Aino Kinnunen

COLLAGEN XVIII REGULATES BASEMENT MEMBRANE INTEGRITY

SPECIFIC EFFECTS OF ITS ISOFORMS ON THE CHOROID PLEXUS, KIDNEY AND HAIR FOLLICLE

UNIVERSITY OF OULU, FACULTY OF MEDICINE, INSTITUTE OF BIOMEDICINE, DEPARTMENT OF MEDICAL BIOCHEMISTRY AND MOLECULAR BIOLOGY;
UNIVERSITY OF OULU, BIOCENTER OULU, CENTER FOR CELL MATRIX RESEARCH
AINO KINNUNEN

COLLAGEN XVIII REGULATES BASEMENT MEMBRANE INTEGRITY
Specific effects of its isoforms on the choroid plexus, kidney and hair follicle

Academic dissertation to be presented with the assent of the Faculty of Medicine of the University of Oulu for public defence in Auditorium F202 of the Department of Pharmacology and Toxicology (Aapistie 5), 20 May 2011, at 12 noon

UNIVERSITY OF OULU, OULU 2011
Abstract

Collagen XVIII is a multidomain basement membrane proteoglycan with three tissue-specific isoforms. Endostatin, the C-terminal part of collagen XVIII, has antiangiogenic properties, while the frizzled-like domain of the longest isoform is suggested to be capable of inhibiting the Wnt/β-catenin signaling network. This study utilized several genetically modified mouse lines and electron microscopy to achieve new information on the biological role of collagen XVIII, its different isoforms, and the frizzled domain.

Lack of collagen XVIII was found to affect the integrity of basement membranes of various tissues, leading to an abnormally loosened network structure. In the choroid plexus, the change in the basement membrane ultrastructure caused alterations in the production of the cerebrospinal fluid and predisposed to the development of hydrocephalus. In the kidney, broadening of the proximal tubular basement membrane was shown to be due specifically to the lack of the short isoform, while the lack of the two longer isoforms led to podocyte foot process effacement. Moreover, lack of collagen XVIII was found to cause softening of the kidney glomeruli and the levels of serum creatinine were elevated in the mutant animals, indicating altered kidney function.

The hair follicle cycle was used as a model to study the possible role of the frizzled domain of collagen XVIII in the Wnt/β-catenin signaling cascade. The longer collagen XVIII isoforms were shown to be expressed in the basement membrane facing the dermal papilla and in the hair follicle bulge, containing the follicular stem cells. Lack of the long isoforms led to abnormalities in the progression of the first hair cycle, and the phenotype could be rescued via transgenic delivery of the frizzled domain of the longest isoform, suggesting its involvement in the regulation of the Wnt/β-catenin signaling network during the cyclic growth of the hair.

Keywords: basement membrane, choroid plexus, collagen type XVIII, electron microscopy, hair follicle, hydrocephalus, kidney defects, podocytes, Wnt signaling
Kinnunen, Aino, Kollageeni XVIII tyvikalvojen eheyden ylläpitäjänä. Eri
isomuotojen vaikutukset suonipunokseen, munuaiseen ja karvatuppiin
Oulun yliopisto, Lääketieteellinen tiedekunta, Biolääketieteen laitos, Lääketieteellinen biokemia
ja molekyylibiologia; Oulun yliopisto, Biocenter Oulu, Center for Cell Matrix Research, PL
5000, 90014 Oulun yliopisto
Oulu

Tiivistelmä
Kollageeni XVIII on useista toiminnallisista osista koostuva tyvi kalvojen proteoglykaani, jolla
on kolme eri kudoksissa esiintyvää isomuotoa. Sen C-terminalisella endostatiini-osalla on veri-
suonten kasvua estäviä vaikutuksia, kun taas pisimmän isomuodon frizzled-osan uskotaan estä-
vän Wnt/β-kateniini signalointireitin toimintaa. Tässä tutkimuksessa saatiin uutta tietoa kolla-
geeni XVIII:n, sen eri isomuotojen sekä frizzled-osan biologisesta merkityksestä useiden geeni-
muunneltujen hiirimallien sekä elektronimikroskopian avulla.

Kollageeni XVIII:n puutoksen todettiin vaikuttavan tyvikalvojen rakenteen eheyteen useissa
eri kudoksissa, johtaa epänormaali löytyvään verkkorakenteeseen. Suonipunoksessa tämä
 tyvikalvon hienorakenteen muutos vaikuttui aivo-selkäydinneteen tuottumiseen ja altisti vesi-
pään kehitysmallin kehittymiseen. Suonipunoksissa proksimaalisen munuaistehyyn tyvikalvon levenemisen osoi-
tettu johtavan syynä hienyön isomuodon puutoksesta, kun taas kahden pidemmän isomuodon puuttu-
minen aiheutti podosyyttien jalkalisäkkeiden leviämistä. Lisäksi kollageeni XVIII:n puuttumi-
sen osoitti johtavan hiirimallien munuaiskerästen pehmemiseen sekä veren kreatiniinita-
son kohoamiseen, viitaten munuaistoinnin häiriöihin.

Karvatuppien syklistikasvua käytettiin mallina tutkittaessa kollageeni XVIII:n frizzled-osan
mahdollisia vaikutuksia Wnt/β-kateniini signalointireittiin. Pidempien kollageeni XVIII isomuo-
tojen osoitettiin tuottavan karvanystyn tyvikalvossa sekä karvatupin kantosolut sisältävällä pul-
listuma-alueella. Pitkien isomuotojen puuttuminen johti karvojen ensimmäisen kasvukierren
epänormaaliin etenemiseen. Tämä voitti esiä siirtogeemisen frizzled-osan avulla, mikä viitatti
sen osallisuuteen Wnt/β-kateniini signalointireittiin säätelevyn karvan syklisen kasvun aikana.

Asiasonat: elektronimikroskopia, karvatuppi, kollageeni XVIII, munuaisvika,
podosyytti, suonipunos, tyvikalvo, vesipää, Wnt signalointi
Acknowledgements

This research was carried out at the Institute of Biomedicine, Department of Medical Biochemistry and Molecular Biology, University of Oulu.

I wish to express my gratitude to my supervisor Professor Taina Pihlajaniemi for giving me the opportunity to be part of her excellent research group and for introducing me to the science community and the field of extracellular matrix research. I also want to state my appreciation for Emeritus Professors Kari Kivirikko and Ilmo Hassinen, Professors Johanna Myllyharju and Seppo Vainio, and Docents Peppi Karppinen and Minna Männikkö, the other keypersons of the department, for creating great facilities and an inspiring atmosphere for all scientific work.

I am very grateful to Professors Nicolai Miosge and Sylvie Ricard-Blum for their valuable comments on this thesis. I also wish to thank Sandra Hänninen for her careful revision of the language of my thesis manuscript.

I owe my deepest gratitude to Docent Raija Sormunen, the head of the Electron Microscopy Core Facility of Biocenter Oulu, without whose great scientific expertise, guidance, support, and personal friendship this thesis would never have been born. I am also very grateful to senior scientists Ritva Heljasvaara and Pirkko Huhtala, the true backbone of our group during the whole length of my thesis work, whose help I have been able to rely on in all practical matters. I wish to thank Dr. Lauri Eklund for his excellent guidance in the beginning of my studies and for all help and support later on. Also Docent Raija Soininen is owed my special respect and gratitude for always sharing her great scientific knowledge with me when needed.

A crucial factor for my thesis has been the collaboration between different laboratories and researchers, and I want to thank all my co-authors for their contribution to the original papers. Special gratitude is expressed to Docent Marja Mikkola for help in writing the part of the thesis concerning the hair follicles and Dr. Lotta Seppinen for cooperation in the hair studies. Moreover, my thesis work has required a huge amount of effort from Päivi Tuomaala within our laboratory, Sirpa Kellokumpu, Anna-Liisa Oikarainen, and Tarja Piispanen from the Biocenter Oulu Electron Microscopy Core Facility, as well as from Irja Leinonen, Tuula Måkinen, Eija Nissinen, Tanja Sankala, and other personnel of the Laboratory Animal Shelter of University of Oulu, and I want to acknowledge them all for their excellent technical assistance. In addition, Auli Kinnunen, Marja-Leena Kivelä, Pertti Vuokila, Marja-Leena Karjalainen, Risto Helminen,
and Seppo Lähdesmäki are appreciated for all their help in practical matters. Furthermore, I wish to thank all my colleagues in our department: it has been a true privilege to work in such company. My special thanks go to Anu Muona for originally introducing me to the group, Maarit Rossi for great company during various scientific courses and meetings, and Mari Aikio, Harri Elamaa and Joni Mäki for their support and friendship. I should also mention the great influence of a student visiting our group, Lorenza S. Carvalhaes, whose positive attitude and enthusiasm still affects all my scientific work. Last but definitely not least, I wish to thank all the girls “downstairs”: Merja, Inderjet, Vanessa, Riitta, Karolina, Jenni, and Hongmin, for all the moments of enthusiasm and despiration that they have shared with me over the years.

My warmest gratitude belongs to my mother Laila Utriainen, who raised me and my brother alone after the sudden death of my father Tuomo Utriainen. One of the main issues she always wanted to teach her children was “to learn how to use their own brains”. This lesson has carried me this far. I am also thankful to my little brother Pekka Utriainen, who as a member of the “three musketeers” has been one of the most important people through my childhood and has now increased our family by his beautiful wife Jenni and their cute little daughter Vilma. Moreover, I am deeply grateful for our babysitters: my mother, Kaija, Anni, Anna, Minna, and other friends and family members, without your kind help this thesis book would have never been written. The furry members of our family, passed and present, have also had a significant role in my life outside the lab, and deserve lots of hugs and treats for their loyal companionship and endless understanding, as well as for trying to keep me fit by taking me on our daily walks and particularly for all the friends they have helped me to meet.

Above all, I owe my greatest gratitude to my husband Mika Kinnunen for his love, care, and patience during the years I spent on this thesis work and for sharing every day life as a great father to our two little daughters Lotta and Liina – wonderful gifts who have given my life a totally new purpose.

This work was supported by the Finnish Center of Excellence Program (2000–2005) and the Research Council for Health of the Academy of Finland (grants 44843 and 115237), the Sixth EU Framework Program (Integrated Project Angiotargeting, contact number 504743), the Sigrid Jusélius Foundation, Biocenter Oulu Graduate School, and Kuopion Luonnon Ystävät Yhdistys.

Kuopio, March 2011

Aino Kinnunen
Abbreviations

BM  basement membrane
BMP  bone morphogenetic protein
C-  carboxy-
CSF  cerebrospinal fluid
E  embryonic day
ECM  extracellular matrix
EM  electron microscopy
ERK  extracellular signal-regulated kinase
FGF  fibroblast growth factor
FS  freeze substitution
GAG  glycosaminoglycan
GBM  glomerular basement membrane
HPF  high pressure freezing
HSPG  heparan sulfate proteoglycan
IEM  immunoelectron microscopy
ILK  integrin-linked kinase
kb  kilobase
kDa  kilodalton
LEF-1  lymphocyte enhancing factor-1
LRP  lipopolysaccharide receptor-related protein
MMP  matrix metalloproteinase
MRI  magnetic resonance imaging
N-  amino-
NC  noncollagenous
P  postnatal day
PP2A  serine/threonine protein phosphatase 2A
Shh  sonic hedgehog
TGF  transforming growth factor
TIMP  tissue inhibitor of metalloproteinases
TSP-1  thrombospondin-1
VEGF  vascular endothelial growth factor
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  
Tiivistelmä  
Acknowledgements  7  
Abbreviations  9  
List of original articles  11  
Contents  13  

### 1 Introduction  15

#### 2 Review of the literature  17

2.1 Extracellular matrix  
2.2 Basement membranes  18  
2.3 The collagen family of proteins  19  
2.4 Collagen XVIII  21

2.4.1 Structure of collagen XVIII  21  
2.4.2 Posttranslational glycosylation of collagen XVIII  22  
2.4.3 Tissue distribution of collagen XVIII  24  
2.4.4 Biological function of collagen XVIII  26  

2.5 Endostatin  28

2.5.1 Biosynthesis  29  
2.5.2 Structure versus function  29  
2.5.3 Effects on tumor growth  31  
2.5.4 Other biological functions  33  
2.5.5 Mechanism of action  34  

2.6 The Kidney  36

2.6.1 Glomerulus  37  
2.6.2 Glomerular basement membrane  38  
2.6.3 Podocytes  39  
2.6.4 Mesangium  41  
2.6.5 Renal tubule  41  
2.6.6 Role of collagen XVIII in kidney  43  

2.7 Hydrocephalus  43

2.7.1 Choroid plexus  44  
2.7.2 Ependyma  46  
2.7.3 Meninges  46  
2.7.4 Genetics of hydrocephalus  47
2.8 Hair Follicles

2.8.1 Hair follicle structure, development, and cycling

2.8.2 Role of ECM in hair follicle development and cycling

2.8.3 Hair follicle stem cells

2.8.4 Wnt/β-catenin signaling in hair follicle development and cycling

2.8.5 Hair follicle-associated angiogenesis

3 Outlines of the present study

4 Materials and methods

5 Results

5.1 Col18a1−/− mice manifest hydrocephalus (I)

5.2 Localization of collagen XVIII in the brain (I)

5.3 Ultrastructural changes in the choroid plexus epithelium (I)

5.4 Broadening of BMs in Col18a1−/− mice (I)

5.5 Improved preservation of mouse kidney ultrastructure using HPF-FS (II)

5.6 Localization of collagen XVIII isoforms in kidney (I, II)

5.7 Structural and functional defects in the knockout kidney (I, II)

5.8 Localization of collagen XVIII isoforms in hair follicles (III)

5.9 Lack of collagen XVIII longer isoforms in the C57BL/6 background leads to a faster start of the first hair cycle (III)

5.10 Delayed start of anagen in FVB/N Col18a1−/− mice (III)

5.11 Lack of clear defects in early hair follicle morphogenesis (III)

5.12 Transgene-derived frizzled domain rescues the phenotype of Col18a1−/− mice (III)

6 Discussion

6.1 Hydrocephalus in the Col18a1−/− mouse line

6.2 HPF-FS method for EM preparation in ECM research

6.3 Kidney abnormalities in the Col18a1−/− mice

6.4 Role of collagen XVIII in BMs

6.5 Frizzled domain of collagen XVIII affects the hair cycle

7 Future perspectives

References

Original Papers
1 Introduction

The collagen-based extracellular matrix (ECM) provides structural and mechanical support for all multicellular animals. It has vital effects on cell behavior, such as proliferation, apoptosis, shape, migration and differentiation. Basement membranes (BMs) are highly specialized ECM structures lining most epithelial and endothelial cell layers. In addition to their supporting and separating functions, the BMs and their components are involved in a variety of biological signaling cascades, developmental processes and disease progression.

Collagen XVIII is one of the BM components that are highly conserved during evolution. It is a multi-domain heparan sulfate proteoglycan (HSPG) expressed as three tissue-specific variants. Endostatin, the C-terminal (carboxy-terminal) globular domain of all three collagen XVIII variants, has antiangiogenic properties. The longest collagen XVIII variant contains a cysteine-rich domain in its N-terminus (amino-terminus), closely resembling the ligand-binding domain of the frizzled-receptors of Wnt-signaling molecules. Both the C-terminal endostatin domain and the N-terminal frizzled-domain can be proteolytically cleaved from the parent molecule to produce soluble factors with inhibitory effects on either angiogenesis or the Wnt/β-catenin signaling cascade, respectively.

The aim of this thesis work was to achieve more in vivo information on the role of the full-length collagen XVIII molecule in BMs and to clarify the specific functions of the different isoforms. Particular interest was placed on the potential role of the frizzled domain in the Wnt/β-catenin signaling network. To accomplish these aims, we utilized various genetically modified mouse lines and found collagen XVIII to be critical for the normal ultrastructure of the BMs of various tissues, with lack of it leading to abnormal loosening of the BM scaffold. The loosening of the BM network predisposed the mutant animals to diseased conditions, such as hydrocephalus and kidney malfunction. Moreover, this study suggested that the frizzled domain of the longest collagen XVIII variant affects the murine hair follicle cycling, possibly via control of Wnt/β-catenin signaling during the normal events of organ function.
2 Review of the literature

2.1 Extracellular matrix

The evolution of multicellular organisms required the development of mechanisms for cell-to-cell communication and cohesion. This need has been solved by collagen-based ECMs, characteristic of all multicellular animals (reviewed by Huxley-Jones et al. 2009). The ECM provides structural support for organs and tissues, for cell layers in the form of BMs, and for individual cells as substrates for migration. ECM influences cell proliferation, apoptosis, shape, migration, and differentiation through adhesion receptors such as integrins (reviewed by Barczyk et al. 2010). The ECM is formed by hundreds of proteins, many of which are conserved during evolution, such as the members of the BM network molecules collagen IV, laminins, nidogen, perlecan and collagen XVIII (Whittaker et al. 2006, reviewed by Huxley-Jones et al. 2007). Many of the ECM proteins are large and complex with multiple distinct domains. Most of them are glycosylated and there is also a significant amount of proteoglycans in the ECM. The ECM varies from tissue to tissue by its precise composition (reviewed by Kolácná et al. 2007), as well as in terms of its mechanical characteristics, such as stiffness and elasticity (reviewed by Buxboim et al. 2010). Cells spread, change shape, orient themselves, and organize their adhesions and cytoskeleton according to ECM stiffness, strain, and stress. The effects of the mechanical characteristics of the ECM are converted directly to the actin-myosin cytoskeleton of the cells by focal adhesions that are linked to various ECM proteins, such as collagens, fibronectin and laminins (reviewed by Zaidel-Bar et al. 2007).

The ECM is being constantly remodeled, particularly during normal processes of development, differentiation, and wound repair (reviewed by Daley et al. 2008). ECM binds several growth factors, acting as a reservoir, and may establish stable gradients of growth factors, which play vital roles in patterning developmental processes. The proteolytic cleavage of ECM components by proteases, including those in the matrix metalloproteinase (MMP), serine protease, and cysteine protease families, releases the growth factors from the ECM by degradation of the ECM proteins during both normal tissue remodeling and pathological situations. In addition, the ECM proteins themselves often contain fragments that can be released by proteolysis, after which they can act as soluble growth factor-like ligands (reviewed by Hynes 2009). Dysregulation of the ECM
assembly or degradation can lead to various diseases; for example, misregulated protease activity is a common characteristic of metastatic tumors.

2.2 Basement membranes

Mammalian BMs are sheet-like, highly specialized ECM structures produced by endothelial and epithelial cells and many mesenchymal cells. The BMs separate cell layers from the fibrillar matrix and in addition, they surround some cells, such as skeletal muscle. The BM networks provide structural integrity to tissues, serve as scaffolds for the assembly of other macromolecular components, and act as ligands for integrin cell-surface receptors that mediate cell adhesion, migration, growth, and differentiation (reviewed by Barczyk et al. 2010). Three of the common BM components – perlecan, collagen XVIII, and agrin – are HSPGs, meaning they carry heparan sulfate side chains. They contribute to storing and preserving the biological activity of various cytokines, and growth factors and abnormal expression or dysregulated function of these proteoglycans affects cancer growth and angiogenesis (reviewed by Iozzo et al. 2009). In addition, some of the constituents of the BM, such as collagens IV, XV, and XVIII and perlecan, have proteolytically cleaved fragments (tumstatin and arresten, restin, endostatin, and endorepellin, respectively), that are able to inhibit the growth of blood vessels (reviewed in Ortega & Werb 2002 and Bix & Iozzo 2005).

