways.

2.7.3 Intestinal epithelial cells in intestinal immunity

The single layer of gut epithelium is the primary cellular barrier that prevents
antigens from encountering the immune system. Nevertheless, the gut epithelial
barrier does not completely prevent luminal antigens from entering the tissues.
Thus, intact food proteins can be detected in plasma (Husby et al. 1985), and a
few gut bacteria can be detected in the MLNs draining the gut of healthy animals
(Berg 1995). Antigens can cross the epithelial surface through breaks in tight
junctions, perhaps at villus tips where epithelial cells are shed, or through the
follicle-associated epithelium (FAE) that overlies the organized lymphoid tissues of the intestinal wall (Neutra et al. 2001). FAE contains specialized epithelial cells termed M cells, whose function is to transport luminal antigens into the dome area of the follicle (Neutra et al. 2001). Antigen-presenting dendritic cells (DC) also send processes between gut epithelial cells without disturbing tight junction integrity and sample commensal and pathogenic gut bacteria (Rescigno et al. 2001, Niess et al. 2005). The gut epithelial barrier therefore represents a highly dynamic structure that limits, but does not exclude, antigens from entering the tissues, whereas the immune system constantly samples gut antigens through the FAE and DC processes. The commensal flora reciprocally affects the gut epithelium to maintain epithelial integrity. For example, recognition of TLR2 or TLR9 ligands by epithelial cells increases gut barrier function (Madsen et al. 2001, Cario et al. 2004).

The gut epithelium itself can also directly sense commensal bacteria and pathogens via pattern recognition receptors (PRRs), which recognize conserved structures of bacteria and viruses, and generally activate proinflammatory pathways alerting the host to infection (Philpott & Girardin 2004). Two different classes of PRRs are involved. The Toll-like receptors (TLRs) are usually associated with cell membranes and the nucleotide-binding oligomerization domain (Nod) molecules, Nod1 and Nod2 [also known as CARD4 and CARD15 (caspase activation and recruitment domain)], are present in the cytosol of epithelial cells and immune cells. Signaling through Nod or TLR activates transcription factor NF-κB, leading to proinflammatory gene expression. (Takeda & Akira 2004, Inohara & Nunez 2003.)

Paneth cells are secretory epithelial cells that reside at the base of the small intestinal crypts, and these cells express a collection of antimicrobial substances, stored in large cytoplasmic granules and released into the crypt lumen (Porter et al. 2002). In mammals, the predominant group of antimicrobial peptides are defensins (Lehrer et al. 2004). Mature defensin peptides are active as antibiotics at micromolar concentrations, and their mechanism of action is a consequence of their ability to disrupt the structure and function of microbial membranes (Zasloff 2002). Reduced Paneth cell defensin expression has been linked with susceptibility to the chronic inflammation in ileal Crohn’s disease (Wehkamp et al. 2005, Wehkamp et al. 2004).

The defensins can be divided into three subgroups, designated α-, β- and θ-defensins (Lehrer et al. 2004), based on structural features of the gene, and precursor and mature peptides. Defensins expressed in the small intestine are
α-defensins (Ouellette 2004), which are mainly provided by Paneth cells (Porter et al. 2002, Bevins 2004). In addition to α-defensins, Paneth cells also produce other antimicrobial proteins including lysozyme and secretory phospholipase A2 (Ouellette & Bevins 2001). Mice express six or more α-defensins (also termed cryptdins, for ‘crypt defensins’) (Selsted et al. 1992) and numerous cryptdin-related peptides (Hornef et al. 2004) depending on the particular mouse strain (Ouellette & Bevins 2001).

Paneth cells secrete cytoplasmic secretory granules into the crypt lumen in response to bacterial products, such as the muramyl dipeptide, a component of bacterial peptidoglycan (Qu et al. 1996, Ayabe et al. 2000), but not in response to fungal or protozoal stimuli (Ayabe et al. 2000). Cholinergic agonists can also stimulate secretion by a mechanism that appears to involve both increased cytosolic Ca$^{2+}$ (Satoh et al. 1995) and Ca$^{2+}$ activated K$^+$ channels (Ayabe et al. 2002).

Small intestinal α-defensins, like all defensins, are initially synthesized as larger precursor propeptides. An important step in the expression of active Paneth cell α-defensins is proteolytic processing, which liberates the active peptide from its precursor molecule. Interestingly, the pathways of processing are quite different in mice and humans (Ouellette & Bevins 2001). In mice, matrilysin [MMP-7 (matrix metalloproteinase-7)] activity is essential for production of active cryptdin peptides (Wilson et al. 1999). MMP-7 is a metalloproteinase expressed at high levels by murine Paneth cells. In vitro, MMP-7 processes procryptdins to active mature α-defensins. In contrast, human Paneth cells do not express MMP-7 and the orthologous human prodefensins are processed by the serine proteinase trypsin (Ghosh et al. 2002).

### 2.7.4 Regulation of the immune response by the LAIR-1 receptor implicates cell-matrix interactions

The immune response needs to be tightly controlled by the opposing actions of activating and inhibitory signals to avoid excessive inflammation and the development of autoimmunity due to hyperreactivity to antigenic challenges. Multiple mechanisms exist to dampen the immune response: programmed cell death, anergy, regulatory T cells, and engagement of inhibitory receptors on the surface of immune cells (Van Parijs & Abbas 1998, Ravetch & Lanier 2000, Shevach et al. 2001). In tissues, immune cells are potentially exposed to multiple activating signals and inhibitory receptors are required to set a threshold for cell
activation and thus prevent unwanted immune reactions (Ravetch & Lanier 2000, Lebbink et al. 2006).

Lebbink et al. (2006) showed that various collagens, including collagen XIII, are high affinity ligands for the broadly expressed inhibitory leukocyte-associated immunoglobulin-like receptor-1 (LAIR-1). LAIR-1 is a 32-kDa transmembrane glycoprotein expressed on almost all human peripheral blood mononuclear cells, including natural killer (NK) cells, T cells, B cells, monocytes and dendritic cells (Meyaard et al. 1997). The interaction between collagens and the LAIR-1 receptor is dependent on the conserved Gly-X-Y collagen sequence, where X is frequently proline (P) and Y hydroxyproline. The corresponding nonhydroxylated sequence Gly-P-P does not bind to LAIR-1. Binding of collagens to the LAIR-1 receptor can directly downregulate immune responses. All earlier documented ligands for immune inhibitory receptors are membrane molecules, implying cell-cell interactions in the regulatory role of immune cell function. Inhibitory immune receptors binding to extracellular matrix collagens reveal a novel mechanism of peripheral immune regulation by cell-matrix interaction. (Lebbink et al. 2006.)

Under physiological conditions, immune cells present in blood are not exposed to immobilized collagens, although they encounter full-length collagen XVIII, which exists as a soluble plasma protein as well as an immobilized ECM protein (Farndale et al. 2004, Musso et al. 2001). In the extravasation, leukocytes move through subendothelial structures, such as the BM and connective tissue, which are rich with immobilized collagens. This may increase the threshold for activation needed to keep these potentially dangerous cells in an inactive state. In the inflammatory locus, the presence of specific and strong activating stimuli introduced by antigen-presenting cells, cytokines or pathogens will override the threshold and allow cells to become activated and mediate their function. Regulation of collagen-LAIR-1 interaction can also occur by modulating LAIR-1 expression at different stages of differentiation or activation of immune cells, as demonstrated for B cells (van der Vuurst de Vries, A.R. et al. 1999), T cells (Maasho et al. 2005) and neutrophils (Verbrugge et al. 2006).

