Collagen XVIII in tissue homeostasis and dysregulation — Lessons learned from model organisms and human patients

Ritva Heljasvaara[a,b], Mari Aikio[c,d], Heli Ruotsalainen[a] and Taina Pihlajaniemi[a]

a - ***Oulu Center for Cell-Matrix Research,*** *Biocenter Oulu, Faculty of Biochemistry and Molecular Medicine, University of Oulu, FIN-90014 Oulu, Finland*

b - ***Department of Biomedicine,*** *Centre for Cancer Biomarkers, University of Bergen, Bergen N-5009, Norway*

c - ***Dana-Farber Cancer Institute,*** *Harvard Medical School, Boston, MA 02115, USA*

d - ***Department of Cell Biology,*** *Harvard Medical School, Boston, MA 02115, USA*

Correspondence to Ritva Heljasvaara: *Faculty of Biochemistry and Molecular Medicine, University of Oulu, PO Box 5400, FIN-90014 Oulu, Finland.* *ritva.heljasvaara@oulu.fi*

http://dx.doi.org/10.1016/j.matbio.2016.10.002

Abstract

Collagen XVIII is a ubiquitous basement membrane (BM) proteoglycan produced in three tissue-specific isoforms that differ in their N-terminal non-collagenous sequences, but share collagenous and C-terminal non-collagenous domains. The collagenous domain provides flexibility to the large collagen XVIII molecules on account of multiple interruptions in collagenous sequences. Each isoform has a complex multi-domain structure that endows it with an ability to perform various biological functions. The long isoform contains a frizzled-like (Fz) domain with Wnt-inhibiting activity and a unique domain of unknown function (DUF959), which is also present in the medium isoform. All three isoforms share an N-terminal laminin-G-like/thrombospondin-1 sequence whose specific functions still remain unconfirmed. The proteoglycan nature of the isoforms further increases the functional diversity of collagen XVIII. An anti-angiogenic domain termed endostatin resides in the C-terminus of collagen XVIII and is proteolytically cleaved from the parental molecule during the BM breakdown for example in the process of tumour progression. Recombinant endostatin can efficiently reduce tumour angiogenesis and growth in experimental models by inhibiting endothelial cell migration and proliferation or by inducing their death, but its efficacy against human cancers is still a subject of debate. Mutations in the *COL18A1* gene result in Knobloch syndrome, a genetic disorder characterised mainly by severe eye defects and encephalocele and, occasionally, other symptoms. Studies with gene-modified mice have elucidated some aspects of this rare disease, highlighting in particular the importance of collagen XVIII in the development of the eye. Research with model organisms have also helped in determining other structural and biological functions of collagen XVIII, such as its requirement in the maintenance of BM integrity and its emerging roles in regulating cell survival, stem or progenitor cell maintenance and differentiation and inflammation. In this review, we summarise current knowledge on the properties and endogenous functions of collagen XVIII in normal situations and tissue dysregulation. When data is available, we discuss the functions of the distinct isoforms and their specific domains.

Introduction

Collagen XVIII is a widely expressed, non-fibrillar collagen that is found in association with various basement membranes (BM) of practically all tissues [1–5]. Together with the structurally similar BM-associated collagen XV, it constitutes a separate multiplexin (multiple triple-helix domains with interruptions) subgroup within the collagen superfamily [6–9]. The fact that highly conserved collagen XVIII