The great importance of the BMs is supported by the fact that the key members of the BM scaffold, collagen IV α-chains, laminin subunits, nidogen, perlecan, and collagen XVIII, are also present in the sea urchin *Strongylocentrotus purpuratus* (Whittaker et al. 2006) as well as in the sea squirt *Ciona intestinalis*, a primitive chordate (reviewed by Huxley-Jones et al. 2007). In addition, all of these basic BM components are highly conserved among *C. elegans*, *Drosophila*, and vertebrates, indicating that some early common metazoan ancestor of nematodes, arthropods, and vertebrates developed a BM (reviewed by Hynes & Zhao 2000). In fact, even the simplest extant multicellular organisms, sponges, express both BM and fibrillar type collagen genes, but their ECMs exhibit no apparent organization to the BM and fibrillar matrices (Exposito & Garrone 1990, Boute et al. 1996). In humans, mutations in the genes coding for BM proteins can lead to various diseases affecting different tissues (reviewed by Yurchenco & Patton 2009 and Agtmael & Bruckner-Tuderman 2010).

In ultrastructural studies, the BMs are often seen as thin sheets consisting of two layers: the electron dense layer *lamina densa*, which is separated from the
cell surface by a looser layer, called the lamina lucida. However, studies utilizing different freezing techniques have shown that the clearly visible lamina lucida is only an artefact caused by extraction of tissue components during the classical preparation of samples for electron microscopy (EM) (Goldberg & Escaig-Haye 1986, Chan et al. 1993, Chan & Inoue 1994, Keene et al. 1997, Reipert et al. 2004) (Fig. 1). The central BM scaffold is believed to consist of a network composed of collagen IV, laminins and nidogens. Assembly of the BM is initiated by laminins, which self-assemble into heterotrimers that bind to the cell surface via their cell surface receptors, which include integrins and other proteins (reviewed by Li et al. 2003b). The laminin network is then crosslinked to the collagen IV network. The network-forming collagen IV α-chains are assembled by oligomerization of triple-helical protomers that are covalently crosslinked to stabilize the networks (Vanacore et al. 2009). Nidogen, which interacts with specific domains of laminin, also links collagen IV with the BM (McKee et al. 2007). All BMs also contain several other molecules, including perlecan, agrin, fibulin, fibronectin, and the proteoglycan collagens XV and XVIII. In addition to variations in molecular composition, the fine structure of BMs varies from tissue to tissue (reviewed by Erickson & Couchman 2000, Yurchenco et al. 2004, and Kruegel & Miosge 2010).

Cells adhere to BMs by binding to specific receptors. This adhesion is primarily mediated by a subfamily of β1-integrins, which act as binding partners to several laminins, collagen IV, perlecan, and other BM components (reviewed by Yurchenco et al. 2004, and Barczyk et al. 2010). In the epidermal BM of the skin, where extra strength is needed in dermal-epidermal adhesion, specific molecules such as collagens XVII and VII link cells to the BM and also the BM to the adjacent ECM (reviewed by McMillan et al. 2003 and Breitkreutz et al. 2009). The function of the outer zone of the BMs is less well understood, although its important role in connecting the cells to the matrix is clear. Among the ubiquitous BM components, collagen XVIII is the only one that has been shown to localize specifically to the fibrillar matrix interface (Marneros et al. 2004, Elamaa et al. 2005), which suggests a possible anchoring function.

2.3 The collagen family of proteins

Collagens form a large family of trimeric ECM proteins that maintain the biological and structural integrity of tissues, and regulate cell adhesion and migration as well as tissue remodelling during development and in many
pathological stages (reviewed by Ortega & Werb 2002 and Gelse et al. 2003). They are the most abundant proteins, constituting approximately 30% of the human protein mass. The collagens consist of three α-chains carrying at least one collagenous domain with repeating peptide triplets of Glycine-X-Y, X and Y being often proline and hydroxyproline (reviewed by Myllyharju & Kivirikko 2004 and Gordon & Hahn 2010). The collagenous domains are flanked by noncollagenous (NC) domains, which often contain peptide modules found in other matrix molecules. Each of the three α-chains coils into a left-handed helix, which are then wound around a common axis to form the collagenous triple helix. The three α-chains can be identical, or alternatively the triple helix can be formed by two or even three different α-chains. At the moment, 28 different human collagen types containing a total of 49 different α-chains have been reported. Collagen synthesis requires many posttranslational modifications, including hydroxylation of certain lysine and proline residues, glycosylation, and cleavage of the N- and C-terminal propeptides. The collagen superfamily can be divided into families on the basis of supramolecular assemblies and other features: fibril-forming collagens (I, II, III, V, XI, XXIV and XXVII), fibril-associated collagens (VII, IX, XII, XIV, XVI, XIX, XX, XXI and XXII), network-forming collagens (IV, VI, VIII and X), transmembrane collagens (XIII, XVII, XXIII and XXV), multiplexins (XV and XVIII), and other collagens (XXVI and XXVIII) (reviewed by Myllyharju & Kivirikko 2004 and Gordon & Hahn 2010) (Fig. 1).

Mutations in collagen genes and in their modifying enzymes have been shown to lead to various disorders (reviewed by Kuivaniemi et al. 1991 and Myllyharju & Kivirikko 2001). Mutations in the collagen I gene or in genes coding for its posttranslational modifier prolyl hydroxylases (Basel & Steiner 2009, Willaert et al. 2009) can lead to osteogenesis imperfecta, a disease characterized by brittle bones. Mutations in collagens II, IX, X, or XI can cause chondrodysplasias, intervertebral disc disease, and osteoarthritis. Moreover, mutations in collagen I, III, or V or in a posttranslational modifier enzyme lysyl hydroxylase (Yeowell & Walker 2000) can cause Ehlers-Danlos syndrome, characterized by joint hypermobility, skin hyperextensibility, and thinness. Mutations in the gene coding for the α3-, α4-, or α5-chains of the BM network-forming collagen IV can lead to Alport syndrome with renal failure, deafness and eye lesions (reviewed by Heidet & Gubler 2009), and in addition, mutations in the α1-chain of the same collagen have been reported in patients with porencephaly (Breedweld et al. 2006). Moreover, mutations in collagen VI can lead to a muscle disease called Bethlem myopathy, and mutations in the genes coding for collagens
VII or XVII can cause *epidermolysis bullosa*, a skin blistering disease. Finally, mutations in the collagen XVIII gene have been shown to be the cause of Knobloch syndrome, characterized by ocular abnormalities and encephalocele.

![Image of EM image of the mouse epidermal BM and the underlying ECM](image)

**Fig. 1.** An EM image of the mouse epidermal BM and the underlying ECM. The localizations of various collagen types are illustrated.

### 2.4 Collagen XVIII

#### 2.4.1 Structure of collagen XVIII

Collagens XV and XVIII belong to a separate subfamily of collagens named multiplexins (*multiple* triple-helix domains and interruptions) (Oh *et al.* 1994a, Rehn & Pihlajaniemi 1994b). The collagen XVIII gene is localized to mouse chromosome 10 and human chromosome 21 (21q22.3) (Oh *et al.* 1994b). It has 10 collagenous, triple helical domains separated by noncollagenous domains, an
N-terminal noncollagenous domain and a C-terminal noncollagenous domain (NC1) which contains an association region, a protease-sensitive hinge region, and a C-terminal endostatin domain (Oh et al. 1994b, Rehn et al. 1994a, Rehn & Pihlajaniemi 1994b, Muragaki et al. 1995, Sasaki et al. 1998) (Fig. 2). The NC1 domain of collagen XVIII contains a structurally novel trimerization domain with a high trimerization potential (Boudko et al. 2009) and thus, collagen XVIII aggregates as trimers typical for all collagens. The N-terminal, noncollagenous domain common to all three variants contains a thrombospondin-1 (TSP-1) -like domain (Oh et al. 1994b, Rehn & Pihlajaniemi 1994b). The highest identity between the human, mouse, zebrafish, Drosophila, and Xenopus laevis collagen XVIII genes is in the endostatin domain (Saarela et al. 1998a, Elamaa et al. 2002, Haftek et al. 2003, Meyer & Moussian 2009). Both the human (COL18A1) and mouse (Col18a1) collagen XVIII genes as well as the Drosophila and Xenopus laevis collagen XVIII are expressed as three variants, which differ in their N-terminal noncollagenous domains (Muragaki et al. 1995, Rehn & Pihlajaniemi 1995, Saarela et al. 1998a, Elamaa et al. 2002, Elamaa et al. 2003, Meyer & Moussian 2009) (Fig. 2). The mouse Col18a1 gene is over 102 kb long and consists of 43 exons, with exons 4–43 being common to all three variants (Rehn et al. 1996). The variants originate from two alternative promoters. The shortest isoform is transcribed upstream of promoter 1 in conjunction with exons 1 and 2, while the downstream promoter 2, separated from the promoter 1 by 50 kb, encodes for the two longer isoforms in conjunction with exon 3. Exon 3 is in turn alternatively spliced to form the middle variant. The longest variant contains a cysteine-rich area homologous with the ligand-binding part of the frizzled receptors. Utilization of an intraexonic alternative splice site causes an in-frame deletion of this 247 residue (frizzled) portion to form the middle variant. Both the C-terminal endostatin domain and the N-terminal frizzled domain can be proteolytically cleaved from the parent molecule to produce soluble fragments (Sasaki et al. 1998, Heljasvaara et al. 2005, Quélard et al. 2008).

2.4.2 Posttranslational glycosylation of collagen XVIII

Collagen XVIII contains heparan sulfate glycosaminoglycan (GAG) side chains (Oh et al. 1994b, Halfter et al. 1998, Saarela et al. 1998b). It has three Ser-Gly consensus sequences carrying heparan sulfate side chains in the middle and N-terminal part of the core protein (Dong et al. 2003) (Fig. 2). All three sites are conserved in human, mouse, and Xenopus collagen XVIII. Thus, collagen XVIII
belongs to a family of proteins called proteoglycans, carrying GAG side chains of repeating disaccharide units. Heparan sulfate GAG chains consist of N-acetylated or N-sulphated glucosamine units and uronic acids. The GAG chains of proteoglycans may be used for storing and preserving growth factors and cytokines and presenting them to their cell-surface receptors. They also participate in selective filtration of biological fluids, in establishment of cellular barriers, and in modulation of angiogenesis (reviewed by Iozzo et al. 2009 and Kirn-Safran et al. 2009).

**Fig. 2.** A schematic picture of collagen XVIII variants. The short variant has its own signal sequence, while the other two variants share a common signal sequence. All three variants contain a TSP-1-like domain, three binding sites for heparan sulfate side chains, and a C-terminal endostatin domain. The longest variant has a cysteine-rich frizzled domain in its N-terminus.

The N-terminal heparan sulfate side chain of collagen XVIII has been shown to be involved in the BM binding of collagen XVIII (Dong et al. 2003). In addition, the GAG chains of collagen XVIII have been reported to bind L-selectin, a C-type lectin involved in leukocyte migration, as well as to interact with monocyte chemoattractant protein-1, inducing α4β1 integrin activation of monocytes in leukocyte infiltration during inflammation (Kawashima et al. 2003, Celie et al. 2005). Moreover, the heparan sulfate chains of collagen XVIII have been reported to bind to receptor protein tyrosine phosphatase σ, a cell adhesion molecule involved in nervous system development (Aricescu et al. 2002). Mice lacking the heparan sulfate chains of perlecan show abnormalities of the lens capsule and these defects are more severe and progress faster in double mutants also lacking collagen XVIII. Thus, the heparan sulfate chains of these BM components have a
structural function and are essential in the insulation of the lens capsule (Rossi et al. 2003).

### 2.4.3 Tissue distribution of collagen XVIII

Collagen XVIII is a ubiquitous BM component (Oh et al. 1994b, Muragaki et al. 1995, Halfter et al. 1998) expressed in most endothelial, epithelial and mesenchymal cells throughout mouse development (Miosge et al. 2003). The shortest variant is the dominant collagen XVIII isoform, being the sole isoform of e.g. endothelial BMs (Muragaki et al. 1995, Saarela et al. 1998a and 1998b). Thus, detailed studies of the localization of collagen XVIII mostly reveal the tissue distribution of this dominant isoform. For comprehensive studies of the localization of collagen XVIII in various tissues, refer to the following papers (Inoue-Murayama et al. 2000, Pollheimer et al. 2004, Pufe et al. 2004, Väänänen et al. 2004, Määttä et al. 2007, Errera et al. 2008, Tilki et al. 2008).

The middle collagen XVIII variant is expressed most abundantly in the liver (Murakagi et al. 1995, Saarela et al. 1998a and 1998b), where hepatocytes are reported as the main source of collagen XVIII (Schuppan et al. 1998). In the normal human liver, collagen XVIII is localized in persinusoidal spaces and BMs and its expression level is increased in cirrhosis and highest in neoplastic livers (Musso et al. 1998, Schuppan et al. 1998). In contrast, during hepatocellular tumor progression, low collagen XVIII expression is associated with large tumor size, while tumors expressing high collagen XVIII levels are smaller and have low microvessel density (Musso et al. 2001a). The longest isoform of collagen XVIII can be detected in most of the tissues, although the expression level is relatively low (Muragaki et al. 1995, Saarela et al. 1998a and 1998b). It is expressed at the highest levels in kidney, moderately in liver, placenta, enamel, and lung, and low levels can be detected elsewhere (Elamaa et al. 2003, Väänänen et al. 2004, Quélard et al. 2008). Its expression is lowered in several human cancer types and higher expression levels may be associated with less aggressive tumors. In addition, a single nucleotide polymorphism within the frizzled motif (c.1136C>T) has been reported to increase susceptibility to obesity in type 2 diabetes (Errera et al. 2008).

There are only a few studies concerning the transcriptional regulation of collagen XVIII. The promoter 2 of collagen XVIII, which directs the transcription of the two longest variants, has been shown to contain liver-specific regulatory elements, such as binding sites for hepatocyte nuclear factor-3 (Liétard et al. 2008).
It has also been reported to contain five conserved regions between human and mouse (Armelin-Correa et al. 2005), and comparisons between the zebrafish and human genes revealed four conserved, independent non-coding areas that are involved in the regulation of transcription (Kague et al. 2010). In addition, the tumor suppressor molecule p53 has been shown to activate the production of collagen XVIII (Miled et al. 2005) and also to increase the production of α(II) collagen prolyl-4-hydroxylase, a posttranslational modifier, leading to increased synthesis of collagen XVIII (Teodoro et al. 2006). In contrast, expression of ornithine decarboxylase, a molecule involved in the biosynthesis of polyamines and associated with promoted tumor growth, in cancer cell lines has been shown to lead to suppression of collagen XVIII expression (Nemoto et al. 2002).

In Caenorhabditis elegans, the collagen XVIII homolog cle-1 is expressed in all BMs but accumulates in the nervous system, where it concentrates near the synapse-rich regions (Ackley et al. 2001 and 2003). In Xenopus laevis, all three collagen XVIII variants are expressed during the development, the short variant having the highest and the longest variant the lowest expression level (Elamaa et al. 2002). During development, the zebrafish homolog is expressed in several locations, such as the neural tissues, epidermis, optic vesicles, and pronephric ducts (Thisse et al. 2001, Haftek et al. 2003, Kague et al. 2010). At later stages, zebrafish collagen XVIII expression can be detected in cartilage, blood vessels, and liver (Kague et al. 2010).

Within the BMs, collagen XVIII has a polarized orientation, the C-terminal part being located within the lamina densa and the N-terminal part facing towards the ECM (Marneros et al. 2004, Elamaa et al. 2005). In the elastic fibers of vessel walls, endostatin has been reported to be colocalized with fibulin-1 and -2 and nidogen-2, but not with perlecain (Miosge et al. 1999). Conversely, in the kidney BMs endostatin is partly co-localized with perlecain but not with nidogen-1 (Miosge et al. 2003). Recombinant mouse endostatin was shown to be able to bind to the fibulins and nidogen-2 (Miosge et al. 1999). Together the co-localization and binding data indicate that the endostatin part has a possible role in connecting collagen XVIII to the BM scaffolds (Miosge et al. 1999 and 2003). This view was supported by a study by Elamaa et al. (2005), where the epidermal BM of mice overexpressing monomeric endostatin in keratinocytes was shown to become widened due to dispensation of the full-length, native collagen XVIII from the BM. Interestingly, these mice also showed degeneration of lens epithelial cells and progressive development of cataracts (Elamaa et al. 2005).
2.4.4 Biological function of collagen XVIII

Mutations in the COL18A1 gene lead to a rare, recessive condition called Knobloch syndrome (Passos-Bueno et al. 1994, Sertié et al. 1996 and 2000). It is characterized by high myopia, vitreoretinal degeneration, retinal detachment, and midline encephalocele, mainly in the occipital region (Knobloch & Layer 1971, Cook & Knobloch 1982, Seaver et al. 1993, Sniderman et al. 2000). In addition, patients often suffer from other, variable symptoms, including a duplicated kidney collecting system, bifid ureter, and carious teeth (Czeizel et al. 1992), as well as ventricular dilatation of the brain (Kliemann et al. 2003), central nervous system malformations (Keren et al. 2007), bilateral duplex kidneys with grade-IV vesicoureteric reflux and patent right processus vaginalis (Williams et al. 2008), ventricular septal defect, a patent ductus arteriosus, and abnormal chest radiograph (Wilson et al. 1998). Persistent fetal vasculature and abnormal retinal vasculature in the eyes of a young patient have also been reported, in support of the findings in the Col18a1−/− mice (Duh et al. 2004). Recently, patients of Knobloch syndrome as well as the the Col18a1−/− mice were also shown to present lower than normal plasma lipoprotein lipase mass and activity and exhibit fasting hypertriglyceridemia (Bishop et al. 2010). Lack of only the short collagen XVIII isoform is sufficient to cause the Knobloch syndrome, while the lack of all three isoforms leads to a more severe ocular phenotype and a possible predisposition to epilepsy (Suzuki et al. 2002 & 2009b, reviewed by Passos-Bueno et al. 2006).

Lack of collagen XVIII in mice leads to abnormalities in the eye with delayed regression of blood vessels in the vitreous and abnormal outgrowth of retinal vessels (Fukai et al. 2002). Mice lacking collagen XVIII (Col18a1−/−) also show anterior ocular defects including rupture of the iris epithelial cell layers, atrophy of the ciliary body epithelial cells and accumulation of ECM in the BMs of the iris and ciliary body (Marneros & Olsen 2003, Ylikärppä et al. 2003a). The average intraocular pressure was measured to be lower in aged mutant animals compared to controls (Ylikärppä et al. 2003a). Aged Col18a1−/− mice also show additional eye alterations, such as abnormal retinal pigment epithelium with subretinal pigment epithelium accumulation of BM-like deposits (Marneros et al. 2004). The persistent hyaloid vessels of Col18a1−/− animals abnormally vascularize the retinas of the mice by day 10 after birth (Hurskainen et al. 2005), and high oxygen-induced neovascularization is less intense in the knockout animals compared to wild-type. The effect of the endostatin domain on
angiogenesis was further supported by a study, where aortic explants of Col18a1−/− mice showed a twofold increase in microvessel outgrowth compared to wild-type and the increase could be reduced to a normal level by addition of recombinant endostatin (Li & Olsen 2004). In addition, there is an increase in the amount of retinal astrocytes in the knockouts, and lack of both collagens XV and XVIII has been shown to lead to migration of astrocytes on persistent hyaloid vessels (Hurskainen et al. 2005). However, despite the similarity of these collagen molecules, double knockout mice for collagens XV and XVIII show only minor compensatory effects; thus, the biological roles of the collagens are separate (Ylikärppä et al. 2003b). Moreover, healing of wounds is accelerated in Col18a1−/− mice, while it is delayed in mice overexpressing endostatin in keratinocytes (Seppinen et al. 2008). Within the wounded areas, vascularization rate was accelerated in knockouts but not affected in the transgenic animals, although abnormal capillaries were detected. In addition, in the endostatin overexpressing transgenics, the formation of the epidermal BM was delayed, the structure of the epidermal and endothelial BMs was more disorganized, and cases of detachment of the epidermis from the granulation tissue were observed. Furthermore, in the teeth, the enamel of Col18a1−/− mice had slightly increased total mineral, calcium, and phosphorus contents, without any visible defects in the enamel or the dentin structures (Väänänen et al. 2004). The Col18a1−/− and endostatin overexpressing mice also had mild abnormalities in bone development; knockout mice showed a delay in the formation of secondary ossification centers, and the bones of endostatin overexpressing mice grew more slowly in length than the bones of control mice (Sipola et al. 2009).