The identification of collagens as ligands for LAIR-1 brought up an interesting scenario for the regulation of the immune system in tumors. Tumor cells and/or tumor-associated stroma cells are known to upregulate the expression of ECM collagens and transmembrane collagens (Bode et al. 2000, Fischer et al. 2001, Parikka et al. 2003, Väisänen et al. 2005). This can lead to downregulation of the immune response directed against tumors.
2.7.5 Microbial infection, inflammation and cancer

Clinical and epidemiologic studies have revealed links between pathogens and chronic inflammatory disorders, and cancer. *Helicobacter pylori* infection can produce chronic gastritis and peptic ulcers, and appears to be a predisposing factor in 60%–90% of gastric carcinomas. However, most individuals with *Helicobacter pylori* infection develop no clinical symptoms. Humans may also be lifelong carriers for the genetically unrelated hepatitis B and C viruses without any symptoms, but their infection can also result in chronic hepatitis and liver cirrhosis. Ultimately, hepatitis B and C infections are collectively believed to trigger at least 50–70% of cases of hepatocellular carcinomas. Endemic Burkitt’s lymphoma in Central Africa and nasopharyngeal carcinoma in Southeast Asia are linked to Epstein-Barr (EBV) virus infection. However, a vast majority of EBV-carrying individuals have a lifelong asymptomatic infection or simple mononucleosis. The flatworm *Schistosoma haematobium* exists in endemic foci within Africa. It infects the urinary tract, and yet a subset of patients develops a chronic granulomatous inflammatory response to parasite eggs and consequently is prone to development of bladder carcinoma. In these cases it is the host response in chronic infection rather than the toxins or onco- gens produced by the pathogen which causes the pathology. (Karin et al. 2006.) Thus, inflammatory cells and the innate immune system are important mediators of tumor promotion and progression (Karin & Greten 2005). Leukocytes, including normal tissue macrophages, tumor-associated macrophages (TAMs), dendritic cells (DCs), neutrophils, mast cells and T cells, are recruited to the tumor microenvironment through interactions with local stromal cells and malignant cells. In the tumor microenvironment, leukocytes produce proteins that allow tumor cells to proliferate, invade and metastasize. These proteins include cytokines, growth and angiogenic factors as well as matrix-degrading proteases (such as the matrix metalloproteinases MMP1, MMP3 and MMP9) and their inhibitors. (Balkwill & Mantovani 2001, Coussens & Werb 2002.)

The contribution of the NF-κB activation pathway to acute inflammation and cell-survival mechanisms is well accepted, and sustained NF-κB activation in various malignancies has been described (Karin et al. 2002). Several proinflammatory cytokines and chemokines, such as TNF, interleukin-1 (IL-1), IL-6 and IL-8, are encoded by target genes of the classical NF-κB-activation pathway and they also are associated with tumor development and progression in humans and mice (Balkwill & Mantovani 2001). Furthermore, many oncogenes
43

and carcinogens cause activation of NF-κB, whereas chemicals with known chemopreventive properties can interfere with NF-κB activation (Bharti & Aggarwal 2002). Importantly, recent mouse studies provide strong and direct genetic evidence that the IKK-β-dependent (inhibitor of NF-κB kinase-β-dependent) NF-κB-activation pathway is a crucial mediator of tumor promotion. In these models of cholangitis (bile duct inflammation) and colitis-associated cancer (CAC), NF-κB promotes the survival of initiated or more advanced malignant cells when functioning in the cancer cells themselves. It also provides cells with growth and survival factors when it functions in the tumor stromal cells or tumor-associated inflammatory cells (Greten et al. 2004, Pikarsky et al. 2004.)

2.8 Collagens in autoimmune diseases

Several collagen types are implicated in human autoimmune diseases. Collagen XVII is an autoantigen in an acquired blistering disorder, bullous pemphigoid. Both full-length collagen XVII (Ghohestani et al. 1997, Zillikens et al. 1997a, Zillikens et al. 1997b, Haase et al. 1998) and the shed ectodomain (Schumann et al. 2000) are recognized by patient autoantibodies. Collagen II is an autoantigen in rheumatoid arthritis and systemic lupus erythematosus (Gioud et al. 1982) and collagen VII in epidermolysis bullosa acquisita (Woodley et al. 1984, Chen et al. 1997). Autoantibodies against the α3-chain of collagen IV, present in the glomerular BM of the kidney, have been detected in patients with Goodpasture syndrome (Butkowski et al. 1987, Kalluri et al. 1994). Collagen XIII antibodies are present in sera of Graves’ patients with active ophthalmopathy (De Bellis et al. 2005). This progressive eye disorder is characterized by immune-mediated inflammation of the extraocular muscles and orbital connective tissue. According to the findings of Lebbink et al. (2006), autoantibodies targeted to the various collagen molecules could interfere with the interaction of collagen with LAIR-1 and thereby contribute to the pathology of these autoimmune diseases.

2.9 Diffuse large B-cell lymphoma

Diffuse large B-cell lymphoma (DLBCL) is the most common lymphoid malignancy in adults. Approximately 50-55% of DLBCL patients can be cured with appropriate therapies (Abramson & Shipp 2005). DLBCLs are thought to arise from normal antigen-exposed B cells that have migrated to or through germinal centers (GCs) in secondary lymphoid organs (Kuppers et al. 1999).
NF-κB signaling supports survival of a subset of DLBCLs (Davis et al. 2001). The NF-κB signaling pathway regulates the survival of both normal and malignant B cells by controlling the expression of cell death regulatory genes (Karin et al. 2002, Karin & Lin 2002). Gene expression profiling has revealed that existing diagnostic categories of human lymphomas are comprised of multiple distinct diseases based on both clinical and molecular criteria. By whole genome arrays, Monti et al. (Monti et al. 2005) indicated that discrete subsets of DLBCLs, namely oxidative phosphorylation, B-cell receptor/proliferation and host response (HR) tumors, can be identified by their unique transcriptional profiles and associated clinical and genetic features. HR tumors have a brisk host immune/ inflammatory response and increased expression of T/NK cell receptor and activation pathway components, complement cascade members, and macrophage/dendritic cell markers. Consistent with this signature, primary HR DLBCLs contain significantly higher numbers of morphologically distinct tumor-infiltrating lymphocytes and interdigitating dendritic cells. In spite of the brisk inflammatory infiltrates in HR tumors, patients with these DLBCLs do not have a more favorable outcome.

Dave et al. (2004) showed that in the case of follicular lymphoma, the length of survival following diagnosis can be predicted by gene expression profiling at the time of diagnosis. In this method, a survival model in follicular lymphoma was created using two survival-associated signatures, one from the good prognosis gene set and one from the bad prognosis gene set. These signatures reflected gene expression by nonmalignant tumor-infiltrating immune cells. Therefore, in the case of follicular lymphoma an interplay between the malignant cells and the host immune system influences the clinical behavior of follicular lymphoma.
3 Outlines of the present study

When this study was started, the function of collagen XIII was unknown and a transgenic mouse line was chosen to illuminate its function. The strategy of overexpression of mutant collagen XIII in transgenic mice was used to provide information about the function of the corresponding endogenous collagen.

As a model organism to understand human biology, the mouse provides several advantages. One is that nearly every gene in the human genome has a counterpart in the mouse. Mice are small, easy to maintain in the laboratory and they have a short breeding cycle (about 2 months). Importantly, techniques for generating genetic modifications are well established.

The following goals were set for this doctoral project;

1. Chain association, trimer formation and stabilization are not well understood in the family of collagenous transmembrane proteins. Here, collagen XIII was used as a prototype to unravel these issues. To study the biosynthesis of collagen XIII by analyzing the capacity of the different cysteines along its molecule to form interchain disulfide bonds, and to study the significance of the NC3 coiled-coil domain for the biosynthesis of stable type XIII collagen molecules.