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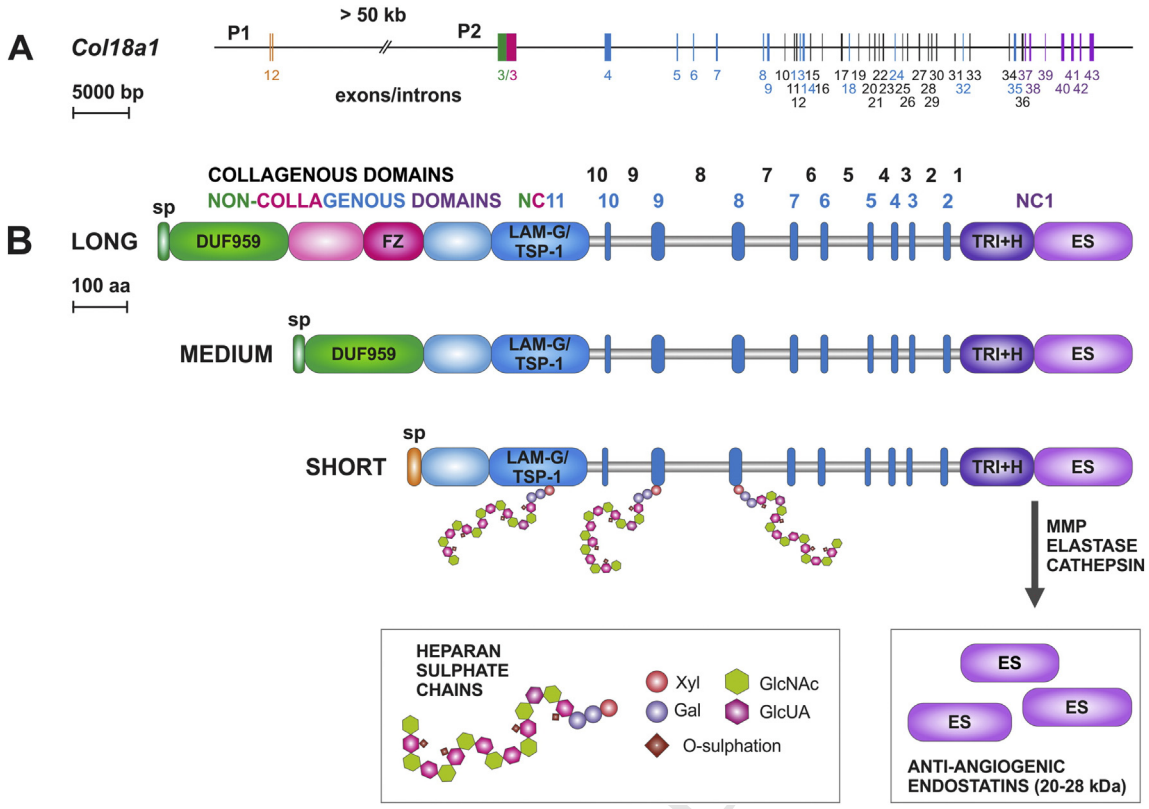

Fig. 1. Schematic structure of mouse *Col18a1* gene (A), and the domain structures of the three isoforms of mouse α1(XVIII) collagen chains (B). The *Col18a1* gene includes two alternative promoters (P) and 43 exons. The external P1 directs the expression of the short collagen XVIII, which consists of a signal peptide (sp), an N-terminal non-collagenous domain 11 (NC11), including laminin-G-like/thrombospondin-1 homology region (LAM-G/TSP-1), ten triple-helical collagenous domains (1–10) interrupted by nine short NC sequences (NC2-NC10), and a C-terminal NC1 with a trimerisation (TRI) domain, a protease-sensitive region (H) and an endostatin (ES) domain. The internal promoter, P2, directs expression of the long and medium collagen XVIII isoforms, which contain the same collagenous and NC1-NC10 domains as the short form as well as the LAM-G/TSP-1 region. The medium and long isoforms share a signal peptide distinct from that in the SHORT isoform and an N-terminal domain of unknown functions (DUF959). Unique to the NC11 of the long isoform is a frizzled (FZ) domain flanked by the DUF959 and LAM-G/TSP-1 domains. Conserved glycosaminoglycan attachment sites at the common NC11, NC9 and NC8 domains carry mainly heparan sulphate (HS) side chains (shown here only for the short isoform), and these are O-sulphated at least in some tissues. The release of endostatin-containing fragments by various proteases, and the magnification of a typical HS chain structure are illustrated in separate boxes. The colours depict the various protein domains of collagen XVIII, and the corresponding exons in *Col18a1*. Collagenous regions are shown in grey. MMP, matrix metalloprotease; Xyl, xylose; Gal, galactose; GlcNAc, *N*-acetylgalactosamine; GlcUA, glucuronic acid. Scale bars: 5000 bp (A), 100 amino acids (B).

homologues can be found in organisms such as *Xenopus laevis*, *C. elegans*, zebrafish and chick suggests a fundamental role for this BM collagen [2,10–12]. In humans, mutations in the *COL18A1* gene result in Knobloch syndrome, a rare genetic disorder characterised mainly by severe eye and skull defects, but occasionally a spectrum of other manifestations appear in isolated cases [13–15].