Collagen XVIII has been shown to be one of the few (37) genes with elevated expression in different stem cell types (hair follicle bulge, hematopoietic, embryonic, and neural stem cells), thus being one of the candidates for “stemness genes”, which play a role in self-renewal and differentiation (Blanpain et al. 2004). The actions of collagen XVIII on development have also been studied in a few tissues. The COL18A1 gene is located in chromosome 21, and thus is expressed with an extra copy in Down’s syndrome (chromosome 21 trisomy). The gene is located within a region on chromosome 21 associated with a congenital heart disease in Down’s syndrome patients. Interestingly, collagen XVIII has been shown to be highly expressed throughout the connective tissue core of the endocardial cushions and forming atrioventricular valve leaflets during mouse heart development, and moreover, was localized around migrating mesenchymal
cushion tissue cells (Carvalhaes et al. 2005). Thus, overexpression of collagen XVIII in Down’s syndrome may have an effect on cardiac valve morphogenesis. Moreover, collagen XVIII has been shown to be expressed in the epithelial buds during both lung and kidney branching morphogenesis, displaying a reverse pattern: in the lung it localizes to the epithelial tips, while in the kidney it is confined to the epithelial stalk region (Lin et al. 2001b). Alteration in collagen XVIII expression is accompanied by a shift in sonic hedgehog (Shh) expression, which has an effect on the tissue morphogenesis (reviewed by Vainio et al. 2003). Blocking of collagen XVIII leads to a reduction in the expression of Wnt2, suggesting a regulatory interaction between these molecules (Lin et al. 2001b). The possible role of collagen XVIII in regulating Wnt-signaling was supported by a study, where the N-terminal frizzled domain of collagen XVIII was reported to be proteolytically cleaved from the parent molecule; after cleavage it localized to the cell surface, where it pulled down Wnt3a in vitro and suppressed Wnt-dependent β-catenin activation (Quélard et al. 2008). Thus, the frizzled domain is cryptic and becomes activated only after proteolytic cleavage. The cleaved frizzled-containing fragment has also been shown to inhibit tumor growth of β-catenin-activated tumor cells both in vitro and in vivo (Quélard et al. 2008, Lavergne et al. 2010).

2.5 Endostatin

Endostatin is a C-terminal, 20 kDa fragment of collagen XVIII, first described by O’Reilly et al. (1997). It is present in the human blood circulation (Ständker et al. 1997), where it can be stored in platelet α granules and released by regulation of G-protein-mediated signaling pathways (Italiano et al. 2008, Klement et al. 2009). Endostatin has antiangiogenic properties by inhibiting endothelial cell proliferation (O’Reilly et al. 1997) and migration (Yamaguchi et al. 1999) and inducing apoptosis of these cells (Dhanabal et al. 1999b); thus, it is capable of regressing primary tumors in animal models (O’Reilly et al. 1997). These antitumor effects of endostatin have led to extensive studies of the molecule. However, the antimigratory effects of endostatin are not restricted to endothelial cells, since its inhibitory effects on migration have been shown in several other cell types in mammals (Karihaloo et al. 2001, Nyberg et al. 2003, Wilson et al. 2003, Pollheimer et al. 2004 and 2005, Feng et al. 2005, Al Ahmad et al. 2010). In fact, the endostatin domain of collagen XVIII may have a conserved role in regulating cell migration, since lack of it in both Caenorhabditis elegans and
Drosophila has been shown to result in defects in cell migration and axon guidance (Ackley et al. 2001 & 2003, Meyer & Moussian 2009).

2.5.1 Biosynthesis

Endostatin can be cleaved from its parent molecule, collagen XVIII, from a protease-sensitive hinge region by several cathepsins (L, B and K), MMPs (-2, -3, -7, -9, -12, -13, -14 and -20) and elastase with variable efficiencies (Wen et al. 1999, Felbor et al. 2000, Ferreras et al. 2000, Lin et al. 2001a, Chang et al. 2005, Heljasvaara et al. 2005). The endostatin-containing fragments of corresponding sizes (25–32 kDa) are present in human tissues and plasma and have anti-proliferative and anti-migratory effects on endothelial cells (Ferreras et al. 2000, Heljasvaara et al. 2005). Moreover, the tumor suppressing molecule p53 has also been reported to accelerate the proteolytical processing of endostatin from its parent molecule (Teodoro et al. 2006).

The NC1 domain of collagen XVIII contains a structurally novel trimerization domain with a high trimerization potential (Boudko et al. 2009) and thus, it aggregates into trimers, while the endostatin part is monomeric (Sasaki et al. 1998). Both the NC1 domain and the single endostatin can bind to heparin and fibulin-1 and -2. The binding of the whole NC1 domain to laminin-111 and perlecan is stronger compared to endostatin alone. Thus, proteolytic release of endostatin may lead to a switch from a matrix-associated to a more soluble endocrine form (Sasaki et al. 1998).

2.5.2 Structure versus function

The crystal structures of both the mouse and human endostatin domains have been solved and shown to contain a zinc-binding site (Ding et al. 1998, Hohenester et al. 1998 and 2000). Zinc-binding has been reported to have a structural, stabilizing role and therefore has potential effects on endostatin’s antiangiogenic activity, possibly by protecting it from proteolytic degradation or by promoting correct conformation needed for the interaction of endostatin with its receptors (Boehm et al. 1998, Hohenester et al. 2000, Tjin Tham Sjin et al. 2005, Han et al. 2007). However, the importance of zinc in the biological roles of endostatin is still a matter of debate (Yamaguchi et al. 1999, Ricard-Blum et al. 2004). The circulating forms of endostatin have been reported to be glycosylated, the importance of which remains unclear at the moment (John et al. 1999 and
Endostatin also has two pairs of nested disulphide bonds (cysteine residues 1–3 and 2–5), which stabilize its conformation (Ständker et al. 1997, Ding et al. 1998). Mutated forms lacking the disulphide bonds lose nearly their entire tertiary structure and most of their inhibitory activities both on the migration and proliferation of endothelial cells (Zhou et al. 2005). Deficiency in the formation of the disulphide bonds leads to the formation of amyloid-like fibrils and granular aggregates of endostatin, with cytotoxic effects (He et al. 2006). Recombinant endostatin has been shown to be able to form amyloid-like fibrils that bind and are cytotoxic to murine neuroblastoma cells in vitro (Kranenburg et al. 2003) and to induce endothelial cell detachment by stimulation of the plasminogen activation system (Reijenkerk et al. 2003). This ability to form amyloid-like fibrils may be related to the findings that endostatin binds to the amyloid-β peptide (Faye et al. 2009b) and accumulates in amyloid plaques in Alzheimer’s disease (Deininger et al. 2002b).

In a chick chorioallantoic membrane assay, the inhibitory effects of endostatin on angiogenesis were found to be dependent on heparin sulphates (Sasaki et al. 1999). Endostatin binds heparin at two sites: arginines 155/158/184/270 (major site) and arginines 193/194 (minor site). Both of the sites are required for inhibition of fibroblast growth factor-2 (FGF-2)-induced angiogenesis, while only the minor site is essential for the inhibition of vascular endothelial growth factor-A (VEGF-A)-induced angiogenesis (Olsson et al. 2004). In addition, a synthetic peptide covering the minor heparin-binding site is enough to inhibit endothelial cell chemotaxis and tumor vascularization. Endostatin binds heparin and heparan sulfate with moderate affinity (K_D ~ 2µM) and the binding is dependent on divalent cations (Ricard-Blum et al. 2004). The structure and size of the heparan sulfate saccharides binding to endostatin are limited by the fact that they need to be able to interact with endostatin’s two separate arginine clusters (Kreuger et al. 2002, Blackhall et al. 2003). The same arginines are needed also for endostatin’s binding to transglutaminase-2, an enzyme involved in regulating angiogenesis (Faye et al. 2010).

The importance of endostatin’s ability to bind to heparan sulfates has been reported in a number of studies. In a study by Kuo et al. (2001), oligomeric endostatin had a crucial role in ECM-dependent endothelial migration and morphogenesis, and these effects were mediated by laminin-111 in BMs and heparan sulfates on the cell surface (Javaherian et al. 2002). Endostatin was also reported to compete with the proangiogenic growth factor bFGF for binding to heparan sulphate, and thus inhibit its proliferative effects on endothelial cells
(Reis et al. 2005). The function of the heparan sulfate binding site of endostatin was also shown to be essential for endostatin’s antiangiogenic effect on a bone angiogenesis model, and heparan sulfates increased the binding of endostatin to endothelial cells (Gaetzner et al. 2005). Moreover, the effects of the oligomeric NC1 domain of collagen XVIII on endothelial cell migration can be inhibited by addition of exogenous GAGs, and the morphological changes induced by the NC1 domain on epithelial cells are dependent on heparan sulfates (Clamp et al. 2006). However, some opposite results have also been reported. In a study by Yamaguchi et al. (1999), detailed analysis of mutated endostatin fragments showed that endostatin’s inhibitory effect on endothelial cell migration and tumor growth is independent of both zinc and heparin binding. In addition, endostatin’s binding to blood vessels has been shown to be independent of HSPGs (Chang et al. 1999, Rychkova et al. 2005). Thus, it is likely that in addition to the heparan sulfate binding site, endostatin also contains other sites for binding its variable ligands. In fact, this was suggested in a study by Stahl et al. (2005), where endostatin was shown to have two surface-exposed hydrophobic phenylalanines at positions 31 and 34, and that they defined a receptor binding site acting independently from heparan sulfate binding.

**2.5.3 Effects on tumor growth**

The growth of tumors is limited by the cellular oxygen requirements and thus, the tumor must recruit new blood vessels in order to grow beyond a critical size (reviewed by Carmeliet & Jain 2000). Endostatin treatment has been shown to inhibit the growth and reduce the size of tumors by reducing the vascular density within the tumor tissue in a variety of animal models (reviewed by Folkman 2006). The inhibitory effects of recombinant endostatin on angiogenesis have been shown to occur by inhibition of the proliferation and migration of endothelial cells (Dhanabal et al. 1999a and 1999c) and in addition, by inducing apoptosis in endothelial cells (Dhanabal et al. 1999b and 1999c). Endostatin treatment has also been reported to inhibit the formation of distant metastases in animal models (Yoon et al. 1999, Perletti et al. 2000, Sauter et al. 2000, Yokoyama et al. 2000, Li et al. 2006). Surgical removal of primary, endostatin-producing tumors can lead to a marked decrease in levels of circulating endostatin, promoting the growth of distant metastases (Peeters et al. 2005, Sun et al. 2005). This activation of liver and lung metastases has been shown to be inhibited by intramuscular endostatin gene transfer leading to high levels of circulating
endostatin (Sun et al. 2005). Endostatin’s inhibitory effects on the development of metastases may be explained by its inhibitory effects on lymphangiogenesis, since endostatin has also been shown to inhibit the migration and proliferation of lymphatic endothelial cells in a dose-dependent manner (Shao & Xie 2005). In support of this in vitro data, although mice overexpressing endostatin in keratinocytes developed similar tumor incidence and multiplicity with control mice after an experiment of carcinogen-induced skin tumors, there was a significant reduction in lymphatic vessels in the papillomas and squamous cell carcinomas, leading to inhibition of lymph node metastasis in the transgenic mice (Brideau et al. 2007). Endostatin overproduction inhibited the accumulation of tumor-infiltrating mast cells expressing VEGF-C, which in turn inhibited the lymphangiogenesis of the tumors.

The actions of endostatin have also been studied in various human cancer types. Serum endostatin levels are known to be elevated in patients of various cancer types (Feldman et al. 2000b, Feldman et al. 2001, Hata et al. 2001, Ohlund et al. 2008, Babkina et al. 2009). High expression of collagen XVIII is associated with poor prognosis in patients of some cancer types (Kim et al. 2009, Lee et al. 2010). In gliomas, the amount of endostatin staining was shown to be significantly lower in the tumor parenchyma of human glioblastomas than in WHO Grade II astrocytomas (Strik et al. 2001). In mild oral epithelial dysplasias, collagen XVIII localizes in BMs as a continuous signal, however, in severe dysplasias and in the invasive areas of oral squamous cell carcinoma collagen XVIII is absent (Väänänen et al. 2007).

Interestingly, Down’s syndrome patients have a reduced risk (50–90%) of developing solid tumors (Hasle et al. 2000, Yang et al. 2002) as well as vascular anomalies, such as infantile hemangiomas and vascular malformations (Greene et al. 2008). One of the hypothesized explanations is the presence of an extra copy of endostatin and indeed, the serum levels of endostatin have been measured to be 48% higher in patients with Down’s syndrome compared to other populations (Zorick et al. 2001). In support of the hypothesis, transgenic mice overproducing endostatin in endothelial cells have a 1.6-fold endostatin increase in circulating endostatin levels compared to normal mice, mimicking the situation in Down’s syndrome, and a 3-fold reduction in the rate of tumor growth (Sund et al. 2005). In contrast, whether Knobloch syndrome patients have an increased risk of developing cancer is not known, although a recent paper reports of a patient with acute lymphoblastic leukemia (Mahajan et al. 2010).
Endostatin was accepted as a drug in studies of cancer treatment in 2005 due to its nontoxic, inhibitory effects on tumor growth in a wide variety of mouse models (reviewed by Folkman 2006). However, after more than a decade of basic and clinical research, the clinical role of endostatin in cancer therapeutics is still not clear (Karamouzis & Moschos 2009). In phase I studies recombinant human endostatin has been proven to be safe and well-tolerated, but with only minor antitumor activity in the treatment of patients with various malignancies, advanced neuroendocrine tumors, and melanomas (Eder et al. 2002, Herbst et al. 2002, Thomas et al. 2003, Kulke et al. 2006, Moschos et al. 2007). To improve endostatin’s antitumor effects in human patients, several proposals have been made. In animal models, continuous administration of endostatin has been shown to be more efficient in regressing tumors, compared to single daily doses (Kisker et al. 2001). In addition, the effect of endostatin on endothelial cell proliferation and migration as well as its efficacy in tumor inhibition has been shown to exhibit a U-shaped curve, where the maximum activity was observed within a certain critical concentration range, suggesting that an optimum dose range may be required to achieve therapeutic efficacy (Celik et al. 2005, Tjin Tham Sjin et al. 2006). Moreover, several attempts have been made to increase the half-life of endostatin, including PEGylation and addition of a metal chelatin sequence at the N-terminus of endostatin, leading to a derivative called Endostar that has been approved for the treatment of non-small-cell lung cancer in 2005 in China (reviewed by Zheng 2009 and Ricard-Blum & Ballut 2011).

2.5.4 Other biological functions

In addition to effects on tumor growth, endostatin’s actions have been studied in several other conditions. A summary of these with references is listed in Table 1.
Table 1. Conditions with endostatin association.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alzheimer’s disease</td>
<td>Deininger et al. 2002b, van Horssen et al. 2002</td>
</tr>
<tr>
<td>Asthma</td>
<td>Suzaki et al. 2005</td>
</tr>
<tr>
<td>Atherosclerotic lesions</td>
<td>Moulton et al. 1999 &amp; 2004, Zeng et al. 2005</td>
</tr>
<tr>
<td>Aortic valve stenosis</td>
<td>Chalajour et al. 2004</td>
</tr>
<tr>
<td>Arterial injury</td>
<td>Hutter et al. 2003</td>
</tr>
<tr>
<td>Bone development</td>
<td>Sipola et al. 2006, 2007 &amp; 2009</td>
</tr>
<tr>
<td>Brain injury</td>
<td>Deininger et al. 2006, Mueller et al. 2007b Tian et al. 2007, Hou et al. 2010</td>
</tr>
<tr>
<td>Bullous scleroderma</td>
<td>Santos et al. 2005</td>
</tr>
<tr>
<td>Cerebral malaria</td>
<td>Deininger et al. 2002a</td>
</tr>
<tr>
<td>Cystitis</td>
<td>Beecken et al. 2004</td>
</tr>
<tr>
<td>Myocardial infarction</td>
<td>Isobe et al. 2010</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>Matsuno et al. 2002, Yin et al. 2002</td>
</tr>
<tr>
<td>Spinal cord injury</td>
<td>Mueller et al. 2007a</td>
</tr>
</tbody>
</table>

2.5.5 Mechanism of action

Several studies suggest that the constituents of the ECM surrounding endothelial cells have an effect on endostatin’s actions in inhibiting endothelial cell migration and proliferation, leading to different effects on angiogenesis depending on the maturation state of the endothelial cells (Boye et al. 2001, Schmidt et al. 2004a, 2004b, 2004c & 2005a, Delaney et al. 2006, Khan et al. 2006). In fact, it has been suggested that the mechanism by which endostatin inhibits the formation of new blood vessels is through the stabilization and maturation of newly formed endothelial tubes (Ergün et al. 2001). This view has been supported by a few studies (Schuch et al. 2003, Li & Olsen 2004, Huang & Chen 2010), while on the contrary, in wound healing experiments endostatin treatment resulted in impaired blood vessel maturation (Berger et al. 2000, Bloch et al. 2000).

The molecular mechanism of endostatin’s actions has been under heavy investigation during the last decade, but no clear route or signaling cascade has emerged. Instead, a broad range of molecular targets have been reported suggesting that multiple signaling systems are involved in mediating endostatin’s...
Table 2. The interacting partners of endostatin.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidic phospholipids</td>
<td>Zhao et al. 2005</td>
</tr>
<tr>
<td>Amyloid peptide Aβ-(1-42)</td>
<td>Faye et al. 2009a</td>
</tr>
<tr>
<td>β-catenin</td>
<td>Dixielus et al. 2002, Hanai et al. 2002a &amp; 2002b</td>
</tr>
<tr>
<td>Biglycan</td>
<td>Zeng et al. 2005</td>
</tr>
<tr>
<td>Ceramide</td>
<td>Zhang et al. 2005</td>
</tr>
<tr>
<td>Chondroitin and dermatan sulphate</td>
<td>Faye et al. 2009a</td>
</tr>
<tr>
<td>Collagens I, IV, V and VI</td>
<td>Ricard-Blum et al. 2006a &amp; 2006b, Faye et al. 2009a</td>
</tr>
<tr>
<td>E-selectin</td>
<td>Yu et al. 2004</td>
</tr>
<tr>
<td>Fibulins</td>
<td>Miosge et al. 1999</td>
</tr>
<tr>
<td>Glypicans</td>
<td>Karhaloo et al. 2001, Karumanchi et al. 2001</td>
</tr>
<tr>
<td>Laminin-111</td>
<td>Javaherian et al. 2002</td>
</tr>
<tr>
<td>Low density lipoprotein</td>
<td>Zeng et al. 2005</td>
</tr>
<tr>
<td>Matrix metalloproteinases</td>
<td>Kim et al. 2000, Nyberg et al. 2003</td>
</tr>
<tr>
<td>Nerve growth factor</td>
<td>Al Ahmad et al. 2010</td>
</tr>
<tr>
<td>Nidogens</td>
<td>Miosge et al. 1999</td>
</tr>
<tr>
<td>Nucleolin</td>
<td>Shi et al. 2007, Fu et al. 2009</td>
</tr>
<tr>
<td>Perlecan/endorepellin</td>
<td>Miosge et al. 2003, Mongiat et al. 2003</td>
</tr>
<tr>
<td>SPARC</td>
<td>Faye et al. 2009a</td>
</tr>
<tr>
<td>Thrombospondin-1</td>
<td>Faye et al. 2009a</td>
</tr>
<tr>
<td>Transglutaminase-2</td>
<td>Faye et al. 2009a</td>
</tr>
<tr>
<td>Tropomyosins</td>
<td>MacDonald et al. 2001</td>
</tr>
<tr>
<td>VEGF receptors</td>
<td>Kim et al. 2002, Kojima et al. 2008</td>
</tr>
<tr>
<td>Zinc</td>
<td>Ding et al. 1998, Hohenester et al. 1998 &amp; 2000</td>
</tr>
</tbody>
</table>

Endostatin can bind α5- and αv-integrins (Rehn et al. 2001) and the binding of endostatin to integrins α5β1 and αvβ3 is mediated by two arginines (Arg27 and Arg139) in a heparin/heparan sulfate dependent fashion (Wickström et al. 2004, Faye et al. 2009b). A fraction of cell-surface-bound endostatin associates with lipid rafts with caveolin-1, where it interacts with α5β1 integrin to downregulate RhoA (Ras homolog A) activity and induce phosphatase-dependent activation of caveolin-associated Src kinases and disassembly of actin stress fibers and focal adhesions (Wickström et al. 2002 and 2003). Endostatin’s binding to α5β1 integrin has also been reported to lead to inhibition of the focal adhesion kinase/c-Raf/MEK1/2/p38/extracellular signal-regulated kinase (ERK) mitogen-activated...
protein kinase pathway (Sudhakar et al. 2002). These actions of endostatin with α5β1 integrin in endothelial cells lead to impaired ability of the cells to deposit fibronectin to their ECM and inability to migrate in response to bFGF. In addition, endostatin has been reported to inhibit endothelial cell adhesion to collagen I via α2β1 integrin (Furumatsu et al. 2002).