2. To characterize the phenotypic consequences of expression of mutant collagen XIII in heterozygous Col13a1del mice.

3. To analyze collagen XIII tissue distribution at the protein level and its localization in selected tissues.

4. To further address the effect of the Col13a1del transgene in mice without coexpression of the endogenous collagen XIII, the Col13a1del transgenic mouse line was crossed with the collagen XIII knockout line Col13a1− to analyze the mutant mice.
4 Materials and methods

A detailed description of the materials and methods used in the studies can be found in the original articles.

4.1 Construction of recombinant baculoviruses (I)

The pVLdel1-38 construct (del1-38 cloned into pVL1392 (Invitrogen)) (Snellman et al. 2000a) was used as a template for generating human variants Cys-1S2S, Cys-3S4S, Cys-5S6S, E430P and A431P. A QuikChange™ site-directed mutagenesis kit (Stratagene) was used to convert cysteines 45 and 53, 117 and 119, and 213 and 221 in pairs to serines and variants Cys-3S and Cys-4S were produced by separately mutating the corresponding cysteines to serines. In E430P and A431P variants the glutamic acid 430 and alanine 431, respectively, were mutated to prolines. The sequences were verified on an automated DNA sequencer (ABI Prism 377; Applied Biosystems). The constructs together with modified Autographa californica nuclear polyhedrosis virus DNA (BaculoGold, Pharmingen) were co-transfected into Spodoptera fugiperda Sf9 insect cells by calcium phosphate transfection (Gruenwald & Heitz 1993). The amplification of the viruses was performed twice and amplified viruses were used for recombinant protein production.

To obtain a mouse COL2del construct for recombinant expression in insect cells, the KpnI-XbaI fragment was removed from the mouse collagen XIII cDNA moXIII (670) (Peltonen et al. 1997) and replaced with a comparable sequence from the clone 3VPL6 (Sund et al. 2001b). This sequence contains a deletion in the COL2 domain and a point mutation in the COL3 domain, leading to a lack of the extreme 38 C-terminal amino acids. The 5' sequence originates from the cDNA moXIII (670). The resulting COL2 del construct was digested with EcoRI and ligated into pVL1392. The COL2 del variant was sequenced and co-transfected as above for the human constructs. The recombinant virus was then amplified twice, plaque-purified and amplified three times. The previously described recombinant baculovirus coding for both subunits of human prolyl 4-hydroxylase (4PHαβ) was also used (Nokelainen et al. 1998).
4.2 Analysis of recombinant proteins by Western blotting (I)

Sf9 or High Five insect cells were infected at a density of 6×10^5 cells/ml with viruses coding for the different collagen XIII variants and with the virus 4PHαβ. In virus infections, the COL2del virus was used at a multiplicity of infection (m.o.i.) of 1, the control del1-38 virus at m.o.i. 5, whereas the 4PHαβ virus was used at m.o.i. 1. Ascorbate at 80 μg/ml (Wako Pure Chemical Industries Ltd.) was added daily to the culture medium. After 47–48h of infection, the cells were washed with phosphate-buffered saline (PBS), homogenized in 67 mM Tris-HCl, pH 7.5, 267 mM NaCl, 0.2% Triton X-100 supplemented with Complete Protease Inhibitor Cocktail (Boehringer Mannheim), and centrifuged at 8000 g for 10 min. The supernatants were separated from the pellets, which were suspended in 1% SDS. The medium samples were also supplemented with Complete Protease Inhibitor Cocktail. Samples of the different fractions were analyzed by Western blotting with a polyclonal antibody against a bacterial fragment corresponding to the NC1 domain of mouse collagen XIII, excluding the transmembrane domain [antibody XIII/NC1-Q610, (Hägg et al. 2001)], part of the NC2 domain of human collagen XIII [antibody XIII/NC2-55, (Snellman et al. 2000a)], the whole NC3 domain of human collagen XIII [antibody XIII/NC3-1, (Hägg et al. 1998)], or the C-terminal end of the COL3 domain and the whole NC4 domain of human collagen XIII [antibody XIII/NC4-SO, (Snellman et al. 2000a)] and detected with enhanced chemiluminescence.

4.3 Amino acid sequence analysis and secondary structure prediction (I)

The amino acids essential for the coiled-coil structure in the NC3 domain of collagen XIII were determined by changing individual residues to proline and running the COILS program [version 2.1, (Lupas et al. 1991)]. The COILS program was also used to predict coiled-coil regions for collagen XIII variants and other proteins. Furin-type cleavage site predictions were obtained using the ProP 1.0 Server (Duckert et al. 2004). Predictions of transmembrane helices were obtained using the TMHMM Server v. 2.0 (Sonnhammer et al. 1998). Sequence alignment was performed using the Clustal W method (Thompson et al. 1994) and compiled into a figure using BOXSHADE.
4.4 Generation of Col13a1\(^{del}\) and Col13a1\(^{del}\);Col13a1\(^{−/−}\) transgenic mice, their health monitoring and pregnancy terminations (II, III)

Col13a1\(^{del}\) and Col13a1\(^{−/−}\) mice were prepared and genotyped as previously described (Sund et al. 2001b, Latvanlehto 2004). In order to obtain Col13a1\(^{−/−}\);Col13a1\(^{del}\) mice, we crossed the Col13a1\(^{del}\) transgenic mouse line with the collagen XIII knockout line (Col13a1\(^{−/−}\)). The health monitoring in the specific pathogen-free (SPF) barrier unit was performed according to FELASA recommendations (Nicklas et al. 2002). In the conventional animal facility, bred mice were tested for all viruses recommended by FELASA and for several bacteria known to commonly afflict mouse colonies. Mice reared in the conventional unit were positive for the mouse hepatitis virus, the Theiler’s murine encephalomyelitis virus, Helicobacter pylori and Pasteurella pneumotropica, while mice in the SPF were negative for these and other microbes specified by FELASA. The Animal Care and Use Committee of the University of Oulu approved all the animal experiments performed.

Heterozygotes for the endogenous collagen XIII gene; Col13a1\(^{del}\) transgene bearing mice were mated with collagen XIII-null mice and the appearance of a vaginal plug in the morning was designated as day 0.5 of gestation. Fetuses were dissected from the uterus at E9.5 and E10.5.

Col13a1\(^{−/−}\);Col13a1\(^{del}\) mice and fetuses were genotyped for both the Col13a1\(^{del}\) transgene and the endogenous collagen XIII gene as described previously (Sund et al. 2001b, Latvanlehto 2004).

4.5 Tissue preparation, histological and immunofluorescence analysis (II, III)

At the age of 18 months, heterozygous mice and wild-type littermates were necropsied for the occurrence of tumors. Tissue samples were either used fresh or rapidly frozen in liquid nitrogen and stored at −70ºC until used. For histological and immunohistochemical analysis, tissues were embedded in Tissue Tec cryoprotectant (Sakura Finetek) and frozen in isopentane cooled with liquid nitrogen or on dry ice. Alternatively they were fixed in 10% PBS and embedded in paraffin. For general histology, 5 μm-thick paraffin or frozen sections were stained with hematoxylin-eosin according to standard procedures. Antibodies against CD31/PECAM (Pharmingen), CD45 (Pharmingen), CD68 (Serotec),
collagen IV (Chemicon), desmin (Sigma), perlecan (Pharmingen) and collagen XIII (Hägg et al. 1998) were used.

4.6 Flow cytometry (FACS) (II)

Tumor tissues were homogenized in a Medimachine homogenizer (DAKO) and the cells were counted and analyzed on a FACSCalibur flowcytometer (Becton Dickinson) using CELL Quest software (Becton Dickinson). Forward and side-scatter gating were used to exclude dead cells from the analysis. Cells were stained using standard protocols with FITC or PE-conjugated monoclonal antibodies (Becton Dickinson) against CD3, CD19 and isotype-matched negative controls.