Collagen XVIII is expressed as three variant polypeptides, or isoforms, namely short, medium and long isoforms, which differ from each other in terms of their N-terminal non-collagenous (NC) terminus and tissue distribution [5,12,16,17] (Fig. 1). Each isoform has a complex modular structure, which is typical of extracellular matrix (ECM) proteins [18]. The long and the medium isoforms also contain some unique segments that are not found in other ECM molecules. An additional feature of collagen XVIII is that it is highly glycosylated by heparan sulphate glycosaminoglycan (GAG) side chains, which further increase the functional complexity of this collagen [1,2,19–21].

Collagen XVIII has attracted much interest because of its endostatin domain, the first identified ECM-derived endogenous angiogenesis inhibitor [22,23]. This domain, which shares sequence homology and antiangiogenic activity with the restin domain of collagen XV [24], can efficiently inhibit the migration

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and proliferation of endothelial cells and induce their apoptosis, and thereby, the growth of experimental tumours. After the initial discovery of the anti-angiogenic and anti-tumourigenic properties of endostatin, other biological activities have now also been associated with it [25–28]. It has been reported, for instance, that endostatin induces the autophagy of endothelial cells [29,30], affects tissue fibrosis [31–34], and plays a role in synapse formation [35].

While the physiological and pathological roles of endostatin have been extensively studied and well documented, considerably less knowledge is available on the functions of the three collagen XVIII isoforms and their N-terminal domains. In this review, we summarise current knowledge on the structural features of mammalian collagen XVIII, discuss some of its physiological and pathological roles, which have been discovered mainly using genetically modified mice as models, and supplement this information with data obtained using human samples and other model organisms. When data is available, we highlight the functions of different N-terminal domains and isoforms, the data which largely stems from the use of recombinant fragments [36,37], isoform- and domain-specific antibodies [1,17,37,38], and the initial characterisation of the isoform-specific mutant mice lacking expression exclusively of the short isoform (referred later in this document as "*Col18a1*$^{P1/P1}$" mice), or, alternatively, the medium and long isoforms ("*Col18a1*$^{P2/P2}$") [37,39,40]. As with endostatin, the N-terminal NC domains may also exhibit different biological activities depending on whether they are embedded in or proteolytically cleaved from the full-length collagen XVIII molecule [17,41]. With respect to endostatin, we briefly summarise published data on its activities in selected tissues. For more information about the multiple biological functions and mechanisms of action of endostatin, as well as its potential use as a cancer biomarker or therapeutic agent, the reader can consult a number of other published reviews [22,25–28,42].

Gene and protein structure of mammalian collagen XVIII

The three differing α1(XVIII) chains of collagen XVIII are encoded by a single gene, which localises on chromosome 21 in humans and chromosome 10 in mice [43]. Both the human *COL18A1* and murine *Col18a1* genes span a region of more than 100 kb and show high structural similarity [17,43,44] (Fig. 1A). They contain 43 exons and have two active promoters, which are separated by a large intronic region of approximately 50 kb.

Transcription from the two promoters results in the formation of the collagen XVIII isoforms, which differ from each other in terms of their size, N-terminal NC sequences, tissue distribution and functions (Fig. 1B). The promoter one (P1), which is upstream of exon 1, encodes the short variant, and the ensuing transcript contains exons 1, 2 and 4–43, while the transcript of the long isoform contain exons 3–43 and is encoded by the promoter two (P2), which is located within the large second intron upstream of exon 3 (Fig. 1A). The alternative splicing of exon 3 in the long transcript gives rise to the medium isoform [3,5,17,44,45].