Endostatin can also inhibit VEGF-mediated signaling by binding to VEGF receptors and blocking the phosphorylation of endothelial NO synthase possibly via the serine/threonine protein phosphatase 2A (PP2A), leading to inhibition of the proliferation and migration of endothelial cells (Kim et al. 2002, Urbich et al. 2002, Kojima et al. 2008). PP2A was also reported to be involved in endostatin’s downregulating effects on soluble guanylate cyclase in endothelial cells (Schmidt et al. 2005b). The effects of endostatin on the morphology of endothelial cells were shown to be dependent on ERK1/2, and endostatin induces dephosphorylation of the ERK1/2-kinase via PP2A (Schmidt et al. 2006).

Endostatin was also shown to promote the integrity of the retinal endothelial barrier by upregulating the levels of occludin, a constituent of tight junctions, and phosphorylating it on specific regions by activation of p38 mitogen-activated protein kinase and ERK1/2 (Brankin et al. 2005, Campbell et al. 2006).

Endostatin downregulates several genes in growing endothelial cells, including immediate early response genes, cell-cycle-related genes, and genes regulating apoptosis inhibitors, mitogen-activated protein kinases, focal adhesion kinase, G-protein-coupled receptors mediating endothelial growth etc. (Shichiri & Hirata 2001). In another study, endostatin has been shown to downregulate several proangiogenic signaling pathways such as Isds, hypoxia-inducible factor 1-α and ephrins, and upregulate many antiangiogenic genes such as TSP-1 and vasostatin in human microvascular endothelium (Abdollahi et al. 2004).

2.6 The Kidney

The nephron is the structural and functional unit of the kidney that eliminates wastes from the body, regulates blood volume and blood pressure and controls levels of electrolytes and metabolites (Ross et al. 2003). One human kidney contains 800 000 to one million nephrons. Each nephron is composed of an initial filtering component consisting of the glomerulus and the Bowman’s capsule, and a renal tubule specialized for reabsorption and secretion (Fig. 3A). Correct function of the nephrons is vital to life and any disturbances lead to a decrease in
kidney function and to development of nephropathies, often leading to end-stage renal disease.

Fig. 3. Structure of the nephron and the glomerular filtration barrier. A) Schematic picture of the nephron showing the urinary filtration route from the glomerulus to the proximal tubule, loop of Henle, distal tubule, and to the collecting duct. B) An EM image of mouse glomerulus showing the constituents of the glomerular filtration barrier. The glomerular ultrafiltrate travels from the vascular lumen through the fenestrated capillary wall (black arrows), the GBM, and the slit diaphragms of the podocyte foot processes (P) to the urinary space (U), as indicated by the white arrow. C) Higher magnification showing the slit diaphragm (arrow) between the podocyte foot processes. E = endothelial cell.

2.6.1 Glomerulus

The glomerulus is a capillary tuft that performs the first step in filtering blood to form urine. The traditional constituents of the glomerular filtration barrier of the kidney are the fenestrated endothelial cells, the glomerular BM (GBM), and the podocyte foot processes with slit diaphragms (reviewed by Patrakka & Tryggvason 2010) (Fig. 3B). The importance of the podocyte and endothelial cell glycocalyx consisting of mostly proteoglycans has been highlighted in recent studies (reviewed by Salmon et al. 2009). The plasma molecules are sieved through the glomerular filter based on their size and charge (reviewed by Haraldsson et al. 2008). The size-selectivity of the renal filtration is widely accepted, whereas the charge-selectivity is still under investigation (Goldberg et
The precise location of the size- and charge-selection is currently a matter of debate. Present data suggests that all three layers of the glomerular capillary wall are needed to maintain normal filtration function. However, recent studies have underlined the role of the podocytes in the filtering process (reviewed by Patrakka & Tryggvason 2009). Disruption of the glomerular filtration barrier results in loss of permselectivity and the appearance of macromolecules, such as albumin, in the urine. Elevated amounts of proteins passing through the glomerular barrier lead to nephrotic syndrome with proteinuria, hypoalbuminemia and edema, most often resulting eventually in end-stage kidney failure through progressive loss of filtration surface. Both genetic and environmental factors contribute to the development of nephrotic syndrome (reviewed by Zenker et al. 2009). Animal models have significantly advanced the understanding of the pathogenesis of renal diseases and the use of genetically modified mice has favored the knowledge of physiologic processes and molecular mechanisms governing common and rare kidney diseases (reviewed by Zoja et al. 2004 & 2006).

2.6.2 Glomerular basement membrane

The GBM is a unique type of BM due to its extensive thickness and its position between two cell layers, podocytes and endothelial cells. The GBM is formed by fusion of two separate layers (glomerular endothelial BM and podocyte epithelial BM) during kidney development (reviewed by Abrahamson 1987 and McCarthy 1997). However, recent evidence suggests that the epithelial and endothelial sides of the GBM differ in their molecular composition, at least in the localization of different collagen IV isoforms (reviewed by Abrahamson 2009a). The mature GBM is known to consist of both the α1α2α1 and α3α4α5 subtypes of collagen IV, the first one produced by endothelium and the latter one by podocytes, and immunolocalization studies have shown that the collagen IV subtypes remain on the side of the GBM, where they have been produced (Abrahamson et al. 2009b).

The GBM has a specific role in the maintenance of the glomerular filtration barrier and mutations in some of its components have been shown to be the cause of several hereditary glomerular diseases (reviewed by Gubler 2008). For example, Alport syndrome, characterized by hematuria, thickening and splitting of the GBM, podocyte effacement and progressive proteinuria, is caused by mutations in the α3-, α4- or α5-chains of collagen IV, which compose the collagenous network of mature GBM (reviewed by Heidet & Gubler 2009). Lack
of the α3(IV) chain in mice has also been shown to result in softening of the glomerular stiffness, most likely contributing to the disease by permitting increased distension with hemodynamic force (Wyss et al. 2010). In the mature GBM, the main laminin is laminin-521 (composed of α5, β2, γ1 chains). Pierson syndrome, with eye abnormalities and progressive nephrotic syndrome, has been shown to be caused by mutations in the laminin β2 gene (reviewed by Matejas et al. 2010). Mice lacking laminin β2 develop proteinuria which precedes podocyte abnormalities, showing that the GBM alone can also act as a protein barrier (Jarad et al. 2006).

HSPGs, carrying negatively charged GAG side chains, provide anionic charge to the GBM. In the GBM three HSPGs, agrin, perlecan, and collagen XVIII, have been identified, the majority of them representing agrin (Kanwar et al. 1980, Harvey et al. 2007). The negative charge of the GBM has classically been considered to form an important part of the glomerular filtration barrier, although the extent of its contribution has lately been re-evaluated as genetically engineered mice lacking podocyte-derived agrin and perlecan heparan sulfate side chains do not develop proteinuria (reviewed by Harvey & Miner 2008, Goldberg et al. 2009).

2.6.3 Podocytes

Podocytes are highly differentiated epithelial cells connected to each other by specific adherens junctions, the slit diaphragms (Fig. 3C). The importance of the slit diaphragms is highlighted by the nephrotic diseases caused by mutations in their components (reviewed by Patrakka & Tryggvason 2010). The interdigitating foot processes of the podocytes are attached to the GBM (reviewed by Mundel & Kriz 1995). The mechanical properties of podocytes are unusual, being extremely stiff compared to most other cell types (Tandon et al. 2007). The high stiffness is most likely needed to permit podocytes to maintain their shape and structure, particularly the slit diaphragm, and the integrity of glomerular capillaries, in the presence of capillary hemodynamic forces of the glomerulus. Podocyte injury and loss have been observed in various glomerular diseases leading to kidney failure (reviewed by Shankland 2006). For example, recent evidence suggest that the onset of albuminuria in diabetic nephropathy is associated with podocyte injury, caused by hyperglycemia and mechanical stress due to glomerular hypertension, early in the progression of the disease, leading to further kidney damage (reviewed by Lewko & Stepinski 2009 and Stitt-Cavanagh et al. 2009). A
common final pathway of the podocyte’s response to injury is a change in shape called effacement, during which the foot processes widen and shorten leading to a flat and elongated cell shape with a reduced number of filtration slits. Effacement is an active phenomenon initiated by changes in the podocyte’s cytoskeleton (reviewed by Chuang & He 2009). Correct interaction of podocytes with the ECM is crucial in maintaining the order of foot process architecture (reviewed by Kretzler 2002).

Podocytes are connected to the underlying GBM through two major cell adhesion complexes: α-β-dystroglycans and α3β1-integrin (Adler 1992, Raats et al. 2000), both capable of binding several common BM components (Cybulsky et al. 1992, Hohenester et al. 1999). The dystroglycan complex links the cytoskeleton to the underlying matrix by its polyanionic binding site for the cationic laminin globular-binding domain common to several matrix proteins (laminins, agrin, perlecan, etc) (Hohenester et al. 1999). Splitting of α-dystroglycan from its binding partners in the GBM leads to BM abnormalities and podocyte effacement (Kojima et al. 2004). The quantity and distribution of dystroglycan is altered in minimal change nephritic syndrome in humans (Regele et al. 2000) and in experimental animal models of glomerulopathies (Raats et al. 2000), all characterized by podocyte foot process effacement. Podocytes are known to express several integrin types (reviewed by Kreidberg & Symons 2000), the major podocyte form being integrin α3β1. It is known to bind collagen IV, fibronectin, laminin, and nidogen in the GBM (Cybulsky et al. 1992). Mice with a podocyte specific deletion of integrin β1 show severe glomerular BM defects with podocyte foot process effacement (Kanasaki et al. 2008) and lack of α3 integrins in mice leads to disorganized GBM and failure in mature foot process formation (Kreidberg et al. 1996, Sachs et al. 2006).

Action of the Wnt signaling pathway has been reported in the kidney podocytes. Induction of integrin-linked kinase (ILK), which links the integrins to the actin cytoskeleton, has been demonstrated in renal diseases with podocyte foot process effacement (Kretzler et al. 2001). ILK has been shown to affect the Wnt signaling pathway by inducing translocation of β-catenin to the nucleus and induction of lymphocyte enhancing factor-1 (LEF-1) (Novak et al. 1998, Wu et al. 1998). Also in cultured podocytes, overexpression of active ILK induced translocation of β-catenin to the cell nucleus and nuclear colocalization of β-catenin with LEF-1 (Teixeira et al. 2005). Mice with a podocyte-specific deletion of ILK develop kidney failure with podocyte foot process effacement (Dai et al. 2006, El-Aouni et al. 2006, Kanasaki et al. 2008). In addition, in a recent paper
by Dai et al. (2009), overexpression of Wnt1 in podocytes was shown to activate β-catenin and lead to albuminuria, whereas blockade of Wnt signaling ameliorated podocyte lesions. Moreover, a podocyte-specific knockout of β-catenin in mice protected against albuminuria after injury. The authors also observed upregulation of Wnt1 and active β-catenin in podocytes in human proteinuric kidney diseases with podocyte dysfunction.

2.6.4 **Mesangium**

Mesangial cells are a special form of microvascular pericytes, found in the interstitium between endothelial cells of the glomerulus. They provide structural support for the glomerular capillary loops, generate and control the turnover of the mesangial matrix, contribute to the regulation of glomerular filtration, and serve as a source and target of growth factors (reviewed by Schlöndorff & Banas 2009). The mesangial cells have contractile properties generated by anchoring filaments on the GBM opposite the podocyte foot processes. On the capillary lumen side, mesangial cells are in direct contact with endothelial cells without an intervening BM. The importance of crosstalk between the mesangial cells and the podocytes is highlighted by the fact that podocyte injury frequently results in mesangial cell proliferation, whereas mesangial cell injury leads to foot process effacement and proteinuria.

Mesangial cells generate and embed in their own ECM, the mesangial matrix. It is composed of collagen IV (only the α1- and α2-chains), collagen V, laminin, fibronectin, heparan sulfate and chondroitin sulfate proteoglycans, entactin, and nidogen. The components of the mesangial matrix contribute to ECM-cell signaling via integrins and MMPs (Bieritz et al. 2003, Ronco et al. 2007). The composition and amount of mesangial matrix are tightly controlled and they can be markedly altered during disease, such as diabetic nephropathy (reviewed by Couchman et al. 1994 and Mason & Wahab 2003). The mesangial cells play a significant role in the events leading to the development of diabetic glomerulopathy by mesangial cell hypertrophy, proliferation, and expansion of the mesangial matrix.

2.6.5 **Renal tubule**

After filtration through the glomerulus, the primary ultrafiltrate enters the renal tubule, which can be divided into different segments based on their structure and
function: the proximal tubule, loop of Henle, and distal tubule (Ross et al. 2003) (Fig. 3A). The proximal tubule reabsorbs larger substances (glucose, amino acids, protein, etc.) from the glomerular filtrate. The loop of Henle is a U-shaped tube that connects the proximal and distal parts of the renal tubule. It has a crucial role in reabsorbing water from the kidney filtrate. The cells of the distal tubule contain numerous mitochondria to produce energy for active transport of ions. The ion transport is regulated by the endocrine system and is a critical factor in regulating blood pressure.

Renal proximal tubular epithelial cells are involved in a variety of vital functions. Of these, receptor-mediated endocytosis is an essential function of the cells to reabsorb and metabolize proteins and other substances from the glomerular filtrate (reviewed by Saito et al. 2010). The origin of proteinuria is found in either the glomerular filtration device or the proximal tubular reabsorption machinery. In normal situations, small amounts of predominantly low molecular weight proteins are filtered and reabsorbed by the proximal tubules via the receptor complex megalin/cubilin/amnionless (reviewed by Nielsen & Christensen 2010). The reabsorbed constituents are directed to the lysosomes, where proteins are degraded and other components such as vitamins are exported to the circulation for reuse. The receptors reabsorb proteins very efficiently from the primary ultrafiltrate, resulting in an almost protein-devoid urine (< 20 mg/l). During glomerular damage, the reabsorption machinery in the proximal tubule is challenged due to elevated amounts of proteins passing to the primary ultrafiltrate. Protein accumulation in the lysosomes of the proximal tubule, due to increased protein load, is thought to mediate inflammation and fibrosis, eventually leading to renal failure. In contrast, tubular (i.e. low molecular weight) proteinuria develops when the glomerular filtration process works normally, but the endocytic machinery of the proximal tubular cells is malfunctioning either by direct defects, affecting the receptors megalin and cubilin, or indirect causes involving changes in endocytic or recycling processes, such as in Imerslund-Gräsbeck syndrome or Dent’s disease, respectively (reviewed by Nielsen & Christensen 2010). Within the tubular proteinuria, the urinary protein composition mirrors more or less the composition in the primary ultrafiltrate. The proximal tubule also plays a crucial role in the pathogenesis of diabetic kidney disease (reviewed by Magri & Fava 2009). Glucose entry into proximal tubular cells is insulin-independent, which makes proximal tubular cells particularly sensitive to the deleterious effects of chronic hyperglycemia, leading to tubular hypertrophy and atrophy, and reduced organic ion transport (reviewed by Thomas et al. 2005).
2.6.6 Role of collagen XVIII in kidney

Collagen XVIII is expressed in the Bowman’s capsule, glomerular and tubular BMs, and the mesangial matrix of the kidney both in humans (Saarela et al. 1998a, Tomono et al. 2002) and mice (Muragaki et al. 1995, Sasaki et al. 2000, Miosge et al. 2003). In a recent study, collagen XVIII expression was shown to be elevated in the Bowman’s capsule and the GBM in a mouse model of anti-GBM glomerulonephritis, and the Col18a1−/− mice developed more severe glomerular and tubulointerstitial injury with altered matrix remodelling, enhanced inflammatory response and endothelial cell damage, than wild-type mice (Hamano et al. 2010). Interestingly, treatment of the knockout mice with endostatin did not affect the progression of anti-GBM disease, suggesting that the whole collagen XVIII molecule is needed to preserve the integrity of the ECM and capillaries in the kidney, protecting against progressive glomerulonephritis.

However, actions of the endostatin fragment have also been reported in several studies of kidney diseases. For example, plasma endostatin levels have been shown to be highly elevated in patients with end-stage renal disease (Rydzewska-Rosolowska et al. 2009). Endostatin’s actions have also been studied in several murine models of kidney diseases. Administration of endostatin peptide in a murine model of type 1 diabetic nephropathy suppressed glomerular hypertrophy, hyperfiltration, and albuminuria, as well as expansion of the glomerular mesangial matrix, accumulation of collagen IV, and expression of several proangiogenic factors, such as VEGF-A (Ichinose et al. 2005). In a murine ureteral obstruction model, the expression of collagen XVIII and endostatin was increased during the progression of renal fibrosis (Maciel et al. 2008). In contrast, in a murine model of endotoxemic acute renal failure, the expression of endostatin was not increased, although immunohistological examination revealed increased levels of endostatin staining in BMs, possibly through increased cleavage of collagen XVIII (Cichy et al. 2009).

2.7 Hydrocephalus

Human hydrocephalus is a significant medical condition with an estimated incidence of 1 in 1500 births. It is characterized by abnormalities in the production, flow or resorption of cerebrospinal fluid (CSF), resulting in brain ventricular dilatation (reviewed by Zhang et al. 2006). The enlarging ventricles of the hydrocephalic brain cause damage by destroying the ependymal cell lining of
the ventricle surface, compressing small blood vessels in the periventricular white matter, and stretching and eventually destroying axons, leading to loss of connections between neurons (reviewed by Del Bigio 2010b).

The CSF occupies the subarachnoid space and the ventricular system around and inside the brain and spinal cord. The CSF not only provides a protective fluid layer for the brain, but also delivers nutrients and growth factors important for maintenance of neural networks and removes waste products secreted by the brain parenchyma (reviewed by Macaulay & Zeuthen 2010). It is produced by the choroid plexuses within the brain ventricles and circulates from lateral ventricles to the foramen of Monro, third ventricle, aqueduct of Sylvius, fourth ventricle, foramina of Magendie and Luschka; to the subarachnoid space around the brain and spinal cord (Fig. 4A). The CSF is believed to reabsorb back into the bloodstream via two systems: by entering the dural venous sinuses via the arachnoid granulations and by lymphatic outflow (reviewed by Pollay 2010).

### 2.7.1 Choroid plexus

The choroid plexus consists of epithelial cells, fenestrated blood vessels, and the stroma that is rich with ECM (reviewed by Wolburg & Paulus 2010). The choroid plexus epithelial cells produce the CSF, and constitute the blood-CSF-brain barrier (reviewed by Engelhardt & Sorokin 2009). Among these, the choroid plexus also has other important functions, such as participating in repair processes after trauma and changes in the anatomy and physiology of the choroid plexus have been linked to aging and several neurodegenerative diseases (reviewed by Emerich et al. 2005). The choroid plexus is divided into four parts: one in each lateral ventricle, one in the third, and one in the fourth ventricle. The production of the CSF in the choroid plexuses requires several transporter proteins, such as aquaporins (reviewed by Filippidis et al. 2010). The apical surface of the choroid plexus epithelial cells contains extended microvilli, while the basal side forms an extended basal labyrinth that lays on a BM separating the cells from the inner stroma of highly vascularized connective tissue (Fig. 4C). The blood vessels are fenestrated in order to represent permeability. To prevent the diffusion of blood-borne substances into the ventricle, the choroid plexus epithelial cells are connected to each other by tight junctions.