For the activation marker analysis, CD4+ and CD8+ T cells were isolated using MACS separation columns (Miltenyi Biotec) and the cells were stained for BIOTIN, TC or PE-conjugated naïve markers L-selectin and CD45RB and activation markers CD69 and IL2R.

4.7 Molecular analysis (II)

Total RNA was isolated from tumors and used as a template for reverse transcription (RT), followed by PCR as previously described (Sund et al. 2001b). The DNA isolated from the mesenteric lymphomas was digested with EcoRI or HpaI and hybridized with the Ig probe for IgH rearrangements, or digested with restriction endonuclease BamHI or HhaI for T cell receptor β chain (TCRβ) rearrangements. The probes were provided by Dr. Terence Rabbits of the MRC Laboratory of Molecular Biology, Cambridge.

4.8 Electron microscopy (II)

The small intestine samples of control and transgenic mice were fixed in a mixture of 1% glutaraldehyde and 4% formaldehyde in 0.1 M phosphate buffer, postfixed in 1% osmium tetroxide, dehydrated in acetone and embedded in Epon EMBed 812.

A Reichert Ultracut ultramicrotome was used to cut thin sections, which were examined in a Philips CM100 transmission electron microscope (FEI, Eindhoven, Netherlands). Images were captured with a CCD camera equipped with TCL-EM-
4.9 Microarray analysis (II)

Total RNA was isolated using the RNeasy Kit (Qiagen). RNA (5 μg) was used as a template for synthesizing the cDNA and making biotinylated cRNA, according to the manufacturer’s instructions (Affymetrix). The GeneChip Mouse Expression Set 430_2.0 Array representing approximately 45,000 mouse transcripts was used to hybridize the biotinylated cRNA and a GeneChip Scanner 3000 was used to scan the arrays. The resulting expression data were analyzed with the DNA-Chip analyzer (dChip) (Li & Wong 2001) and the Affymetrix GeneChip Operating System. The intensities of the signals for all probe sets were scaled to a target value of 500. The genes in which altered expression was observed in three out of the four small intestines of the transgenic mice compared to two small intestines of their control littermates were listed.

4.10 Western blotting of tissues (III)

Tissue proteins were extracted in QIAzol lysis reagent (Qiagen). The concentration of the isolated protein was determined using a BCA Protein Assay kit (Pierce). An equal amount of protein was analyzed by Western blotting with an anti-collagen XIII antibody (Hägg et al. 1998).
5 Results

5.1 The two cysteines at the end of the first noncollagenous domain (NC1) are responsible for linking the three α1(XIII) chains together by interchain disulfide bonds (I)

Four pairs of cysteine residues are found in human α1(XIII) chains, located in the NC1, COL1/NC2 and NC4 domains (Figure 3). Earlier studies suggested that some of the cysteines in the NC1 domain of human collagen XIII, and possibly those at the junction of the COL1 and NC2 domains, form interchain disulfide bonds, whereas the two cysteine residues in the NC4 domain are likely to form intrachain bonds (Snellman et al. 2000a). In order to identify the cysteine residues forming interchain disulfide bonds, cysteines in the NC1 and COL1/NC2 domains were mutated to serines. In addition, the role of cysteines in the NC4 domain was tested by using a COL2del variant lacking the most C-terminal part of the molecule. The COL2del α1(XIII) chains also lack 83 amino acid residues of the COL2 domain.

![Fig. 3. Schematic structure of the human type XIII collagen α-chain. The noncollagenous domains (NC1-NC4) are shown as black boxes; the transmembrane domain as a gray box; the cleavage site as R4. Cysteine residues (C) are numbered from the N- to C-terminal end.](image)

The human del1-38 variant lacking the intracellular domain of collagen XIII was used as a control, since previous work showed that it forms disulfide-bonded trimers with three triple-helical domains. Also its expression level is far higher than that of the full-length mouse and human α1(XIII) chains (Snellman et al. 2000a). When cysteine-mutated variants were analyzed, disulfide-bonded trimers were formed on Cys-1S2S and Cys-5S6S chains according to the Western blotting of non-reduced samples, while the absence of cysteines 3 and 4 (Cys-3S4S) abolished the formation of disulfide-bonded trimers as well as dimers. In the case of single mutated cysteines, the Cys-3S and Cys-4S variants were able to form disulfide-bonded dimers, but the formation of disulfide-bonded trimers was
diminished, indicating that both of these cysteine residues are needed for the formation of a disulfide-bonded collagen XIII trimer. The COL2del variant assembled into trimeric disulfide-bonded molecules, confirming our previous suggestion that these two cysteines in the NC4 domain form intrachain bonds. A deletion in the interior of the COL2 domain did not affect its capacity to form a triple helix. These results demonstrate that the two cysteines at the end of the first NC domain form interchain disulfide bonds and therefore are responsible for linking the three α1(XIII) chains.

5.2 The NC3 coiled-coil domain is important for correct folding and stability of collagen XIII in vitro (I)

Previous computer analyses predicted that collagen XIII contains potential coiled-coil domains in its NC1 and NC3 domains (Latvanlehto et al. 2003). The coiled-coil structure can be disrupted by proline residues which introduce a kink into the helix (Barlow & Thornton 1988, Chang et al. 1999). Therefore, each residue in the NC3 29-residue coiled-coil domain of del1-38 was changed to a proline and the predicted effect on coiled-coil structure was analyzed using the COILS program, assessing the role of the NC3 coiled-coil domain in the stability of collagen XIII. Residues E430 and A431 were predicted to be important for the coiled-coil structure, as replacement of E430 with proline reduced the probability of coiled-coil formation from nearly 100% to less than 30%, and replacement of A431 dropped the predicted coiled-coil formation to nearly a zero probability.

Based on these in silico predictions, the corresponding residues in the del1-38 variant were mutated to prolines using site-directed mutagenesis to confirm the relevance of the NC3 coiled-coil domain. Recombinant viruses E430P and A431P were produced and used together with 4PHαβ to infect insect cells. The del1-38 virus was used as a control. Expressed proteins analyzed by Western blotting under non-reducing conditions showed del1-38 made disulfide-bonded trimers, but the E430P and A431P products were monomers unable to form disulfide-bonded trimers. Under reducing conditions, the proline-mutant proteins were labile. Furthermore, the A431P protein was more sensitive to degradation than the E430P protein, this being in agreement with its coiled-coil probability of ~ 0% versus ~ 30%. When various collagen XIII domain-specific antibodies were employed to detect degradation the COL1 and COL2 domains were found to be intact, whereas the COL3 domain was degraded as assessed by the absence of antibody reactivity.
5.3 *In silico* analysis of the novel collagenous transmembrane proteins (I)

The COILS program and the ProP 1.0 Server were used to analyze potential coiled-coils and furin cleavage sites of new members of the transmembrane subfamily – namely the colmedins *R. norvegicus* gliomedin, *D. melanogaster* CG6867, and *C. elegans* cof-2 and unc-122 (Franzke et al. 2005). All these molecules were found to contain at least one coiled-coil domain and one or two potential furin cleavage sites.

We also analyzed the recently discovered colmedin proteins *H. sapiens* BAD18742, *M. musculus* CRG-L2, *G. gallus* XP_425097, *T. nigroviridis* CAF99838 and CAG05536 and *A. gambiae* XP_315876 (Zeng et al. 2005) for potential transmembrane domains, coiled-coils and furin cleavage sites using the TMHMM Server v. 2.0, the COILS program and the ProP 1.0 Server, respectively. The C-terminal end of the *H. sapiens* BAD18742 protein was shown to be identical to *H. sapiens* collomin with further 5’ sequence. *H. sapiens* collomin, *M. musculus* CRG-L2 and *T. nigroviridis* CAF99838 and CAG05536 were predicted to be type II transmembrane proteins. All of these proteins were shown to potentially contain multiple coiled-coil domains, one of which was at the beginning of the transmembrane domains. These proteins were predicted to have one furin cleavage site located between the transmembrane and the (first) collagenous domain. The colmedins *G. gallus* XP_425097 and *A. gambiae* XP_315876 are not type II transmembrane proteins, since one was predicted to contain two transmembrane domains, and the other no transmembrane domain.

In a search for related sequences, two more proteins, *P. troglodytes* XP_510405 and *D. rerio* XP_700764, were found to contain three or two collagenous domains, respectively, and one OLF domain. These two type II transmembrane proteins also possessed potential coiled-coil domains beginning at the transmembrane domain, followed by a potential furin cleavage site before the first collagenous domain. Thus, the colmedin subfamily of collagenous transmembrane proteins containing *R. norvegicus*, *D. melanogaster* CG6867, and *C. elegans* proteins is enlarged to include also the *H. sapiens*, *P. troglodytes*, *M. musculus*, *T. nigroviridis* and *D. rerio* proteins.
5.4  Occurrence of lymphomas in Col13a1del mice (II)

The Col13a1del transgenic mouse line was generated in order to study the consequences of the synthesis of mutant collagen XIII. Transgenic mice homozygous for the Col13a1del allele were aborted during pregnancy (Sund et al. 2001b). Mice heterozygous for the Col13a1del transgene raised in a conventional colony exhibited no abnormalities through a year of age. However, we began to observe development of tumors in some mice at older ages which prompted further analysis. At the age of 18 months, altogether 33/209 (15.8%) of the Col13a1del mice had prominent enlargement of the MLN with changes suggestive of lymphoma and there was an association with marked splenomegaly in 15 of these cases. Of littermate control mice, only 3/146 (2.1%) exhibited similar changes, a highly significant difference (p = 0.002). Both control and mutant mice were fostered under identical conditions and they are of the B6D2F1 strain. In the barrier colony, where several pathogenic microbes found in the conventional facility were lacking, 14/190 (7.4%) of Col13a1del but only 1/118 (0.8%) of wild-type mice exhibited enlargement of the MLN. Splenomegaly was seen in 4 of the Col13a1del, although it was not as marked as in affected conventionally reared transgenics.

5.5  Analysis of the lymphomas and preneoplastic changes (II)

The lymphomas were characterized using flow cytometry. In a typical experiment 53% of the total viable lymphocytes were T cells and 32% were B cells in the lymphoma. Immunohistochemical studies showed that the tumors often contained substantial populations of cells with cell surface antigen CD68, a marker for macrophages and activated dendritic cells. In further FACS analysis, we studied naïve and activation markers on purified CD4+ and CD8+ T cells and found that the lymphomas were populated by activated CD4+ cells.

To determine if the T and/or B cell populations present in the tumors were clonally dividing, we examined the organization of immunoglobulin heavy chain (IgH) and T cell receptor beta chain (TCRβ) loci of DNA from the tumors. According to these Southern blot analyses, clonal rearrangements of the Ig gene in 5 of 11 cases were detected, whereas clonal rearrangements of TCRβ were not observed.

In histopathological analysis, the earliest changes in Col13a1del mice without substantial lymphadenopathy or splenomegaly were detected in a few mice at the
age of 4 months and in a higher proportion at 12 months of age. These changes were detected at the paracortical regions of MLNs, at the splenic periarteriolar lymphoid sheaths (PALS) and, less often, at the GC in the nodes and spleens. The lymph node paracortex area and the splenic PALS were populated by cells with a cytology resembling centroblasts or with features of lymphoblasts.

The prominently enlarged spleens and MLNs of Col13a1\textsuperscript{del} mice frequently exhibited features of overt lymphoma containing centroblasts, immunoblasts or plasma cells. Prominent populations of histiocytes were also frequently interspersed throughout these lymphoid neoplasms, suggesting a diagnosis of histiocyte-associated lymphomas.

Altogether 47 lymphomas were observed in the 399 Col13a1\textsuperscript{del} mice necroscoped, of which 10 were characterized histologically. These hematopoietic neoplasms included one histiocytic sarcoma and nine B cell lineage tumors. According to the diagnosis, the B-lineage tumors contained three follicular lymphomas, three centroblastic lymphomas (one histiocyte-associated) and one immunoblastic lymphoma, all of which originate from GC or post-GC B cells. Two cases of mature B cell-originating lymphoblastic lymphomas without thymic enlargement were also diagnosed. In the control mice, only four lymphomas out of 264 mice were found and one was used for histopathological analysis and classified as a follicular B-cell lymphoma.

5.6 Expression and localization of collagen XIII in lymphomas, lymphoid tissues and intestine (II)

Immunofluorescence and \textit{in situ} hybridization studies revealed little if any collagen XIII expression in the lymphomas. Although the tissue stainings lacked the collagen XIII signals, at the mRNA level all the lymphomas in the mesenteric nodes expressed both the transgene and the endogenous collagen XIII, with a higher expression level for the transgene.

The hypothesis that lymphoid neoplasms in the MLN originated from the intestine led us to examine the dense B cell populations of normal Peyer’s patches from wild-type and Col13a1\textsuperscript{del} mice. Peyer’s patches populated by CD45R(B220)-positive B cells were negative for collagen XIII staining. This data suggests that the B cell transformation in the Col13a1\textsuperscript{del} mice does not occur through transgene expression by the B cells. Instead, lymphomagenesis may result from influences within the intestinal environment in which they developed and were activated.
RT-PCR analysis showed that in the large intestine a markedly higher amount of transgene and endogenous collagen XIII mRNA was expressed compared to the lymphomas. In the small intestine, the collagen XIII protein is known to be localized in the stroma of the villi (Hägg et al. 2001). Identical staining patterns of collagen XIII beneath the epithelial cells of the villus area were seen in the small intestines of the control and \( \text{Col13a1}^{\text{del}} \) mice, resembling that seen with staining for collagen IV.

Of the lymphoid tissues analyzed, the lymph nodes and thymus did not express collagen XIII, whereas the spleen showed strong collagen XIII staining colocalizing with the endothelial cell marker PECAM in small vessels, the larger vessels being collagen XIII-negative. However, collagen XIII did not fully colocalize with PECAM but exhibited a similar distribution to desmin, possibly in association with pericytes.

5.7 **Electron microscopy of the small intestine (II)**

The high expression level of collagen XIII in the intestines encouraged us to examine the small intestine at the ultrastructural level by electron microscopy. As expected, the epithelial cells of the small intestines of control mice showed a well-defined BM, but an abnormal appearance of the BM was observed in the jejunum of 12 to 18-month-old \( \text{Col13a1}^{\text{del}} \) mice, namely the normal lamina lucida and lamina densa layers of the BM could not be detected. Instead, the entire BM had an appearance of tightened lamina densa, while cellular changes were not apparent.

5.8 **Gene expression profiles from wild-type and \( \text{Col13a1}^{\text{del}} \) mice differ for small intestinal tissues (II)**

We analyzed the gene expression profiles of small intestines of healthy \( \text{Col13a1}^{\text{del}} \) mice without any macroscopic lymphomas or enlarged MLNs in order to determine if the basis for the enhanced lymphoma susceptibility of the \( \text{Col13a1}^{\text{del}} \) mice correlated with altered gene expression profiles. The RNAs prepared from small intestinal tissue was studied using a gene chip representing 45,000 transcripts.