Hydrocephalus can be caused by overproduction of the CSF from the choroid plexuses. This has been reported in a rare condition called diffuse villous hyperplasia of the choroid plexus, characterized by diffuse enlargement of the
entire choroid plexus (reviewed by Cataltepe et al. 2010), and also in choroid plexus tumors (reviewed by Rickert & Paulus 2001). In mice, there are at least two reports where development of hydrocephalus occurs due to morphological changes in the choroid plexus epithelial cells: deficiencies in the transcription factors Otx2 and E2F-5 (Makiyama et al. 1997, Lindeman et al. 1998).

Fig. 4. The production, flow, and reabsorption of CSF. A) A schematic picture showing the route of CSF flow from the brain ventricles to the subarachnoid space. B) A schematic picture showing the structure of the brain meninges. The CSF is absorbed back into the blood stream from the subarachnoid space via arachnoid granulations making protrusions into the venous sinuses of the dura mater. C) An EM image of a mouse choroid plexus, which produces the CSF from blood-derived substances that are delivered from the vascular lumen, through the fenestrated vascular wall and endothelial and epithelial BMs, to the cell and finally excreted into the brain ventricle by extensive apical microvilli (black arrows), as indicated by the white arrow.
2.7.2 Ependyma

The ependyma is a simple ciliated epithelium that lines the ventricular surface of the central nervous system, extending from the lateral ventricles to the spinal cord (reviewed by Del Bigio 2010a). Ependymal cells lie on a thin BM and are connected to each other by zonula adherens type junctions. The apical surface is covered by microvilli and most of the cells have a central cluster of long cilia that have been shown to beat in a coordinated manner. It has been suggested that ependymal cilia may help to create concentration gradients of guidance molecules in CSF that serve to direct neuroblast migration from the lateral ventricle wall into the olfactory bulb (Sawamoto et al. 2006). Atrophy of ependymal cilia occurs prior to loss of ependymal cells in hydrocephalus due to increased intraventricular pressure. However, primary dysfunction of cilia can also cause hydrocephalus in humans (reviewed by Zariwala et al. 2007). In support of this, several strains of mice with targeted mutations in cilia proteins develop hydrocephalus with or without abnormalities in the cerebral aqueduct (reviewed by Del Bigio 2010a).

2.7.3 Meninges

The meninges covering the brain consist of the dura mater and the leptomeninges (arachnoid and pia mater) (reviewed by Weller 2005) (Fig. 4B). Dura forms an outer, thick layer closest to the skull. It contains large veins carrying blood from the brain. The pia mater is a delicate BM adhering to the surface of the brain and spinal cord, known to be important for cortical histogenesis (Halfter et al. 2002). It contains capillaries that nourish the brain. The arachnoid membrane between the dura and pia is formed by epithelial cells that are folded onto themselves forming a double-layered structure. The subarachnoid space located between the arachnoid and the pia mater is filled with the CSF. Fine filaments called arachnoid trabeculae pass from the arachnoid through the subarachnoid space to be in contact with the pia mater. Arachnoid granulations are protrusions of the arachnoid through the dura. Leptomeningeal cells form channels in the core and apical cap of arachnoid granulations for the drainage of CSF into venous sinuses. Hydrocephalus due to obstruction of the CSF outflow route is a common result of subarachnoid space hemorrhages (reviewed by Germanwala et al. 2010).
2.7.4 Genetics of hydrocephalus

Human hydrocephalus can be classified into two clinical forms, congenital and acquired. The development and progression of congenital hydrocephalus is a dynamic process that is not yet well understood. Congenital hydrocephalus may occur alone (non-syndromic) or as part of a syndrome with other anomalies (syndromic) (reviewed by Partington 2001). In syndromic forms, it is hard to define the defective gene because of the association with other anomalies. Genetic factors are involved in the pathogenesis of hydrocephalus in about 40% of cases (reviewed by Zhang et al. 2006). Besides genetic factors, many other factors influence the development of congenital hydrocephalus, such as congenital malformations, intracerebral hemorrhage, and infections. Most of the known hydrocephalus gene products are important cytokines, growth factors, or related molecules in the cellular signal pathways during early brain development.

Genetic studies in hydrocephalic animal models have increased the knowledge of the underlying pathology of hydrocephalus. For example, the autosomal recessive congenital hydrocephalus (ch) mouse line was reported to have a mutation in the winged helix/forkhead transcription factor gene, Foxc1 (Kume et al. 1998). Mouse models have also shown that hydrocephalus may be caused by disruption of the ECM. In the transforming growth factor (TGF)-β1 overexpression mouse model, the changing expressions of MMP-9 and its specific inhibitor TIMP1 were found to be important factors in the spontaneous development of hydrocephalus by altering the ECM environment (Zechel et al. 2002), and by disrupting vascular ECM remodeling, promoting hemorrhages, and altering the re-absorption of CSF (Crews et al. 2004). In another mouse model, ablation of the nonmuscle myosin heavy chain II-B results in severe hydrocephalus with enlargement of the lateral and third ventricles, possibly due to abnormalities in the cell adhesive properties of neuroepithelial cells (Tullio et al. 2001).

2.8 Hair Follicles

The presence of hair is characteristic for mammals, in which it exerts a wide range of tasks, including physical protection, thermal insulation, masking, sensory functions, and social interactions. The hair follicle is an exceptional miniorgan that can be used in studies of epithelial-mesenchymal interactions, various biological signaling cascades, stem cells and tissue regeneration, control of cell
growth, differentiation, migration and death, and in regulation of angiogenesis (reviewed by Schneider et al. 2009).

### 2.8.1 Hair follicle structure, development, and cycling

The hair follicle is composed of an outer root sheath that is continuous with the epidermis and lies on the epidermal BM, an inner root sheath, and the hair shaft itself (reviewed by Stenn & Paus 2001) (Fig. 5A). The hair grows from the bulb of the follicle, which contains proliferating progenitor cells, surrounding a pocket of specialized mesenchymal cells, the dermal papilla, which is covered by a BM. Each hair follicle cycles through periods of regression (catagen), rest (telogen), and active growth (anagen) (reviewed by Alonso & Fuchs 2006) (Fig. 5B).

During anagen, the progenitor cells of the hair bulb divide rapidly and move upward to differentiate and give rise to the inner root sheath and the hair shaft. During catagen, the follicle undergoes apoptotic death and the dermal papilla moves upward beneath the hair bulge, where it remains during telogen. The hair bulge contains multipotent epithelial stem cells that not only contain the progenitors for new hair growth (migrating downward) but also participate in re-epithelialization of the epidermis after skin damage (migrating upward) (Taylor et al. 2000). Induction of a new anagen occurs after the dermal papilla activates bulge cells to regenerate the follicle. Hair growth continues in this cyclic fashion throughout postnatal life.

The development of the hair follicles requires coordinated ectodermal and mesenchymal interactions (reviewed by Millar 2002) (Fig. 5B). In an embryo, where the skin is formed by ectodermal cells lying on a BM, signals from the mesenchymal cells beneath the ectodermal cells determine whether ectodermal cells start to differentiate to form epidermis or a hair follicle. Subsequent ectodermal messages lead mesenchymal cells to reorganize to form the dermal papilla. Finally, due to signals from these dermal papilla cells the developing hair follicle starts to grow and differentiate. Several conserved signaling pathways have been identified to play a role in hair development, including members of the Wnt, FGF, Shh, bone morphogenetic protein (BMP), ectodysplasin, and Notch families (reviewed by Millar 2002 and Mikkola & Millar 2006).
Fig. 5. The structure, development, and cycling of hair follicles. A) A schematic picture showing the structure of a mature hair follicle. B) A schematic picture showing the morphogenesis and the following cycle of a hair follicle. Epidermal-mesenchymal interaction is required to form an epithelial thickening (placode), which grows into the dermis and induces the formation of a mesenchymal condensation (dermal papilla), which starts to grow the hair shaft. After the morphogenesis, the hair follicle enters the hair cycle and regresses by apoptosis during the catagen phase, leading to
upward movement of the dermal papilla to close proximity of the hair bulge. The hair follicle stays in the resting (telogen) phase until the activation of new progenitor cells from the bulge to form a new hair, after which the hair follicle enters the anagen (growth) phase (Modified from Seppinen 2009, Acta Universitatis Ouluensis D 1038 by permission of the author).

The mouse back skin contains several subtypes of hair: the primary guard hairs, constituting less than 5% of mouse hair on the back skin, overlay and protect the 95% of secondary hairs, including awl, auchen and zigzag subtypes, having a crucial physiological role as a thermal insulator, compensating for the lack of sweat glands on the mouse body (reviewed by Schlake 2007). Mouse hair follicle development takes place in three waves, starting with the primary (guard) hairs at embryonic day 14 (E14), followed by secondary intermediate (awl, auchene) at E16 and downy (zigzag) hairs between E18 and a few days after birth. Hair placode formation signals vary significantly between the different hair types (reviewed by Schneider et al. 2009). The first two hair cycles are fairly synchronous, after which environmental factors start to influence the length of the telogen phase.

### 2.8.2 Role of ECM in hair follicle development and cycling

Both the morphogenesis and cyclic growth of the hair follicle are characterized by significant remodeling of the ECM, and the ECM molecules have been shown to play a role in these processes. The structure and the components of the BM differ between the upper and the lower portions of the hair follicle, the lower portion (hair bulb area) expressing less common BM proteins and lacking hemidesmosomes, which are present in the upper portion (Chuang et al. 2003, Joubeh et al. 2003, Malgouries et al. 2008). This is possibly due to differences in the stability, the lower portion moving upward in catagen and downward in anagen, while the upper part represents the permanent region of the hair follicle. During hair follicle morphogenesis and cycling, the BM undergoes changes in its laminin composition. In developing hair follicles, laminin-332, normally a prominent component of the epidermal BM (Rousselle et al. 1991), and laminin-111 are both downregulated (Nanba et al. 2000; Hayashi et al. 2002), leaving laminin-511 as the primary laminin of the early developing hair follicle. Lack of this laminin in mice results in failure of hair follicle morphogenesis (Li et al. 2003a) due to impaired production of noggin in the dermal papilla, which leads to epithelial LEF-1 expression and amplification of Shh signaling (Gao et al. 2008).
Within cycling mouse hair follicles, the expression of laminin-511 is increased in the beginning of anagen, while the expression of laminin-332 is increased in late anagen and is then transiently downregulated, indicating a positive role for laminin-511 and a negative role for laminin-332 on hair growth (Sugawara et al. 2008).

The outer root sheath expresses $\alpha_2\beta_1$, $\alpha_3\beta_1$ and $\alpha_6\beta_4$ integrins at different levels according to the region of the hair follicle (Commo & Bernard 1997). Conditional knockout of the $\beta_1$-integrin gene in mouse keratinocytes has been shown to lead to severe hair follicle abnormalities as well as to epidermal BM alterations with skin blistering disease (Brakebusch et al. 2000, Raghavan et al. 2000). Similarly, deletion of the $\alpha_3$-integrin subunit in mouse skin leads to severe abnormalities in the hair follicle morphology (Conti et al. 2003). In addition, conditional knockout of ILK in mouse keratinocytes leads to blistering skin disease and hair loss due to impaired migration of proliferative progenitor cells during follicle morphogenesis (Lorenz et al. 2007, Nakrieko et al. 2008).

The ECM remodeling requires protein degradation, and the actions of proteases and their inhibitors have also been reported during hair follicle morphogenesis and cycling. Hair follicles are known to produce collagenases (Yuspa et al. 1993, McGowan et al. 1994, Scandurro et al. 1995, Obana et al. 1996) and the collagenase activity is increased in early anagen (Paus et al. 1994). MMPs -2, -9 and -19 have been reported to be expressed in the hair follicles (Jarrousse et al. 2001, Sadowski et al. 2003) and the expression of MMPs and their inhibitors are known to reflect hair cycle-dependent changes (Kawabe et al. 1991, Krejci-Papa & Paus 1998). During follicle morphogenesis, collagen VII is absent from the tips of the developing buds, while MMP-2 is strongly expressed in the mesenchymal cells surrounding the downward migrating epithelial cells (Karelina et al. 2000). Both the loss of collagen VII and activation of MMP-2 are suggested to play an important role in the localized ECM turnover required for hair follicle morphogenesis. In addition, lack of proteinase cathepsin L in mice has been shown to result in severe malfunction of hair follicle cycling and morphogenesis, including retarded final stages of morphogenesis and induction of the cycling (Roth et al. 2000, Tobin et al. 2002).

The hair follicles have also been reported to express several proteoglycans (Westgate et al. 1991, Couchman 1993, Kaplan & Holbrook 1994, Malgouries et al. 2008). The GAG side chains of proteoglycans are known to bind to and modulate the functions of a large number of signaling molecules that are important for cellular differentiation or proliferation, including various FGFs.
(Pellegrini 2001), VEGF (Cohen et al. 1995), Shh (Bornemann et al. 2004), BMPs (Takada et al. 2003), and Wnts (Bornemann et al. 2004). All of these growth and morphogenetic factors are known to be involved in hair follicle biology (reviewed by Millar 2002 and Mikkola & Millar 2006). Thus, the network of proteoglycans in the hair follicle could represent a reservoir for growth promoters and/or modulators able to maintain hair follicle development and cycling. The fine composition of the proteoglycans of hair follicles has been reported to be altered during the anagen to catagen transition (Malgouries et al. 2008) and in addition, the expression of heparanase, the predominant enzyme capable of heparan sulfate degradation, has been shown to represent hair cycle-dependent changes (Zcharia et al. 2005). The degradation of heparan sulfates not only releases heparan sulfate-bound growth factors from ECM deposits, but also enables cell movement through extracellular barriers (reviewed by Fux et al. 2009 and Barash et al. 2010). Transgenic mice overexpressing heparanase have been shown to represent enhanced active growth phase of the first postnatal hair cycle and the subsequent growth cycles, simply due to eased migration of the follicular stem cell progeny through the ECM (Zcharia et al. 2005).

2.8.3 Hair follicle stem cells

Epidermis and its specialized compartments are the first line of defense against the various physical traumas of the environment and thus, they must constantly renew themselves. This is achieved through actively proliferating stem cells located in specific epidermal regions (reviewed by Blanpain 2010). In the adult skin, interfollicular epidermis and sebaceous glands are subject to constant self-renewal, whereas hair follicles cycle between growth, regression, and resting phases (reviewed by Alonso & Fuchs 2006). Under normal conditions, these three skin cell populations are each believed to be maintained by their own discrete stem cells (Fuchs & Horsley 2008). When tissue homeostasis is disrupted, however, any of the three stem cell populations is capable of producing all three structures.

In the hair follicle, stem cells reside in the bulge, located at the base of the part of the follicle that is established during morphogenesis but does not degenerate during the hair cycle (reviewed by Blanpain & Fuchs 2009). The bulge contains two distinct populations of multipotent stem cells, the other one attached to the BM while the other one is detached from it (Blanpain et al. 2004). Interestingly, collagen XVIII mRNA has been shown to be enriched in the bulge
stem cells independently of activation state (anagen vs. telogen) or attachment to BM (Blanpain et al. 2004). During the hair cycle, bulge stem cells are stimulated to exit the stem cell niche, proliferate, and differentiate to form the various cell types of mature hair follicles. In addition, the bulge is a reservoir of multipotent stem cells that can be recruited during wound healing to help the repair of the epidermis. Stem cells are located also elsewhere in the hair follicle. The upper part of the hair follicle, which interfaces with the interfollicular epidermis, is thought to be maintained by progenitors located in a hair follicle region known as the isthmus (reviewed by Blanpain 2010). Recently, Snippert et al. (2010) reported a new marker of these stem cells and showed that they can renew sebaceous cells and seed the epidermis throughout life, independently of Wnt signaling.

2.8.4 Wnt/β-catenin signaling in hair follicle development and cycling

The Wnt signaling pathway plays a crucial role in intercellular signaling during hair follicle morphogenesis (reviewed by Millar 2002), as well as during the cycling of mature follicles, where activation of the Wnt signaling is believed to control the telogen-anagen transition (DasGupta & Fuchs 1999, Huelsken et al. 2001, Van Mater et al. 2003, Lowry et al. 2005). Wnts are a large family of signaling molecules that bind to their frizzled receptors, which also form a large protein family. The binding of Wnt molecules to their receptors activates several different pathways that control cellular proliferation, fate, shape, and migration (reviewed by Clevers 2006). In the canonical Wnt/β-catenin pathway, the binding of Wnts to their frizzled receptors together with their co-receptors (LDL receptor-related proteins, LRPs) cause the inhibition of a complex of proteins that normally targets cytoplasmic β-catenin for degradation. As a consequence, β-catenin accumulates in the cytoplasm, translocates to the nucleus, and affects gene transcription via the LEF/T-cell factor family of DNA binding factors. Alternatively, in the non-canonical Wnt-signaling, binding of Wnt-molecules to their frizzled receptors is mediated by Knypek (an HSPG of the glypican family in zebrafish), leading to activation of Rho1.

Multiple Wnt genes and their frizzled receptors are expressed in developing and mature hair follicles (Hung et al. 2001, Reddy et al. 2001 and 2004). The actions of Wnt signaling in hair follicle morphogenesis and cycling have been studied by the aid of several genetically altered mouse lines. Overexpression of
Wnt3 in mouse skin has been shown to lead to a short hair phenotype with cyclical balding due to altered differentiation of the hair shaft precursor cells and defects in hair shaft structure (Millar et al. 1999). A conditional knockout of β-catenin in mouse skin has been reported to prevent hair placode formation (Huelsken et al. 2001, Zhang et al. 2009). Similarly, mice that lack Lef1 function, fail to develop whiskers and most of the pelage hair follicles (van Genderen et al. 1994, Kratochwil et al. 1996). In contrast, mutation of the endogenous epithelial β-catenin to a dominant-active form in mice has been shown to lead to expansion of hair follicle placode formation, the entire epidermis eventually adopting the hair follicle fate (Närhi et al. 2008, Zhang et al. 2008). The effects of constitutively active β-catenin could be suppressed by introducing a conditional, repressing mutation of Shh signaling or of Bmp signaling, demonstrating that the regulation of hair follicle placode formation is dependent on the presence of growth factor signal crosstalk involving β-catenin signaling through Shh and Bmp (Suzuki et al. 2009a). In addition, a complex interplay of Wnt/β-catenin and ectodysplasin/ectodysplasin receptor/NF-kappaB signaling pathways is needed for the initiation and maintenance of primary hair follicle placodes (Zhang et al. 2009). Moreover, inactivation of the β-catenin gene within the dermal papilla of fully developed hair follicles has been shown to result in reduced proliferation of the progenitors and their progeny that generate the hair shaft, premature induction of the catagen phase of the hair cycle, and inhibition of regeneration of the cycling follicle from stem cells (Enshell-Seijffers et al. 2010). Activation of the stabilized β-catenin transgene in the hair bulge of mice has been reported to lead to stimulated proliferation and bulge expansion, with the existing hair follicles entering anagen (Baker et al. 2010).

The role of the Wnt-signaling inhibitors has also been elucidated. Overexpression of the Wnt inhibitor Dickkopf 1 in mouse skin has been reported to prevent hair placode formation (Andl et al. 2002). The mouse mutant Hairless represents normal initial hair follicle morphogenesis, but the hair follicles fail to regenerate after the first regression and the mice lose their hair permanently. The activity of Wnt signaling has been shown to be downregulated in these mice (Beaudoin et al. 2005). In addition, deletion of an intracellular Wnt signaling inhibitor Apc in skin epithelial cells has been shown to result in the formation of an increased number of embryonic hair follicles and hair follicle abnormalities, while the development of interfollicular epidermis was unaffected (Kuraguchi et al. 2006). Recently, the gene mutated in a human disease called hereditary hypotrichosis simplex, characterized by hair loss and hair follicle miniaturization,
was discovered to be adenomatosis polyposis downregulated 1 (Shimomura et al. 2010). The authors found it to be an inhibitor of Wnt signaling in hair follicles containing a conserved frizzled motif, interacting with WNT3A and LRP5.