At first, we performed the gene ontology classification of the data using the dChip Gene Function Enrichment analysis and found a significantly over-represented number of genes in at least three out of four \( \text{Col13a1}^{\text{del}} \) mice, with
altered levels of expression in functional groups defined by the annotation terms “immune response”, “defense response” and “antigen binding”. Further analysis with the Affymetrix GeneChip Operating System indicated markedly higher expression levels of 23 genes in total, and interestingly, 11 of these genes were involved in various aspects of innate and acquired immunity. The immune system genes with the most extensive increase in expression, Defcr4 and Defcr-rs7, encode defensins, mediators of innate immunity with bactericidal activity produced by Paneth cells.

5.9 Expression of collagen XIII protein in mouse tissues analyzed by Western blotting (III)

Very little is known about the levels of collagen XIII expressed in tissues, and whether it is present as shed and/or distinct splice variant forms. In Western blot analyses of various adult mouse tissues, the highest collagen XIII protein expression was detected in lung. Moreover, high expression of collagen XIII was demonstrated in liver, pancreas and epididymis and moderate expression in brain, eyeballs, prostate, testis, adrenal gland, jejunum and spleen. The tissues analyzed contained 1–4 collagen XIII polypeptide bands with somewhat different molecular weights. More specifically, lung, liver, pancreas and jejunum contained 102, 95, 86 and 78 kDa bands, while the other tissues studied contained varying combinations of these four forms. The range of tissues analyzed here excluded bone, cartilage, skin, heart and muscle, which have been analyzed previously (Sund et al. 2001a, Ylönen et al. 2005, Latvanlehto, unpublished) or are in the process of being studied.

5.10 Collagen XIII localization in mouse brain, epididymis, adrenal gland, pancreas and liver (III)

We complemented the Western blot data by studying the collagen XIII localization in selected mouse tissues using immunofluorescent staining. Collagen XIII was found in association with blood vessels in brain, epididymis, adrenal gland, pancreas and liver. In brain, however, the most intense collagen XIII staining was detected in the choroid plexus, and the meninges also stained clearly with the collagen XIII antibody. In the adrenal gland collagen XIII could be detected both in the cortex and the medulla, and the most intense staining was localized to the interface between the medulla and cortex, to the cortex side. In
pancreas, collagen XIII localization to the blood vessels was most obvious in the islets of Langerhans, but part of the vessels in the exocrine pancreas was also collagen XIII-positive. Finally, sinusoidal collagen XIII staining was found in liver, and the most intensive collagen XIII staining was detected in the vicinity of central veins. The central veins were, however, negative for collagen XIII.

5.11 Early embryonal lethality of the Col13a1\textsuperscript{del} transgenic mice in the absence of endogenous collagen XIII (III)

We considered it possible that mutant Col13a1\textsuperscript{del} polypeptides affect the function of endogenous collagen XIII in the Col13a1\textsuperscript{del} transgenic mice. To assess more directly the effects of the mutant protein in the absence of the endogenous protein, we crossed the Col13a1\textsuperscript{del} transgenic mouse line with a collagen XIII knockout line (Col13a1\textsuperscript{−}). Mice with one functional allele for the endogenous collagen XIII and positive for the transgene (Col13a1\textsuperscript{+/-};Col13a1\textsuperscript{del+}) appeared normal and were fertile. Further breeding of Col13a1\textsuperscript{+/-};Col13a1\textsuperscript{del+} mice with Col13a1\textsuperscript{−/-} mice was performed and 73 pups were genotyped. However, no Col13a1\textsuperscript{del+} transgenic mice in the knockout background (Col13a1\textsuperscript{−/-};Col13a1\textsuperscript{del+}) were born, although 18 mice of this genotype (25% of all pups) were expected according to Mendelian inheritance.

Since Col13a1\textsuperscript{−/-};Col13a1\textsuperscript{del+} mice were not born, we examined the genotypes of fetuses at embryonic days 9.5 and 10.5 from breedings of Col13a1\textsuperscript{+/-};Col13a1\textsuperscript{del+} with Col13a1\textsuperscript{−/-}. Even at the earliest time point studied (embryonic day 9.5), we could not find the Col13a1\textsuperscript{−/-};Col13a1\textsuperscript{del+} genotype among 40 embryos studied, although all other expected genotypes were found. Thus, the expression of the transgene without the endogenous collagen XIII seems to have an deleterious effect on mouse embryonic development.
Discussion

6.1 Cysteine residues on the amino terminal side of the collagenous ectodomain of collagen XIII are obligatory for trimer stabilization

Collagen XIII occurs in most tissues at low levels, rendering studies with tissue-derived collagen XIII difficult. To perform protein-level analysis of collagen XIII, including mutagenesis of selected sequences, we have expressed the collagen in insect cells. First, we identified the cysteine residues at the end of the NC1 domain to be required for the stabilization of collagen XIII trimers. Closely related transmembrane collagens, collagen types XXIII and XXV, also harbor cysteines directly preceding the most N-terminal collagenous domain (Hashimoto et al. 2002, Banyard et al. 2003), which are also likely to establish the interchain stabilizing effect. In this respect, collagen XVII is unlike the other transmembrane collagens, since it completely lacks cysteines in its ectodomain region. Also, the conserved nucleation site for triple helix folding in other collagenous transmembrane proteins is not necessary for the formation of a stable triple-helical collagen XVII trimer (Snellman et al. 2000b, Franzke et al. 2004). These characteristics highlight some of the differences between collagen XVII and the other transmembrane collagen proteins.

Although we found cysteine residues at the end of the NC1 domain to be required for the stabilization of collagen XIII trimers, these cysteines alone appear not to be sufficient for trimer formation. The intracellular and transmembrane domains have an important impact on trimer formation and stabilization as well. On the other hand, deletion of 83 amino acid residues within the central COL2 domain, or the lack of the C-terminal cysteine residues had no effect on trimer formation. With low probability, the coiled-coil sequences in the NC1 domain begin within the transmembrane domain (Latvanlehto et al. 2003). The coiled-coil may be incomplete if the transmembrane domain is deleted, leading to the inability to trimerize disulfide-bonded collagen XIII molecules. On the other hand, other sequences have also been shown to contribute to trimerization, either independently or together with coiled-coil regions (Malone et al. 2005). Since the collagen XIII variant lacking only the intracellular domain is fully capable of forming trimeric molecules, the transmembrane domain seems to be additionally important for trimer formation.
6.2 Coiled-coil motifs of the collagenous transmembrane proteins

The transmembrane collagens are type II membrane proteins, and the collagenous ectodomain is folded in an N- to C-terminal direction (Snellman et al. 2000b, Latvanlehto et al. 2003). In the case of collagen XVII, most of its noncollagenous domains have potential coiled-coil features, except for the extreme C-terminal NC16 domain (Latvanlehto et al. 2003). Similarly, all NC-domains of collagen XIII contain coiled-coil features, except the extreme C-terminal NC4 domain. Thus, it is likely that in order to assemble and fold properly, every collagenous domain requires a nearby coiled-coil region. In the case of collagen XIII, the conserved NC4 domain can be considered completely “free” for other purposes.

By computer analysis, the NC3 domain of collagen XIII was predicted to have a coiled-coil conformation important for the correct folding of the corresponding molecule. We distorted the coiled-coil structure by changing a glutamine-430 or alanine-431 for a proline, and in subsequent characterization of the recombinant proteins this prediction was found to be correct. In non-reduced SDS-PAGE analysis, the mutant α1(XIII) chains remained monomeric, demonstrating that the formation of stabilizing interchain disulfide bonds in the NC1 domain was compromised. Moreover, the mutant α1(XIII) chains were found to be labile and the folding of the COL3 domain seemed to be most severely affected by degradation, since this domain could not be detected with a domain specific antibody.