2.8.5 Hair follicle-associated angiogenesis

Active hair growth requires an effective supply of nutrients and oxygen by the blood circulation. During skin development, the hair follicle-linking vasculature is believed to be formed by a specific type of hair follicle bulge stem cells (Amoh et al. 2004). Hair follicle cycling is associated with cyclic expansion and regression of the perifollicular and interfollicular microvasculature (Yano et al. 2001). The anagen phase is coupled with active angiogenesis, inhibition of which leads to retardation of the hair growth (Mecklenburg et al. 2000), while rapid decrease in perifollicular vessel size occurs during catagen and telogen. The perifollicular angiogenesis is correlated with VEGF expression from the outer root sheath and over-expression of VEGF by these cells in mice has been shown to result in enhanced perifollicular vascularization and accelerated hair growth, while blocking of VEGF function led to retardation of hair growth (Yano et al. 2001). In contrast, the expression of TSP-1, a matricellular protein that inhibits endothelial cell proliferation and migration, has been shown to be upregulated in the hair bulb and dermal papilla during the catagen and telogen phases, and mice lacking TSP-1 show prolonged anagen phase with increased perifollicular vascularization. Moreover, overexpression of TSP-1 in the outer root sheath showed delayed hair follicle growth with reduced perifollicular vascularization (Yano et al. 2003).
3 Outlines of the present study

When this thesis work was started, collagen XVIII was known just as the precursor of the antiangiogenic molecule endostatin and little interest was paid towards the whole molecule. However, the mutations found in Knobloch syndrome patients as well as the first reports concerning the knockout mouse line lacking collagen XVIII suggested it to have various biological functions not restricted to angiogenesis. Not much was known about the distinct functions of the three isoforms of collagen XVIII or about the roles of its multiple biologically active domains besides the C-terminal endostatin domain. During the progression of this thesis work, increasing genetic data from a range of species revealed collagen XVIII to be an ancient molecule that is highly conserved in evolution, pointing to a fundamental biological task not restricted to vascular structures. In addition, in vitro studies suggested the longest isoform of collagen XVIII to be involved in the Wnt/β-catenin signaling network.

Utilizing a number of genetically modified mouse lines, this thesis work has aimed to expand the understanding of the in vivo functions of the full-length collagen XVIII molecule. Particular attention has been paid towards its structural role as a conserved BM molecule in a variety of tissues. The study also revealed new aspects of the discrete roles of the three isoforms of collagen XVIII and moreover, brought more evidence of the involvement of the longest isoform in the Wnt/β-catenin signaling cascade. The specific aims were:

1. To investigate the mechanism lying behind the development of hydrocephalus in the mouse line lacking collagen XVIII.
2. To study the ultrastructural role of collagen XVIII in the BMs of various tissues and to further characterize the stabilizing effects of its specific isoforms on kidney BM structures.
3. To study the effects of collagen XVIII and its frizzled-like domain on the development and cycling of hair follicles.
4 Materials and methods

The materials and methods used in this thesis are summarized in Table 3. Detailed descriptions of all the experimental procedures are presented in the original papers I–III.

Table 3. Experimental procedures used in the original publications.

<table>
<thead>
<tr>
<th>Method</th>
<th>Original paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genetically modified mouse lines</td>
<td>I, II, III</td>
</tr>
<tr>
<td>- Breeding, genotyping and collection of tissue samples</td>
<td></td>
</tr>
<tr>
<td>- Generation of transgenic mice overexpressing the N-terminal parts of the middle or the longest variant of collagen XVIII (K14-N-LG and K14-N-FZ, respectively)</td>
<td></td>
</tr>
<tr>
<td>Magnetic resonance imaging (MRI)</td>
<td>I</td>
</tr>
<tr>
<td>- Estimation of the total volume of the brain ventricles</td>
<td></td>
</tr>
<tr>
<td>Serum creatinine measurements</td>
<td>I</td>
</tr>
<tr>
<td>Histological examination of tissue samples</td>
<td>I, II, III</td>
</tr>
<tr>
<td>- Light microscopy</td>
<td></td>
</tr>
<tr>
<td>- Immunohistochemistry</td>
<td></td>
</tr>
<tr>
<td>Electron microscopy</td>
<td>I, II</td>
</tr>
<tr>
<td>- Chemical fixation for epon embedding</td>
<td></td>
</tr>
<tr>
<td>- Immunoelectron microscopy (IEM)</td>
<td></td>
</tr>
<tr>
<td>- Measurement of BM thicknesses</td>
<td></td>
</tr>
<tr>
<td>- High pressure freezing (HPF) &amp; freeze substitution (FS)</td>
<td></td>
</tr>
<tr>
<td>- Polyethyleneimine staining for anionic sites of GBM</td>
<td></td>
</tr>
<tr>
<td>Isolation of mouse kidney glomeruli</td>
<td>II</td>
</tr>
<tr>
<td>Glomerular stiffness measurements</td>
<td>II</td>
</tr>
<tr>
<td>Measurement of hair follicle length</td>
<td>III</td>
</tr>
<tr>
<td>Determination of hair follicle-associated angiogenesis</td>
<td>III</td>
</tr>
<tr>
<td>In situ hybridization</td>
<td>III</td>
</tr>
</tbody>
</table>
5 Results

5.1 Col18a1−/− mice manifest hydrocephalus (I)

Characterization of mice lacking collagen XVIII showed abnormalities in retinal vascularization and regression of hyaloid vessels (Fukai et al. 2002). Subsequently, we found that some of the Col18a1−/− mice in a C57BL/6J genetic background bred with the C57BL/6JOlaHsd strain, developed a significant enlargement of the skull with massive bleeding under the skull. Sectioning of the head revealed contraction of the brain and displacement of the skull bones due to pathologically elevated intracranial pressure, and also extensively dilated lateral ventricles compared with the wild-type. The incidence of overt hydrocephalus in the Col18a1−/− mice was investigated by crossbreeding heterozygous Col18a1+/− mice and observing the offspring for 2 months. Severe hydrocephalus was seen in nine out of 42 Col18a1−/− mice (21.4%) during this period, whereas only five of the 130 heterozygous littermates (3.8%) and none of the 33 wild-type littermates (0.0%) showed any signs of dilated ventricles. To detect small differences in brain ventricle sizes, we resorted to MRI. The brain ventricles of the wild-type mice showed only minor variations in size, whereas the ventricles of the Col18a1−/− mice varied from mildly dilated to massively enlarged. Statistical analysis of the volumes of the ventricles of 2.5-month-old mice without overt hydrocephalus showed Col18a1−/− mice to have slightly larger ventricles, 31.1 ± 5.1 mm³ (n = 5), than the control animals, 25.6 ± 2.8 mm³ (n = 6), the difference being statistically significant (p = 0.026).

5.2 Localization of collagen XVIII in the brain (I)

The localization of collagen XVIII in the brain was studied by immunofluorescence. The pial BM and endothelial BMs of most blood vessels showed clear staining with antibodies against collagen XVIII (J. Saarela et al. unpublished data), the strongest staining being seen in the epithelial BM of the choroid plexus. IEM of the choroid plexus with an antibody against the N-terminus of collagen XVIII revealed clear signals in the endothelial and epithelial BMs that could be localized to the border of the BM with the adjacent matrix.
5.3 Ultrastructural changes in the choroid plexus epithelium (I)

To avoid secondary changes caused by elevated intracranial pressure, only mice with a very mild phenotype (without apparent ventricular dilation and subarachnoid bleeding) were used to study the cause of the hydrocephalus. The brain blood vessels, the pial BM, the ciliated ependymal cell layer, and the subcommissural organ in the Col18a1−/− mice were morphologically similar to the wild-type, and no changes were seen in the structure of the cerebral aqueduct in the cases of mild dilation of the brain ventricles. However, in the mutant mice with severe hydrocephalus the cilia of the ependymal cell layer were atrophic, the cerebral aqueduct was sometimes narrowed, and the choroid plexus epithelium was ruptured. These changes are likely to be secondary to the elevated intraventricular pressure and massively enlarged ventricles.

EM studies of mildly affected mice revealed that the mutant choroid plexus epithelial cells were round in shape rather than cuboidal, with individual cells making protrusions into the ventricle. In addition, instead of being apical, the tight junctions were located more closely on the basal side of the epithelial cells in the choroid plexuses of the null mice, and there were more vesicles inside the mutant choroid plexus epithelial cells than in the wild-type, possibly indicating alterations in the CSF outflow. The apical microvilli were abnormally dilated and contained numerous vacuoles. The most remarkable finding was that the BM underlying the choroid plexus epithelial cells was broader in the null mice than in the wild-type. The thickness of the epithelial BM in the 2-month-old Col18a1−/− mice was found to be 86.4 ± 10.52 nm (n = 6, 19 measurements/mouse), compared with 61.4 ± 6.05 nm in their wild-type littermates (n = 6, 19 measurements/mouse). Thus, the mutant BMs were significantly broader (p < 0.001) and varied significantly in thickness (p < 0.001).

5.4 Broadening of BMs in Col18a1−/− mice (I)

We next expanded the analysis of the ultrastructure of BMs to other tissues. The epidermal BM of the skin was also found to be broadened in the null mice, the measured thicknesses at 12 days and 7–15 months being 59.2 ± 8.3 and 77.8 ± 8.95 nm, respectively, as compared with 46.4 ± 3.77 and 56.8 ± 5.81 nm in wild-type mice of the same ages (p < 0.001 in both the age groups). The BM thickness also varied significantly in the mutants (p < 0.001 in both the age groups). Collagen XVIII is also expressed on the matrix side of the BM
underlying the endothelial cell layer of heart atrioventricular valves. This BM was also shown to be clearly broader in the \textit{Col18a1}^{−/−} mice than that of the wild-type littermates, 128.44 ± 19.13 nm on the atrial side and 132.04 ± 23.66 nm on the ventricular side, as opposed to 64.22 ± 2.54 and 62.9 ± 3.04 nm, respectively. This broadening was also statistically significant ($p < 0.001$) on both the atrial and ventricular sides of the \textit{Col18a1}^{−/−} leaflets. In addition, both mutant BMs varied significantly in thickness ($p < 0.01$).

5.5 Improved preservation of mouse kidney ultrastructure using HPF-FS (II)

The kidney provides an interesting repertoire of BMs, and as the proximal tubular BM of \textit{Col18a1}^{−/−} mice showed significant broadening (see 5.7), we decided to use this tissue in further characterization of the changes seen in the BM ultrastructures of knockout animals. We compared the conventional and HPF-FS sample preparation methods for EM in order to determine which approach would lead to the best results when analyzing the roles of collagen XVIII in the BMs of mouse kidneys. The conventionally fixed samples of both distal and proximal tubular BMs showed a clearly detectable \textit{lamina lucida}, which was not seen in the HPF-FS samples, where the BM is seen as a homogenous network structure slightly broader in appearance than in conventionally fixed samples. The proximal tubular BM of HPF-FS samples showed a broader and looser network than in the distal tubular BM, suggesting different functional characteristics for these BMs. This ultrastructural difference was not as clear in the conventionally fixed samples, however, possibly due to shrinkage of the fine structures of the BMs. The GBM of the HPF-FS sample was broader in appearance than in the conventionally fixed sample and the podocyte foot processes lying on it were more rounded in shape than in the sharp-featured conventionally fixed sample, suggesting shrinkage of the structures. A higher magnification of the podocyte and endothelial cell surfaces in the HPF-FS sample showed a well preserved glycocalyx on both, while this was scarcely visible in the conventionally fixed sample. Thus, we concluded HPF-FS method superior compared to conventional fixation in preserving the kidney tissue ultrastructure.
5.6 Localization of collagen XVIII isoforms in kidney (I, II)

The N-terminal antibody recognizing all three collagen XVIII variants gave clear staining on the matrix side of the kidney tubular BMs and in the BM of the Bowman’s capsule, although some additional staining was detected on the lamina lucida side of the latter. In the GBM, a diverse staining pattern was observed, immunogold labeling being located on both sides. Faint staining was also detected in the mesangial matrix. To test whether collagen XVIII has a polarized orientation in the kidney BMs, HPF-FS kidney samples from wild-type mice were rehydrated to form cryosections and stained with antibodies against the N-terminal part of collagen XVIII or against the C-terminal endostatin domain of collagen XVIII. The N-terminal antibody showed staining at the BM-fibrillar ECM interface in the proximal tubular BM, while the immunosignal of the C-terminal antibody is located in the lamina densa. Similarly, the N-terminal antibody in the GBM showed staining on the edges of the BM, localized to both the podocyte and endothelial sides, while the immunosignal of the C-terminal antibody is localized within the GBM. Thus, the results of the immunolocalization studies suggest that collagen XVIII has a polarized orientation in kidney BMs, the C-terminal part lying within the lamina densa and the N-terminal part facing towards the fibrillar ECM.

To determine which of the three collagen XVIII variants are located in the kidney BMs, HPF-FS samples of the promoter-specific collagen XVIII knockouts were stained with antibodies against all three collagen XVIII variants (anti-all antibody) or against only the two longer variants (anti-long antibody). Both the proximal tubular BM and the distal tubular BM of the wild-type and promoter 2-specific knockout (Col18a1P2/P2) mice (lacking the two longer variants of collagen XVIII) showed a strong staining with the anti-all antibody, while no gold particles could be detected with the anti-long antibody. However, the tubular signal was lost in the promoter 1-specific knockout (Col18a1P1/P1) mice that lacked the short isoform of collagen XVIII. Thus, the IEM studies identified the promoter 1-derived protein as the sole isoform in the tubular BMs.

In the GBM, the anti-all antibody gave a clear staining on both the podocyte and endothelial sides of the GBM of the wild-type sample. However, the staining on the podocyte side was lost in the samples from the Col18a1P2/P2 mice that lacked the two longer variants, while the immunosignal on the endothelial side persisted. In contrast, the signal was seen on the podocyte side but almost lost on the endothelial side of the GBM in the Col18a1P1/P1 mice lacking only the short isoform.
isoform of collagen XVIII both with the anti-all and the anti-long antibodies. Thus, the longer collagen XVIII variants are localized on the podocyte side and the short variant on the endothelial side of the GBM.

5.7 Structural and functional defects in the knockout kidney (I, II)

EM showed clear broadening of the tubular BM of Col18a1−/− mice. To confirm this finding, the thickness of the proximal tubular BM was measured in control and mutant animals at ages of 12 days, 2 months, and 7–11 months (adult). The BMs of the knockout mice proved to be significantly broader in the younger age groups (p < 0.001), the measured thicknesses at 12 days and 7–15 months being 59.2 ± 8.3 and 77.8 ± 8.95 nm, respectively, compared with 46.4 ± 3.77 and 56.8 ± 5.81 nm in wild-type mice of the same ages. In addition, the mutant BM thickness varied significantly in the two oldest age groups (p < 0.05 at 2 months, p < 0.001 at 7–11 months). To further characterize the finding, the kidney cortex areas of total and promoter-specific knockout mice were prepared and processed by the HPF-FS method. Similarly to the conventionally fixed EM samples, the proximal tubular BM of the Col18a1−/− mice was broader than that of the wild-type, and a similar broadening of the proximal tubular BM was also seen in the Col18a1P1/P1 sample lacking only the short isoform of collagen XVIII, while the lack of the two longer variants did not lead to any broadening of the proximal tubular BM. In contrast, neither promoter-specific nor total knockout of collagen XVIII led to alterations in the distal tubular BM structure.

Careful studies of the BMs of the Bowman’s capsule and the glomerulus of the Col18a1−/− mice did not reveal any structural changes. Analysis of the HPF-FS-prepared specimens of the kidney GBM revealed no differences between the Col18a1−/−, Col18a1P1/P1, and Col18a1P2/P2 mice with their controls. However, the Col18a1−/− mice and the Col18a1P2/P2 mice lacking the two longer variants showed podocyte foot process effacement, with a flat, elongated cell shape, while the podocyte structures of both the wild-type control mice and the Col18a1P1/P1 mice lacking only the short isoform were unchanged. Thus, lack of collagen XVIII promoter 2 isoforms leads to podocyte effacement. Since the charge of the GBM can affect podocyte growth and development, we determined whether loss of collagen XVIII affects the GBM charge by visualizing the anionic sites of the GBM in kidney samples from wild-type and Col18a1P2/P2 mice by staining with the cationic probe polyethyleneimine. No differences in staining were found
among the GBMs from these different mice when analyzed for number, size, or intensity of staining.

In addition to the abnormalities seen in the proximal tubular BM and the podocytes, the mesangial matrix was expanded in some of the Col18a1−/− glomerulæ and its structure had altered from clearly defined to somewhat indefinite. Despite an increase in the amount of ECM in the mesangium, collagen IV staining did not give an increased immunosignal in the mutant glomerulæ compared with the wild-type. Similarly to this, comparisons of the localization and intensity of immunostaining with antibodies against classical BM components perlecan and collagen IV between the wild-type and total collagen XVIII knockout samples, in both the proximal and distal tubular BMs and the GBM, showed no difference between the genotypes.

In view of the changes detected in the kidneys of the Col18a1−/− mice, we measured serum creatinine levels in the mutant and control animals, and found these to be significantly elevated in the knockout mice (10.11 ± 1.59 µmol/l) compared with the wild-type (8.42 ± 2.07 µmol/l) (p < 0.025), indicating alterations in kidney filtration capacity.

To determine whether loss of collagen XVIII affects the mechanical properties of the glomeruli, we measured the elasticity of whole glomeruli using microindentation. The force-indentation relations of the wild-type glomeruli are remarkably similar, strongly suggesting that glomerular stiffness is a tightly controlled characteristic. In contrast, the glomeruli from the Col18a1−/− mice were on average significantly softer and more heterogeneous. Comparison of the average slopes of all the wild-type and Col18a1−/− glomerulæ suggests that Col18a1−/− glomerulæ are approximately 30% softer than the wild-type. The deviation of the data for Col18a1−/− glomerulæ from a straight line also suggests that there are structural changes altering their responses to small stresses.

5.8 Localization of collagen XVIII isoforms in hair follicles (III)

To achieve more information on the effects of collagen XVIII and its frizzled-like domain on Wnt/β-catenin signaling, we focused our studies on the well-characterized in vivo biological system of hair follicle development and cycling. To study the localization of collagen XVIII variants in hair follicles, skin samples of wild-type, Col18a1P2/P2 and Col18a1P1/P1 mice were treated with N-terminal anti-all, C-terminal anti-endostatin or anti-long antibodies. With the antibodies recognizing all three collagen XVIII variants, an immunosignal could be detected
in the BMs of the interfollicular epidermis, the hair follicles and dermal papilla, and in the skin capillaries. Most of the immunosignal represented the short isoform, since the staining intensity was not changed in the \textit{Col18a1}^{P2/P2} mice lacking the longer isoforms. However, strong staining of the longer isoforms was detected in the BM facing the dermal papilla and in a specific localization along the upper part of the hair follicle. Transversal sections of the hair follicles showed that within these upper locations the staining could be detected not only in the BM but also around the follicular cells. To see whether this cellular staining of collagen XVIII is localized in the bulge area of hair follicles, the sections were stained with an antibody against a bulge marker, keratin-15. In these experiments the staining with the anti-long antibody indeed colocalized with that of keratin-15, revealing that the long variants of collagen XVIII are localized in the bulge.

5.9 Lack of collagen XVIII longer isoforms in the C57BL/6 background leads to a faster start of the first hair cycle (III)

Histological examination of skin samples during the first hair cycle showed that the catagen of the first hair cycle occurs faster in the C57BL/6 \textit{Col18a1}^{−/−} mice. This can be seen at postnatal day 17 (P17), when the hair follicles of the control mice are only starting to regress, but in the \textit{Col18a1}^{−/−} mice the hair follicles have almost fully regressed, being much shorter than in the controls. At P25, the hair follicles in the \textit{Col18a1}^{−/−} mice have started the next anagen growth stage, while most of the follicles of the control mice are still in telogen. Interestingly, the phenotype of the \textit{Col18a1}^{P2/P2} mice is similar to the \textit{Col18a1}^{−/−} mice, suggesting that this accelerated hair cycle is due to the lack of the longer variants of collagen XVIII. The regression of the hair follicles in the \textit{Col18a1}^{P2/P2} mice is also slightly faster than in the controls, the follicles being slightly shorter both at P17 and P21. However, the accelerated catagen is not as obvious in these mice as it is in the \textit{Col18a1}^{−/−} and \textit{Col18a1}^{P2/P2} mice. At P25, the hair follicles in the \textit{Col18a1}^{P1/P1} mice have also entered anagen, and are thus significantly longer than in the controls, suggesting a faster growth of hair follicles also in these mice.

5.10 Delayed start of anagen in FVB/N \textit{Col18a1}^{−/−} mice (III)

In view of our plans to cross the \textit{Col18a1}^{−/−} mice with transgenic overexpression mice in the FVB/N background (see 5.12), we found it necessary to analyze how lack of collagen XVIII affects the hair cycle also in this background. Studies of
the first hair cycle of Col18a1−/− mice backcrossed to the FVB/N mouse line revealed that in this genetic background, the morphogenesis took place normally and entered the catagen and telogen phases simultaneously with the wild-type controls. However, the initiation of the following new anagen was delayed in the FVB/N Col18a1−/− mice as shown by shorter hair follicles on P25 compared to wild-type controls. To study whether the delay in the start of the new anagen in FVB/N Col18a1−/− could be due to affected hair follicle-associated angiogenesis, we stained skin sections of FVB/N Col18a1−/− and wild-type control mice with a CD31-antibody to visualize the blood vessels and measured the relative area covered by blood vessels from the images taken near the bulbs of hair follicles. At P1 and P17 proceeding the hair growth phases, the CD31-positive area was not changed between the genotypes (p-values 0.219 and 0.793 on P1 and P17, respectively), suggesting that angiogenesis is not impaired in the FVB/N Col18a1−/− mice when the hair follicles are just beginning to grow.

5.11 Lack of clear defects in early hair follicle morphogenesis (III)

To study whether the lack of collagen XVIII also affects the early stages of hair follicle morphogenesis, Col18a1−/− and wild-type embryos were collected from both C57BL/6 and FVB/N backgrounds at E14 when the development of the first hair follicles begins. The primary hair placodes were visualized by whole mount in situ hybridization using two placode-specific probes, Shh and Dkk4. Statistical analysis of the number of placodes per mm² in wild-type and mutant mice in either background revealed that the density of primary hair follicles was not altered in Col18a1−/− embryos. Further, vibratome sections did not highlight any morphological difference in placode formation.

5.12 Transgene-derived frizzled domain rescues the phenotype of Col18a1−/− mice (III)

To study whether the changes seen in the hair cycle of the Col18a1P2/P2 and Col18a1−/− mice are due to lack of the frizzled domain of collagen XVIII, we generated a transgenic mouse line overexpressing the N-terminal part of the longest variant of collagen XVIII containing the frizzled domain in skin (K14-N-FZ). As a control, we generated another transgenic mouse line overexpressing the N-terminal part of the middle variant of collagen XVIII lacking the frizzled domain in skin (K14-N-FZ), under the keratin-14 promoter. Analysis of the
expression of the transgenes showed clear staining in the BM region of the epidermis-dermis junction and in the hair follicles in both $K14-N-FZ$ and $K14-N-LG$ animals.

Both of the transgenic mouse lines were crossed with FVB/N $Col18a1^{-/-}$ mice to produce double mutant mice lacking native, full-length collagen XVIII and expressing only the N-terminal parts of the longer variants ($Col18a1^{-/-}; K14-N-LG$ and $Col18a1^{-/-}; K14-N-FZ$). Studies of the first hair cycle of these mice revealed that transgenic presence of the N-terminus of the longest variant of collagen XVIII, containing the frizzled domain, was able to rescue the phenotype of FVB/N $Col18a1^{-/-}$ mice, since the $Col18a1^{-/-}; K14-N-FZ$ mice showed no difference between the wild-type controls in the follicle lengths on P25. In contrast, the $Col18a1^{-/-}; K14-N-LG$ mice expressing the transgenic N-terminus of the middle collagen XVIII variant, lacking the frizzled domain, showed an even more delayed start of the new anagen compared to wild-type controls, the hair follicles were significantly shorter both on P21 and P25.
6 Discussion

6.1 Hydrocephalus in the Col18a1\(^{-/-}\) mouse line

We backcrossed C57BL/6J inbred Col18a1\(^{+/-}\) mice once with C57BL/6JOlaHsd mice and maintained the ensuing line by means of heterozygous (Col18a1\(^{+/-}\)) matings. Surprisingly, this led to the identification of a previously unidentified phenotypic alteration in the mutant mice as about 20% of these mice developed severe hydrocephalus, and dilation of the brain ventricles was observed in all of the mutant mice by MRI. It has been suggested that differences exist between the C57BL/6 strains and substrains (Wotjak 2003). While the inbred C57BL/6 strain is known to be susceptible to hydrocephalus, with an incidence of 4–6% (Mori 1968), our C57BL/6J mice showed a 3.8% incidence of overt hydrocephalus in heterozygous Col18a1\(^{+/-}\) mice and 21.4% in Col18a1\(^{-/-}\) individuals, compared with 0% in controls. Thus, the incidence of severe hydrocephalus was significantly increased in the Col18a1\(^{-/-}\) mice (\(P < 0.001\)), whereas the low occurrence of hydrocephalus in the heterozygous mice closely corresponds to the rate of spontaneous hydrocephalus development reported previously. Thus, the lack of collagen XVIII enhances the vulnerability of inbred C57BL/6 mice to dilation of the brain ventricles and the development of hydrocephalus.

The development of hydrocephalus may be caused by overproduction of CSF, obstruction of its free flow, or its impaired absorption in the subarachnoid space. Previous work and this study show that collagen XVIII expression in the brain takes place in the BMs of the blood vessels, the pia, and the choroid plexuses (Halfter et al. 1998, van Horssen et al. 2002). Attempts to investigate the mechanism lying behind the development of hydrocephalus revealed no differences in the pial BM, in the amount and localization of the brain vessels, or in the composition of the cortex between the wild-type mice and those with mildly dilated brain ventricles. However, significant broadening was observed in the epithelial BM of the choroid plexuses, which are composed of a tight epithelium with tight apical junctions that provide a basis for the blood–CSF barrier (Strazielle & Ghersi-Egea 2000). The Col18a1\(^{-/-}\) mice have tight junctions in their choroid plexuses, but the cell morphology is balloon-shaped rather than cuboidal, so that the tight junctions seem to be localized closer to the basal side, even in the mildly affected animals. Moreover, the microvilli of the apical surface of the choroid plexus epithelium in the null mice contain more
vacuoles than in the wild-type, as do the choroid plexus epithelial cells, possibly indicating alterations in CSF production. Investigations into whether the abnormal BM of the Col18a1−/− mice affects the expression of Na⁺K⁺-ATPase and aquaporin 1, which are involved in active Na⁺ transport and water flux from the blood across the choroid plexuses and into the ventricles (Ernst et al. 1986, Venero et al. 2001), revealed no differences in the immunolocalization of these molecules between the wild-type and Col18a1−/− samples. As the tissues responsible for the production of the ocular and cerebral fluids, the ciliary processes and choroid plexuses, respectively, are developmentally, structurally, and functionally comparable (Tripathi 1977), we propose here that in addition to malformation of the ciliary body of the eye (Ylikärppä et al. 2003a), lack of collagen XVIII in mice also results in ultrastructural changes in the BMs of the choroid plexuses in the brain. It has been suggested that the BM may promote the organization of the choroid plexus epithelial cells into a functional epithelium and thus maintain the integrity of the blood–CSF barrier (Stadler & Dziadek 1996). We consider it likely that the morphological changes detected in the choroid plexus epithelial cells and the alterations in the production of CSF in the Col18a1−/− choroid plexuses are due to changes in the epithelial BM, which lead to altered filtration of the brain fluid components from the choroid plexus capillaries into the epithelial cells, and not to changes in the major fluid transporters.

The Col18a1−/− mice are similar to Knobloch patients in terms of their eye abnormalities (Sertié et al. 2000, Fukai et al. 2002, Suzuki et al. 2002, Ylikärppä et al. 2003a), but encephalocele seems to be typical only of human patients. Kliemann et al. (2003) have reported on four patients with central nervous system defects, two of whom also had ventricular dilation with subependymal heterotopic nodules. In the light of these reports and our new findings with regard to Col18a1−/− mice, dilation of the brain ventricles seems to be a new, additional phenotype caused by collagen XVIII deficiency.

6.2 HPF-FS method for EM preparation in ECM research

In this thesis study, we compared the HPF-FS method with the conventional chemical fixation method for EM preparation in mouse kidney studies. Components of the ECM are known to be significantly altered by the standard techniques used to prepare samples for EM. These artifacts include the extraction or collapse of ECM proteins and proteoglycans, together with shrinkage of the
whole tissue compositions (Keene et al. 1997). The methodology of HPF-FS for EM sample preparation has been shown to restore the tissue fine structures close to the living state (reviewed by Studer et al. 2008). In a study of murine epidermal BM, Reipert et al. (2004) showed that the use of the HPF-FS method results in a very densely packed dermal matrix and absence of a clear lamina lucida. The lack of lamina lucida has also been presented with other cryotechniques (Goldberg & Escaig-Haye 1986, Chan et al. 1993, Chan & Inoue 1994). Thus, it is considered likely, that in standard EM preparations the lamina lucida is artificially created by shrinkage of the basal cell membrane and extraction of BM components, and the remaining lamina densa may be representing a precipitation of otherwise loosely arranged proteins (collagen IV, laminin, and others) (Keene et al. 1997, Reipert et al. 2004).

In our hands, the BMs of the HPF-FS mouse kidney samples were lacking a prominent lamina lucida. Instead, the BM zones were composed of a highly organized protein network structures. The samples prepared by the HPF-FS showed structural differences between BMs of different locations, the distal tubular BM being more compact than the proximal tubular BM. This supports the fact that BMs of different tissues demonstrate molecular and structural heterogeneity enabling them to perform specialized functions, proximal tubules being able to absorb larger substances (glucose, amino acids, protein, etc.) from the glomerular filtrate than distal tubules, which transport mostly ions. These structural differences are efficiently revealed in tissue samples prepared by HPF-FS, in which the overall BM structures are preserved with minimal extraction artefacts.

The importance of the podocyte and endothelial cell glycocalyx as a constituent of the glomerular filtration barrier has been highlighted in recent studies (reviewed by Salmon et al. 2009). The role of the glycocalyx has long been ignored largely due to its sensitiveness to standard fixations procedures making it difficult to visualize in EM. The overall tissue preservation in the HPF-FS samples of kidney glomerulus was significantly better than in the chemically fixed samples, and even the proteoglycans of the glycocalyx were nicely retained. Thus, the HPF-FS method has great potential in the future investigations of this highly interesting but poorly studied part of the glomerular filtration system.
6.3 Kidney abnormalities in the Col18a1<sup>−/−</sup> mice

Our IEM results indicating collagen XVIII staining along the tubular, Bowman’s capsule, and glomerular BMs and in the mesangial matrix agree well with previous findings. In addition, given that messenger RNAs corresponding to all three collagen XVIII isoforms are expressed in the kidney (Murakagi et al. 1995, Elamaa et al. 2003, Quélard et al. 2008), we show here that the shortest isoform is virtually the sole collagen XVIII variant in the tubular BMs, while the longer variants are also present in the GBM. Interestingly, we also found that the endothelial side of the GBM contains the promoter 1-derived short variant of collagen XVIII, while the podocyte side contains only the promoter 2-derived longer variants of collagen XVIII. It should be noted that it was not possible for us to distinguish with the methods available, whether one or both of the promoter 2 isoforms of collagen XVIII is localized on the podocyte side of the GBM. Previous studies at the mRNA level nevertheless indicate that both the middle form and the longest form (containing a frizzled domain) are present in the kidney (Murakagi et al. 1995, Elamaa et al. 2003). Recent evidence suggests that the epithelial and endothelial sides of the GBM differ in their molecular composition, at least in the localization of collagen IV isoforms (reviewed by Abrahamson 2009a). Altogether, we show here that collagen XVIII is also expressed as different isoforms on each side of the GBM.

Ultrastructural analysis of HPF-FS samples revealed an abnormally loose, network-like BM in the proximal tubules of Col18a1<sup>−/−</sup> mouse kidneys, a change that could be attributed to a specific lack of the short isoform of collagen XVIII. BMs from different regions of the nephron were distinct in their ultrastructure, the distal tubular BM being more compact than the proximal tubular BM. This difference probably reflects molecular and structural heterogeneity, enabling them to perform specialized functions, the proximal tubules being able to absorb larger substances (such as glucose, amino acids, and proteins) from the glomerular filtrate than the distal tubules, which transport mostly ions. Although the short isoform of collagen XVIII is normally present at similar levels in both the proximal and distal tubular BMs, its absence affects the fine structure, and hence possibly the function, only of the former.

No clear difference within the GBM ultrastructure was detected in either of the promoter-specific knockout mouse lines, nor in the Col18a1<sup>−/−</sup> mice lacking all the isoforms. Instead, we found that the total knockout and promoter 2-specific knockout mice were affected by podocyte foot process effacement while the
podocyte structure of the promoter 1-specific knockout was normal. Thus, loss of the two longer collagen XVIII variants evidently leads to abnormalities in podocyte structure, while loss of the short variant has no effect on the podocytes. Correct interaction of the podocytes with the ECM is crucial for maintaining the order of the foot process architecture (reviewed by Kretzler 2002). The podocytes are connected to the underlying GBM through two major cell adhesion complexes, αν/β3-dystroglycans and α3β1-integrin (Adler 1992, Raats et al. 2000), both capable of binding several common BM components (Hohenester et al. 1999, Cybulsky et al. 1992). Alterations in the binding properties of either one of these adhesion complexes have been shown to lead to glomerular defects characterized by podocyte foot process effacement (Adler 1992, Raats et al. 2000, Regele et al. 2000, Kojima et al. 2004, Kanasaki et al. 2008).

Thus, it is possible that the podocyte effacement in the Col18a1−/− and Col18a1P2/P2 kidneys may be due to defects in the interaction between the podocytes and the GBM. Endostatin, the C-terminal domain of collagen XVIII, has been shown to bind α5β1- and αvβ3-integrins (Wickström et al. 2002 & 2004, Faye et al. 2009b) and could be required for podocyte-GBM interaction. However, considering the polarized orientation of collagen XVIII molecules in the GBM, the endostatin domain appears rather to be employed for interactions with molecules within the BM. We therefore favor the possibility that the N-termini of the middle and long isoforms interact with the podocytes. In the light of the multidomain nature of the N-termini, several possibilities exist. Both the middle and long variants contain a 247 amino acid residue domain of unknown function (Rehn & Pihlajaniemi 1995), and so far there is no information on its binding activities. The N-termini of all three variants contain a TSP-1 homology domain (Rehn & Pihlajaniemi 1994b), which may possess biological activities. TSP-1 itself is known to bind many growth factors, proteases, cell surface receptors, and ECM proteins, thereby mediating specific cellular responses (reviewed by Kyriakides & Maclachlan 2009). Last but not least, the frizzled module of the longest isoform has been shown to be capable of binding Wnt3a in vitro and suppressing the stabilization of β-catenin in tumor cell lines (Quélard et al. 2008). Interestingly, Wnt-1 upregulation and active β-catenin have been observed in podocytes in cases of human proteinuric kidney disease with podocyte dysfunction (Dai et al. 2009). It is thus also possible that the podocyte effacement seen in the Col18a1−/− and Col18a1P2/P2 kidneys could have been due to a lack of the inhibitory effects of the frizzled module of collagen XVIII on Wnt signaling, leading to anomalous activation of the pathway.
Regardless of whether the function of collagen XVIII is to crosslink other BM components or to interact with cell receptors, loss of this collagen could alter the mechanical properties of the BM. If this is the case, the podocyte pathology could be a response to an abnormal mechanical environment. The mechanical environment is known to have dramatic effects on cell behavior in podocytes (Tandon et al. 2007) and in other situations (Discher et al. 2005). At this time, we cannot separate the mechanical characteristics of the whole glomerulus from those of the GBM, but the *Col18a1*−/− glomeruli are clearly softer than normal, and to a degree that is physiologically significant (Cortes et al. 1996, Levental et al. 2010). The softening of the glomeruli in *Col18a1*−/− mice could have contributed to podocyte effacement and organ dysfunction. A number of recent findings show that changes in the elastic modulus of cells or matrix can have direct effects on cell structure and function, leading to the hypothesis that modulation of the factors that control the elastic modulus of organs and tissues both in the cytoskeleton and in the ECM not only help us identify disease mechanisms, but also have potential therapeutic applications (Discher et al. 2005, Georges et al. 2007).

Despite the ultrastructural changes found in the podocytes and proximal tubular BMs, *Col18a1*−/− mice have a normal lifespan without any obvious signs of kidney malfunction. During our studies, however, the serum creatinine levels of the *Col18a1*−/− mice were shown to be elevated (10.11±1.59 mmol/l versus 8.42±2.07 mmol/l in controls). Creatinine is a muscular waste product which is filtered out of the serum by both glomerular filtration and proximal tubular secretion (10–20% of urine creatinine). Thus, in the light of the ultrastructural findings, the elevated creatinine levels of the *Col18a1*−/− mice could reflect minor malfunction of the glomeruli, proximal tubules, or both.

### 6.4 Role of collagen XVIII in BMs

We show that collagen XVIII has a structural role in BMs and that lack of it seems to affect BMs in a tissue-specific manner, so that some tissues show no obvious structural abnormalities, whereas others are clearly broadened. The BM abnormalities in the eye lead to defoliation of the posterior portion of the iris and atrophy of the ciliary body and retinal pigment epithelial cells (Ylikärppä et al. 2003a, Marneros et al. 2004), whereas those in the choroid plexus and the kidney tubules can cause hydrocephalus and altered kidney filtration, respectively. In most tissues, such as in the epidermal and atrioventricular valve BMs, however,
the altered BMs do not lead to obvious symptoms. It is significant that many of the patients with typical Knobloch syndrome symptoms have other, additional clinical findings, including kidney defects (Knobloch & Layer 1971, Czeizel et al. 1992). As in human patients, the most crucial defects in mice lacking collagen XVIII are detected in the eyes (Fukai et al. 2002, Ylikärppä et al. 2003a), but as shown here, abnormalities may also occur in other tissues. We consider it likely that, by rendering BM structures weaker, the lack of collagen XVIII may predispose the individual to a variety of symptoms, the severity of which is greatly dependent on the particular genetic environment.

Immunostaining with the classical BM components perlecan and collagen IV showed no differences in either staining intensity or localization between the wild-type and the collagen XVIII mutants, suggesting a true loosening of the BM scaffold rather than thickening due to abnormal accumulation of other BM components in these mice. It is possible that the broadening of the BMs in the Col18a1−/− mice seen at the EM level may be due to a minor increase in the amount of other BM components not detectable by immunostainings, or that some other BM components not tested so far may have altered the staining intensities in the mutant BMs. On the other hand, it is conceivable that collagen XVIII could have a role in binding to other BM components, as demonstrated by the binding of its C-terminal NC1 and endostatin domains to laminin-1, perlecan, heparin and fibulin-1 and -2 (Sasaki et al. 1998). Little is known about the binding activities of the N-termini. Studies of the longest isoform, containing the frizzled domain, suggest that this part of the molecule can be proteolytically released, and when released from the parent molecule can affect Wnt signaling (Elamaa et al. 2003, Quélard et al. 2008).