Most members of the collagen superfamily contain α-helical coiled-coil domains of 2–4 heptad repeats. These repeats exist in all fibrillar collagens, FACIT collagens, transmembrane collagens, and also collagens VI, VII, XV, XVIII and XXVI. In addition, the collectins, the emilins and the collagen-like tail subunit of acetylcholinesterase contain coiled-coil motifs. These motifs can reside in the N- or C-terminus of the collagenous sequence, or in between the triple-helical regions. The wide occurrence of the coiled-coil domains in collagens and collagen-like proteins suggest they have a general role in triple-helical assembly (McAlinden et al. 2003.) On the other hand, studies with collagen I and other proteins have shown that in addition to a coiled-coil domain, other sequences might be involved and important in trimerization, acting either independently or together with the coiled-coil region (Steinmetz et al. 1998, Kammerer et al. 1998, Frank et al. 2000, Malone et al. 2005).

Computer analysis revealed that the colmedin subfamily of collagenous transmembrane proteins, consisting of R. norvegicus, D. melanogaster and C.
elegans proteins, can now be enlarged to contain also the *H. sapiens*, *P. troglodytes*, *M. musculus*, *T. nigroviridis* and *D. rerio* proteins. All in all, in the structurally varied group of collagenous transmembrane proteins, molecular assembly is facilitated by a coiled-coil motif following the transmembrane domain, and these trimerization domains appear to be associated with each of the collagenous domains. In the case of collagen XIII, the trimeric molecule has interchain disulfide bonds at the junction of the NC1 and COL1 domains, and several of the other collagenous transmembrane proteins have a pair of cysteines in the same location. Moreover, most of these proteins contain a predicted furin cleavage site in the NC1 domain.

### 6.3 Increased incidence of B-cell lymphomas in Col13a1del transgenic mice

The mutant collagen XIII lacking part of the COL2 domain as well as the entire NC4 domain formed disulfide bonded trimers. The Col13a1del transgenic mice expressing both the mutant polypeptides and the endogenous collagen XIII were initially considered unaffected when heterozygous for the transgene, while homozygous mutant mice died during fetal development (Sund et al. 2001b). However, some of the heterozygous transgenic mice were found to develop peritoneal tumors as they reached 1 year of age. The tumors were identified as B-cell lymphomas.

Different mouse strains develop mature B-cell lymphomas with varying frequencies at older ages (Ward 2006). The Col13a1del transgenic mice were generated in the B6D2F1 background, which had a ~2% incidence of lymphoma when housed under conventional conditions, but an incidence of only 0.8% when raised in SPF conditions. The incidence of lymphomas increased in the transgenic Col13a1del mice to 15.8% under conventional housing conditions and 7.4% under SPF conditions, respectively. The decreased lymphoma incidence and the decreased size of the lymphomas under SPF conditions suggests that the environment contributes to the initiation and/or progression of the lymphomagenic process.

The lymphomas in the Col13a1del mice were almost all follicular, DLBCL or plasma cell neoplasms originating from GC or post-GC B cells. In addition to these clonally dividing B cells, these lymphomas were associated with remarkable populations of activated, non-clonal T cells and macrophages. Importantly, histological and molecular studies of human B cell lineage tumors have revealed
subsets with similar features, namely they contain inflammatory/immune cell infiltrates (Dave et al. 2004, Monti et al. 2005, Jaffe et al. 2001).

Several studies have demonstrated that changes in the intestinal flora and inflammatory stimuli can profoundly influence the development of different types of B cell lineage neoplasms in mice (Byrd et al. 1991, Potter et al. 1985, Enzler et al. 2003, Hartley et al. 2000). The reduced incidence of lymphomas in Col13a1<sup>del</sup> mice under SPF conditions provides a striking parallel to these earlier findings.

The effects of the activated CD4<sup>+</sup> T cell and histiocyte (macrophage) populations on lymphoma initiation, progression or maintenance are not known. CD4<sup>+</sup> T cells recognizing antigens expressed on the surface of B cells have been shown to drive lymphoma development in mice (Zangani et al. 2007). In gastric mucosa-associated lymphoid tissue (MALT) lymphomas of humans, CD4<sup>+</sup> T cells engage Helicobacter pylori antigens on antigen-presenting cells and stimulate chronic proliferation of autoreactive B cells and their eventual transformation (Suarez et al. 2006). Also, the immunoproliferative small intestinal disease, recognized as one of the prototypes of MALT lymphoma but with features of atypical plasma cells, is driven by T cell recognition of Campylobacter jejuni antigenic peptides (Lecuit et al. 2004).

Surprisingly, collagen XIII protein was below the limits of detection in the lymphomas, even though transcripts for both the endogenous and mutant genes were present. Other lymphoid tissues did not express collagen XIII either, except the spleen, where collagen XIII was found in the small blood vessels, possibly in association with pericytes. The lack of mutant as well as endogenous collagen XIII expression in the lymphomas prompted us to study more closely the gut, the source of lymphatic fluid drained by the MLNs. In the intestine, as shown before (Hägg et al. 2001), collagen XIII was expressed at high levels in both the villi and the crypts of the basal aspect of the epithelium. Studies of transcripts suggest that the expression levels of the endogenous and mutant proteins were approximately the same. The epithelial cells were abnormally attached to the BM as assessed by ultrastructural studies, while the BM in older mice lacked the normal lamina lucida and lamina densa layers. In the jejunums of the macroscopically normal mice, altered gene expression levels were only detected in the functional groups immune response, defense response and antigen binding.

We postulated that in the intestine of the Col13a1<sup>del</sup> mice, the coexpression of the endogenous and mutant proteins could result in the formation of greatly perturbed supramolecular complexes of collagen XIII and its potential extracellular partners such as fibronectin, heparin and two BM components,
nidogen-2 and perlecan, all known to bind with high affinity to collagen XIII (Tu et al. 2002). The normal barrier function of the epithelial cells may be compromised due to the marked alteration in the structure of the BM underlying intestinal epithelial cells. The persistent activation of antigen-responsive T cells and B cells could be a component of the initiating phase of B-cell lymphoma development.

In addition to other collagens, collagen XIII has also been shown to be a high affinity ligand for the inhibitory leukocyte-associated immunoglobulin-like receptor LAIR-1 (Lebbink et al. 2006). The heterotrimers formed by mutant and wild-type proteins in the Col13a1del mice may result in disruption of the normal triple-helical conformation encompassing most of the collagen XIII ectodomain and reduce its affinity for LAIR-1, possibly leading to a decreased levels of inhibitory signal on immune cells.

6.4 Mice expressing the Col13a1del transgene in the absence of endogenous collagen XIII

The Col13a1del transgene is embryonic lethal in homozygous mice with two distinct phenotypes at days 10.5 and 13.5 of development. The homozygous Col13a1del mice at 13.5 day of development exhibited a decreased number of capillaries in the developing nervous system, although development of the microvessels in other areas was normal. Similarly, the fetal vessels showed less spreading in the labyrinth layer of the mutant placentas when compared to control placentas. The impaired angiogenesis in the Col13a1del placentas is not due to abnormal endothelial cells, but expression of altered collagen XIII in the surrounding tissue invaded by the newly forming vessels. Thus, collagen XIII may have a function in directing or maintaining developmental events. It may have signalling function important in development and/or migration. Fetuses that die at the later time point have been shown to suffer from cardiac dysfunction, possibly due to abnormal adherence junctions in the myocardium. (Sund et al. 2001b.) Here, we found that a lower dose of the transgene also affects cell-matrix homeostasis and leads to the development of lymphomas.

These findings prompted us to explore further the properties of the Col13a1del mutant protein by generating mice producing only the mutant protein (III), when the Col13a1del transgene is expressed in the absence of endogenous collagen XIII. In this mouse model, severe developmental defect occurs, since no Col13a1−/−;Col13a1del−/− embryos could be found, even as early as E9.5. Collagen
XIII mRNA is expressed at day 7 of development onwards in mouse (Sund et al. 2001b), and time points earlier than this have not been examined. Early lethality (before E9.5) of Col13a1<sup>+/−</sup>;Col13a1<sup>del+</sup> embryos suggests that collagen XIII might be involved in orchestrating developmental events early in mouse development.