Collagen XVIII has a polarized orientation in the epidermal BM and Bruch’s membrane BMs of the eye, with its C-terminal endostatin domain embedded in the BM and the N-terminal portion located at the BM-matrix interface (Marneros et al. 2004, Elamaa et al. 2005). We suggest here that collagen XVIII has a similar polarized orientation in the kidney tubular BM (Fig. 6A). Moreover, the orientation seems to be polarized also in the GBM, with both the endothelial and epithelial isoforms having their C-terminal endostatin domains embedded in the BM and the N-termini at the BM-cell interface, in the case of the short form at the BM-endothelial cell junction and in the case of the middle and long forms at the BM-podocyte junction (Fig. 6B). The occurrence of collagen XVIII at the fibrillar matrix interface is suggestive of an anchoring function, and consequently the lack of this collagen entails loosening of the BMs. This seems to cause the critical
consequences in the eye, whereas it only predisposes other tissues to pathological conditions.

Fig. 6. A) Schematic illustration showing the polarized orientation of collagen XVIII in kidney tubular BM. Only the short collagen XVIII isoform is present. The previously reported binding partners of the C-terminal endostatin domain within the BMs are demonstrated. Putative binding partners of the N-terminus connecting collagen XVIII to the fibrillar matrix are suggested.
Fig. 6. B) Schematic illustration showing the polarized orientation of collagen XVIII in GBM. The short collagen XVIII isoform is present on the endothelial side of the GBM, while the podocyte side contains the longer isoforms. The frizzled domain of the longest collagen XVIII isoform is known to bind Wnt molecules.

6.5 Frizzled domain of collagen XVIII affects the hair cycle

Besides its structural role in BMs, collagen XVIII has several potential regulatory domains, including the capability to bind Wnt molecules as shown in in vitro studies (Quélard et al. 2008). Hence, we have studied here the effects of collagen XVIII on hair follicle development and cycling, known to be centrally regulated by Wnt signaling, in several genetically modified mouse lines. We found collagen XVIII to be continuously expressed along the whole length of the BM of the hair follicle as well as in the BM facing the dermal papilla regardless of the cycling phase. The short variant of collagen XVIII is expressed at high levels in both of the follicular BMs as well as in the BMs of interfollicular vasculature, while the
longer variants are expressed prominently in the BM facing the dermal papilla and in the region of the hair follicle bulge, where the staining is seen also around the follicular cells. The first hair cycle was shown to proceed faster in the \textit{Col18a1}\textsuperscript{−/−} mice. A similar result was obtained with the \textit{Col18a1}\textsuperscript{P2/P2} line, suggesting that the changes seen in the \textit{Col18a1}\textsuperscript{−/−} mice are due to the lack of the longer variants. The \textit{Col18a1}\textsuperscript{P1/P1} mice did not show as dramatic changes in the hair cycle as the \textit{Col18a1}\textsuperscript{−/−} and \textit{Col18a1}\textsuperscript{P2/P2} mice, but even in these mice the hair follicles did show a slightly faster regression and significantly accelerated growth compared to the controls. Despite of the changes seen during the first hair cycle, the formation of primary hair follicles is not altered in the \textit{Col18a1}\textsuperscript{−/−} embryos.

The short variant of collagen XVIII is highly expressed in the hair follicle BM that is continuous with the epidermal BM. Since we have shown earlier that lack of collagen XVIII leads to abnormal loosening of the interfollicular epidermal BM, the findings on the hair cycle of the \textit{Col18a1}\textsuperscript{P1/P1} mice could reflect the abnormal structure of the hair follicle BM itself. On the other hand, it must be taken into account that collagen XVIII is a HSPG (Dong \textit{et al.} 2003). The degradation of heparan sulfates enables cell movement through extracellular barriers, and transgenic mice overexpressing heparanase have been shown to have enhanced active growth phase of the first postnatal hair cycle, simply due to eased migration of the follicular stem cell progeny through the ECM (Zcharia \textit{et al.} 2005). Therefore, it is also possible that lack of collagen XVIII, one of the proteoglycans of the follicular BM, could as well enhance the migration of bulge-derived progenitor cells and lead to the slightly accelerated hair growth observed in the \textit{Col18a1}\textsuperscript{P1/P1} mice. Alternatively, the minor changes in hair follicle cycling seen in this mouse line could reflect abnormalities in the interfollicular vasculature, since the short variant of collagen XVIII is highly expressed in the endothelial BMs. It is known that endostatin, the C-terminal fragment of collagen XVIII, can affect angiogenesis (O’Reilly \textit{et al.} 1996). Hair follicle cycling is associated with cyclic expansion and regression of the perifollicular and interfollicular microvasculature (Yano \textit{et al.} 2001). The anagen phase is coupled with active angiogenesis, inhibition of which leads to retardation of the hair growth (Mecklenburg \textit{et al.} 2000), while a rapid decrease in perifollicular vessel size occurs during catagen and telogen. However, analysis of the vessel area near the bulbs of hair follicles of the FVB/N \textit{Col18a1}\textsuperscript{−/−} mice showed no difference to the wild-type, suggesting that there are no major abnormalities in the vessel growth at least in that mouse line. Altogether, as the skin capillaries only express
the short variant of collagen XVIII, it is very unlikely that the changes seen in the *Col18a1*<sup>12/12</sup> and *Col18a1<sup>−/−</sup>* mice are due to changes in angiogenesis.

There is relatively little knowledge on the molecular mechanisms of catagen induction, and moreover, there are very few mouse models in which an accelerated hair cycle has been reported to occur (Guha *et al.* 2004, Mecklenburg *et al.* 2005, Fessing *et al.* 2006). We show that the longer collagen XVIII variants are expressed at high levels in the BM facing the dermal papilla and around the hair follicle bulge. One possibility is that the lack of long variants of collagen XVIII from the BM facing the dermal papilla affects the viability of the hair follicle cells and therefore induces premature apoptosis. As an HSPG, collagen XVIII is capable of binding to and modulating the functions of a large number of signaling molecules, important for cellular differentiation or proliferation, known to be involved in the hair follicle biology (reviewed by Millar 2002 and Mikkola & Millar 2006). Inactivation of the β-catenin gene within the dermal papilla of fully developed hair follicles has been shown to result in reduced proliferation of the progenitors and their progeny that generate the hair shaft, premature induction of the catagen phase, and inhibition of regeneration of the cycling follicle from stem cells (Enshell-Seijffers *et al.* 2010). Thus, lack of one of the proteoglycans of the BM facing the dermal papilla could decrease the amount of Wnt molecules locally and lead to inactivation of the β-catenin and premature induction of catagen.

The upregulation of collagen XVIII in the bulge region of hair follicles has been shown earlier in microarray studies (Tumbar *et al.* 2004, Blanpain *et al.* 2004). Based on our results, the bulge expression of collagen XVIII is suggested to represent the longer variants. This renders it possible that these collagen XVIII variants may regulate the behavior of bulge stem cells, possibly by affecting the Wnt-signaling pathway, since the longest variant is known to contain a cryptic frizzled-domain capable of pulling down Wnt3a *in vitro* and suppressing Wnt/β-catenin signaling and downstream gene expression (Quélard *et al.* 2008, Lavergne *et al.* 2010). Activation of the stabilized β-catenin transgene in the hair bulge of mice has been reported to lead to stimulated proliferation and bulge expansion, the existing hair follicles entering anagen (Baker *et al.* 2010). Thus, lack of a Wnt signaling inhibitor, the longest variant of collagen XVIII, from the bulge area could lead to similar effects, causing the acceleration in the start of the new anagen phase seen in the C57BL/6 *Col18a1<sup>−/−</super>* mice. It is known that only the proteolysed N-terminus of the longest collagen XVIII variant can inhibit Wnt/β-catenin signaling, but not the full-length molecule (Quélard *et al.* 2008).
Our results suggest that there is at least some full-length collagen XVIII in the bulge, as we can see staining with both N-terminal anti-all and anti-long antibodies as well as with the endostatin antibody. However, the staining pattern around the bulge area differs from a typical BM signal and resembles more that of a cell membrane staining, pointing to a proteolysed N-terminus (Quélard et al. 2008). Thus, it is possible that there is also some proteolysed N-terminus in the bulge in addition to the full-length molecule, and it is known that proteolysed and full-length molecules co-exist for example in human plasma (Musso et al. 2001b) and liver tumors (Quélard et al. 2008). It is also possible that the N-terminal part of collagen XVIII is only released in certain stages of the hair cycle to inhibit Wnt/β-catenin signaling. At other stages, the full-length collagen XVIII may act as a coreceptor for the Wnts via its heparan sulfate side chains, as the full-length form has been shown to increase Wnt/β-catenin signaling at least in some cell lines (Quélard et al. 2008).

To study further whether the changes seen in the hair cycle of the Col18a1P2/P2 and Col18a1−/− mice are due to lack of the frizzled domain, we utilized a transgenic mouse line overexpressing the N-terminal part of the longest variant of collagen XVIII containing the frizzled-domain in skin (K14-N-FZ), and as a control another transgenic mouse line overexpressing the N-terminal part of the middle variant of collagen XVIII lacking the frizzled-domain in skin (K14-N-LG). Both of the transgenic mouse lines were crossed with Col18a1−/− mice to produce double mutant mice lacking native, full-length collagen XVIII and expressing only the N-terminal parts of the longer variants (Col18a1−/−; K14-N-FZ and Col18a1−/−; K14-N-LG). Since the transgenic mice were bred in the FVB/N mouse line, instead of C57BL/6 in the promoter-specific knockouts, we compared the results to Col18a1−/− and wild-type mice in this genetic background. To our surprise, the hair follicle cycle proceeded differently in the FVB/N Col18a1−/− mice compared to that seen in the C57BL/6 background. In the FVB/N Col18a1−/− mice, the morphogenesis carried on normally and entered the catagen and telogen phases simultaneously with the wild-type controls. However, the initiation of the following new anagen was delayed in the FVB/N Col18a1−/− mice. The reason for this difference in the hair cycle phenotype in the Col18a1−/− mice is not clear; however, the influence of the genetic background on the phenotypes of mutant mice is a well known fact (reviewed by Yoshiki & Moriwaki 2006). We have discovered the influence of the genetic background on the phenotype of Col18a1−/− mice already during our previous studies in the case of hydrocephalus as well as in ocular findings (Aikio et al. unpublished results).
In the case of the hair follicle cycle, the C57BL/6 mouse line has been extensively characterized, while the FVB/N line is less well studied. It should be noted that according to our results, catagen of the first hair cycle seems to start more quickly in the FVB/N mouse line after morphogenesis compared to that in the C57BL/6 line.

Notably, transgenic addition of the frizzled-containing N-terminus of the longest collagen XVIII variant was able to rescue the phenotype of the FVB/N Col18a1−/− mice, since the Col18a1−/−; K14-N-FZ mice showed no changes in their hair cycle compared to the wild-type controls. This could not be observed in the Col18a1−/−; K14-N-LG mice, which overexpressed the N-terminus of the middle variant. Thus, only the frizzled domain of the longest variant of collagen XVIII is needed to maintain the normal hair cycle, suggesting the involvement of altered Wnt/β-catenin signaling as the cause of abnormal hair cycling in the Col18a1−/− mice. However, whether the levels of active β-catenin are changed in some parts of the mutant hair follicles remains to be elucidated in future studies.
7 Future perspectives

This work has utilized several genetically modified mouse lines to learn more about the biological roles of collagen XVIII and its tissue-specific variants in \textit{in vivo} situations. It has revealed a previously unnoticed, significant task for this protein to provide structural integrity to the BM scaffolds, by showing that lack of this molecule leads to abnormal loosening of the BM network in a number of tissues. The loosening of the BM ultrastructure appears to predispose for pathological conditions, such as hydrocephalus and kidney malfunction. Moreover, this study has shown that the frizzled domain of the longest collagen XVIII variant has a role in the control of hair follicle cycling, suggesting its involvement in the Wnt/\(\beta\)-catening signaling cascade in normal tissues.

Collagen XVIII is one of the conserved members of the BM network molecules, pointing to a vital function of this molecule. However, lack of this collagen in not lethal to life as shown by the patients of Knobloch syndrome and knockout mice. Instead, as shown by this thesis work, deficiency of collagen XVIII exposes tissues to various debilitating, even life-threatening conditions, the severity of which are strongly dependent on the particular genetic environment. This is clearly shown in the development of hydrocephalus in the \textit{Col18a1}\(^{-/-}\) mouse line in a particular genetic background, and in the variable phenotypes of \textit{Col18a1}\(^{-/-}\) mice in hair follicle cycling depending on the inbred mouse line, as well as in Knobloch patients who display a variety of symptoms. The genes causing the phenotypic differences of subjects lacking collagen XVIII are most likely coding for proteins that interact with collagen XVIII in normal situations and remain to be elucidated in later studies, utilizing, for example, large mouse phenome projects characterizing phenotypic and genotypic strain differences.

This thesis work showed hydrocephalus to be one additional phenotype caused by the lack of collagen XVIII. Human hydrocephalus is a significant medical condition with an estimated incidence of 1 in 1500 births. Not much is known about the genetic basis of hydrocephalus, although it is often presented as part of a syndrome with other anomalies. A point mutation in the forkhead transcription factor \textit{foxc1} causes the congenital hydrocephalus (\textit{ch}) mouse mutant (Kume \textit{et al.} 1998), and mice that are heterozygous for a null mutation of this gene have multiple anterior segment disorders closely resembling many of the abnormalities seen in the \textit{Col18a1}\(^{-/-}\) mice (Smith \textit{et al.} 2000). Mutations in the human \textit{FOXC1} gene are found in some cases of the Axenfeld–Rieger syndrome and in Peters’ anomaly patients, who suffer from defects in the anterior segment
of the eye, the hyaloid system, teeth and heart, and in some rare cases also hydrocephalus (Heon et al. 1992, Moog et al. 1998, Lines et al. 2002). In addition, hydrocephalus is a fairly common symptom in Peters’-Plus syndrome, involving other developmental abnormalities as well as Peters’ anomaly (Hennekam et al. 1993). The close similarities in eye phenotypes between the Col18a1−/− mice and the Axenfeld–Rieger syndrome, Peters’ anomaly and Peters’-Plus syndrome patients indicate a possible role for collagen XVIII in these human defects, and the development of hydrocephalus seems to be one additional phenotype connecting this collagen with the human syndromes. Whether the levels of collagen XVIII are affected in patients of hydrocephalic syndromes are subjects for future investigations.

This study also revealed the lack of collagen XVIII to result in kidney defects including the broadening tubular BM and podocyte effacement, caused by the lack of the short or the longer isoforms, respectively. For the first time, the lack of specifically the longer isoforms was shown to affect the phenotype of the knockout animals. In humans, Knobloch syndrome has been reported to be caused by the lack of only the short isoform or by all three isoforms (Suzuki et al. 2009b). Thus, deficiency of only the longer isoforms has not been described so far. It is conceivable that a lack of collagen XVIII may also have disparate effects on kidney function in man, but considering the mild physiological findings in the mutant mice, such effects may manifest themselves only late in life or require other compounding molecular changes. Detailed examination of the kidney filtration capacities of a majority of the Knobloch patients is currently lacking, although based on the results presented here, those would be interesting to perform.

The effect of the longest collagen XVIII isoform and its frizzled domain was also shown by this thesis work in terms of regulating the hair cycle. The hair follicles have become subjects of in vivo studies of various biological events due to their easy accessibility and well-documented signaling cascades, including the Wnt/β-catenin signaling network. This study was able to show for the first time that the frizzled domain of collagen XVIII has an effect on normal tissues, suggesting its involvement in Wnt/β-catenin signaling. Whether the levels of active β-catenin are changed in some parts of the mutant hair follicles remains to be clarified in future studies. Furthermore, identification of further binding partners of the frizzled domain as well as investigations of the mechanisms leading to its proteolytic release are needed to better understand the molecular
mechanisms underlying the kidney abnormalities, the potential anchoring function of collagen XVIII and the role of the frizzled domain in hair cycle.
References


Rehn M & Pihlajaniemi T (1994b) Alpha 1(XVIII), a collagen chain with frequent interruptions in the collagenous sequence, a distinct tissue distribution, and homology with type XV collagen. Proc Natl Acad Sci U S A 91(10): 4234–4238.


Rehn M, Hintikka E & Pihlajaniemi T (1996) Characterization of the mouse gene for the alpha 1 chain of type XVIII collagen (Col18a1) reveals that the three variant N-terminal polypeptide forms are transcribed from two widely separated promoters. Genomics 32(3): 436–446.


Original Papers


Reprinted with permission from The Oxford University Press (I) and The American Society for Biochemistry and Molecular Biology (II).

Original publications are not included in the electronic version of the dissertation.
<table>
<thead>
<tr>
<th>Book Order</th>
<th>Title and Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1079</td>
<td>Hietasalo, Pauliina (2010) Behavioral and economic aspects of caries control</td>
</tr>
<tr>
<td>1080</td>
<td>Jääskeläinen, Minna (2010) Apoptosis-regulating factors in developing and adult ovaries</td>
</tr>
<tr>
<td>1081</td>
<td>Alahuhta, Maija (2010) Tyypin 2 diabetesen riskiryhmään kuuluvien työikäisten henkilöiden painonhallinnan ja elintapamuutosten tunnuspiirteitä</td>
</tr>
<tr>
<td>1082</td>
<td>Hurskainen, Merja (2010) The roles of collagens XV and XVIII in vessel formation, the function of recombinant human full-length type XV collagen and the roles of collagen XV and laminin α4 in peripheral nerve development and function</td>
</tr>
<tr>
<td>1083</td>
<td>Rasi, Karolina (2010) Collagen XV as a matrix organizer: its function in the heart and its role together with laminin α4 in peripheral nerves</td>
</tr>
<tr>
<td>1084</td>
<td>Korkiasangas, Evelina (2010) Aikuisten liikuntamotivaation vaikutavat tekijät</td>
</tr>
<tr>
<td>1085</td>
<td>Mäkelä, Kari Antero (2010) The roles of orexins on sleep/wakefulness, energy homeostasis and intestinal secretion</td>
</tr>
<tr>
<td>1086</td>
<td>Kuusikko-Gaufin, Sanna (2011) Social anxiety and emotion recognition in autism spectrum disorders</td>
</tr>
<tr>
<td>1087</td>
<td>Peltoła, Mirja (2010) Analysis of heart rate variability from 24-hour ambulatory electrocardiographic recordings: significance of preprocessing of R-R interval time series</td>
</tr>
<tr>
<td>1088</td>
<td>Levy, Anna (2011) Lääketieteen asiantuntijasuunnutiskseen eri vaiheissa: Lääketieteen opiskelijoiden yleisorientaatiot, käsitykset hyvän lääkärin ominaisuuksista ja poistustyön hahmotumisesta</td>
</tr>
<tr>
<td>1089</td>
<td>Miettinen, Johanna (2011) Studies on bone marrow-derived stem cells in patients with acute myocardial infarction</td>
</tr>
<tr>
<td>1090</td>
<td>Pyrhönen, Kaisa (2011) Food allergies and hypersensitivities among children in South Karelia: occurrence, inheritance and seasonality</td>
</tr>
<tr>
<td>1091</td>
<td>Stefanius, Karoliina (2011) Colorectal carcinogenesis via serrated route</td>
</tr>
<tr>
<td>1092</td>
<td>Männistö, Tuja (2011) Maternal thyroid function during pregnancy: effects on pregnancy, perinatal outcome and on later maternal health</td>
</tr>
<tr>
<td>1093</td>
<td>Solyom, Szilvia (2011) BRCA/Fanconi anemia pathway genes in hereditary predisposition to breast cancer</td>
</tr>
</tbody>
</table>

Book orders: Granum: Virtual book store
http://granum.uta.fi/granum/
Aino Kinnunen

COLLAGEN XVIII REGULATES BASEMENT MEMBRANE INTEGRITY

SPECIFIC EFFECTS OF ITS ISOFORMS ON THE CHOROID PLEXUS, KIDNEY AND HAIR FOLLICLE