Analysis of collagen XIII distribution at day E10.5 has identified the molecule in the neuroepithelium lining the brain vesicles and in the neural tube. In situ hybridization analysis at E11.5 revealed collagen XIII mRNAs in the neuroectoderm of the developing brain and neural tube as well as in the myocardium. The heart showed intense collagen XIII staining already at E10.5, being present in the developing myocardium of both the ventricles and the atria. (Sund et al. 2001b.) At E12.5 cardiac dysfunction was detected in Col13a1<sup>del</sup> fetuses, probably leading to the embryonic lethality (Tahkola et al. 2008). Developmental defects of the Col13a1<sup>+/−</sup>;Col13a1<sup>del+</sup> embryos might occur in these tissues or in yet unknown collagen XIII expression sites.

### 6.5 Tissue localization and blood vessel association of collagen XIII

In situ hybridization analyses have demonstrated a wide tissue distribution of collagen XIII in muscle, skin, bone and other connective tissues (Sandberg et al. 1989). Much less is known about the tissue localization of the protein. Here, we found by Western blot analysis, doing a survey of adult mouse tissues, that the highest expression was in lung. High collagen XIII expression was also detected in liver, pancreas, epididymis, adrenal gland, jejunum and spleen and moderate collagen XIII expression in brain, eyeball, prostate and testis. Tissues contained 1–4 collagen XIII polypeptide forms, the sizes of which ranged between 78–102 kDa. Collagen XIII has been shown to undergo complex alternative splicing, producing at least 17 different transcripts (Peltonen et al. 1997). Thus, the existence of collagen XIII proteins with different sizes may result from splicing of collagen XIII in particular tissues and/or from shedding of the ectodomain (Snellman et al. 2000b, Väisänen et al. 2004).

Previously, immunostaining of bone and skeletal muscle revealed collagen XIII along osteoblasts (Ylönen et al. 2005) and at the neuromuscular junctions (Hägg et al. 2001). In the tissues studied here, collagen XIII appeared most prominently associated with blood vessels. Blood vessel association of collagen XIII was found in brain, epididymis, adrenal gland, pancreas and liver. In
publication II we detected blood vessel staining of collagen XIII in spleen, possibly in association with pericytes. The function of collagen XIII adjacent to endothelial cells in these tissues is not yet known, but it may stabilize the walls of blood vessels and participate in the regulation of blood flow. Collagen XIII could also have an influence on the life span of endothelial cells.
7 Future perspectives

Our studies have revealed common features in a structurally varied group of collagenous transmembrane proteins, namely coiled-coil motifs, disulfide bonds at the junction of the NC1 and COL1 domains, and a furin cleavage sites in the NC1 domains. In the near future, we will have tools to study the significance of collagen XIII cleavage in vivo. We are generating a gene-targeted mouse line, where the furin cleavage site is mutated, leading to the synthesis of only the transmembrane form of collagen XIII. This will help to study the physiological significance of the collagen ectodomain shedding in different tissues. Also, the possible tissue/cell-specific significance of alternative splicing of collagen XIII transcripts should be considered in future studies.

When we analyzed lymphoma development in the Col13a1del transgenic mice, to our surprise lymphomas did not express collagen XIII, but changes could be found in the intestine from where the lymphomas likely originate. These results suggested that a matrix defects, in the gut in this case, can contribute to lymphoma development in mice. It will be interesting to study whether collagen XIII has a role in human bowel diseases, such as in Crohn’s disease or ulcerative colitis, and in lymphomas.

The wide tissue distribution, particularly in association with blood vessels, will direct studies of these tissues in more detail in collagen XIII-deficient mice. At first, we can analyze these vessels at the ultrastructural level. This has been successful in analyzing the effect of a lack of collagen XIII in the neuromuscular junction, a structure that normally exhibits very intense collagen XIII expression. The absence of collagen XIII in these structures leads to a loose attachment of the nerve terminal to the muscle cell. In the case of blood vessel-associated collagen XIII, we can evaluate if it has a role as an adhesion molecule or whether it has other function(s). In addition, we will analyze the Col13a1LacZ transgenic mice, which express β-galactosidase under collagen XIII promoter. This will enable us to evaluate the exact expression site of collagen XIII in association with blood vessels.

Due to the lack of Col13a1−/−;Col13a1del+ embryos at E9.5, it is important to study earlier stages, and we will analyze mouse embryonic development at the blastocyst stage. If the Col13a1−/−;Col13a1del+ embryos survive to the blastocyst stage, they either lose the ability to implant or else lethality occurs very soon after implantation. These early studies may reveal new functions of collagen XIII in
mouse development and may also be linked with collagen XIII functions in adulthood.
References


Original articles

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


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Original publications are not included in the electronic version of the dissertation.
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<td>Quantitative and semi-quantitative imaging techniques in detecting joint inflammation in patients with rheumatoid arthritis. Phase-shift water-fat MRI method for suppressing fat at 0.23 T, contrast-enhanced dynamic and static MRI, and quantitative 99mTc-nanocolloid scintigraphy</td>
<td>Palosaari, Kari (2008)</td>
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<td>Perkiömäki, Marja Riitta (2008)</td>
<td>981</td>
</tr>
<tr>
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<td>Matrix metalloproteinase MMP-2 and MMP-9 and their inhibitors TIMP-1 and TIMP-2 in bladder carcinoma</td>
<td>Vasala, Kaija (2008)</td>
<td>983</td>
</tr>
<tr>
<td>Placental angiogenesis and angiogenesis related risk factors in severe pre-eclampsia</td>
<td>Järvenpää, Jouko (2008)</td>
<td>984</td>
</tr>
<tr>
<td>Factors affecting the outcome of IVF/ICSI</td>
<td>Veleva, Zdravka (2008)</td>
<td>985</td>
</tr>
<tr>
<td>Tanssiesitys autismienmestumänä dementoituvin vanhusten hoitoyössä</td>
<td>Ravelin, Teija (2008)</td>
<td>986</td>
</tr>
<tr>
<td>Approaches to improving brain protection in cardiac and aortic surgery. An experimental study in a porcine model with hypertonic saline dextran, levosimendan, leukocyte depleting filter and different acid base management strategies</td>
<td>Kasalinen, Hanna (2008)</td>
<td>988</td>
</tr>
<tr>
<td>Thermal, hormonal and cardiovascular responses to single and repeated nonhypothermic cold exposures in man</td>
<td>Korhonen, Ilkka (2008)</td>
<td>989</td>
</tr>
<tr>
<td>Extracapsular hip fractures—aspects of intramedullary and extramedullary fixation</td>
<td>Saarenpää, Immo (2008)</td>
<td>990</td>
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<tr>
<td>Aspects and determinants of children’s dental fear</td>
<td>Rantavuori, Kari (2008)</td>
<td>991</td>
</tr>
<tr>
<td>Genetic, epidemiological and cell culture studies on human resistin</td>
<td>Kunnari, Anne (2008)</td>
<td>992</td>
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<tr>
<td>Collagen XIII in cardiovascular development and tumorigenesis</td>
<td>Tahkola, Jenni (2008)</td>
<td>993</td>
</tr>
</tbody>
</table>
Anne Tuomisto

THE ROLE OF COLLAGEN XIII IN B-CELL LYMPHOMA DEVELOPMENT, AND CHARACTERIZATION OF ITS BIOSYNTHESIS AND TISSUE DISTRIBUTION

Department of Medical Biochemistry and Molecular Biology, University of Oulu; Biocenter Oulu, Center for Cell Matrix Research, University of Oulu.