The core polypeptide of the short collagen XVIII in mice encompasses 1315 amino acid residues and its predicted molecular weight (MW) is 134 kDa. The medium and long isoforms comprise 1527 and 1774 amino acids, respectively, and their predicted MWs are likewise 154 kDa and 182 kDa [5] (Fig. 1B). However, the actual MW for each variant is considerably bigger in tissues and cells, between 200 and 300 kDa, due to extensive post-translational modifications [1–3,36]. The primary protein core structures of human collagen XVIII isoforms and the post-translational modifications are highly similar to those in mice [1,17,45–47].

The C-terminal NC1 domain includes 315 amino acids in mice and is common to all three collagen XVIII isoforms. Endostatin resides at the end of this domain and can be released from the parental collagen XVIII by the actions of several enzymes at cleavage sites within a protease-sensitive hinge region in NC1 [46,48–52] (Fig. 1B). The N-terminus of the NC1-domain contains a trimerisation domain, which is required for triple helix formation and the correct alignment of α1(XVIII) chains. The crystal structure of this particular domain differs from the trimerisation domains found in other collagens, and it exhibits a high degree of specificity and great trimerisation potential at low protein concentrations [53]. The 20 kDa globular endostatin consists of the last 180 amino acid residues of collagen XVIII. It contains two pairs of disulphide bonds and conserved zinc-binding histidine residues that both are critical for the proper structure, stability and biological activity of endostatin [25,54,55]. Additionally, an almost 700 residues' central portion consisting of ten collagenous domains interrupted by nine short NC sequences is shared by the three alpha α1(XVIII) isoforms [3,5,6,8,17,45]. Rotatory shadowing electron microscopy showed that the full-length collagen XVIII can bend in the central NC regions [56].

In mice, the N-terminal NC11 portion of each isoform has a common region of 301 residues that includes a laminin-G-like/thrombospondin-1 (Tsp-1) homology of ~180 amino acids, but otherwise the NC11 sequences differ from each other. The short isoform has its own 25-residue signal peptide and two amino acid residues at the N-terminus of the mature protein, which are not found in the other two polypeptides [5,6,8,43]. The two longer variants share the same 21-residue signal peptide, and an

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N-terminal 218-residue sequence termed DUF959 (domain of unknown function-959) [3,5,41] whose properties and functions remain unknown. They also share a conserved, approximately 30-residue coiled-coil sequence at the N-terminus, which is not present in the short form and which thus may serve as an independent oligomerisation domain affecting the folding, stability or binding activities of the N-terminus of these particular variants [17]. The NC11 portion of the long variant includes a so-called frizzled (Fz) domain flanked by DUF959 and Tsp-1 domains. This domain includes a cysteine-rich area of 110 amino acids, which is homologous to the ligand-binding part of the frizzled receptors for Wnt/Wingless signalling molecules [3,5,16,17,41].

Collagen XVIII-derived matricryptins

Endostatin

Endostatin is the first discovered matrix-derived anti-angiogenetic molecule that can inhibit tumour growth in experimental models [23]. It is generated by proteolytic cleavage in the sensitive hinge region of the NC1 domain with enzymes such as matrix metalloproteases (MMP), elastase and cathepsins, leading to the release of a 20-kDa endostatin fragment as well as endostatin-containing fragments with MWs varying from 24 to 28 kDa [46,48–52] (Fig. 1B). Several factors, for instance hypoxia or p53, have been found to either stimulate or down-regulate endostatin release from collagen XVIII [57–59].

Initially, endostatin's anti-angiogenic activity was found to be due to its ability to inhibit endothelial cell migration and proliferation and induce endothelial cell apoptosis [23,60,61]. The molecular mechanisms whereby endostatin regulates angiogenesis have been extensively investigated; however, a clear picture is still lacking, likely due to the fact that it actually can deploy various receptors and signalling pathways to exert its influence. It binds, for example, to α5 and αv integrins [62–64], glypican [65], caveolin [64,66], vascular endothelial growth factor receptors [67–69] and nucleolin [70], and it modulates the major downstream signal transduction from these receptors to control endothelial cell adhesion, migration, proliferation and survival [25,26,28]. Endostatin also downregulates the transcription of several proangiogenic signalling pathways while upregulating many anti-angiogeneic genes [71]. Recent data shows that endostatin can also induce autophagy in endothelial cells *via* integrin α5β1 and Wnt/β-catenin pathways, thereby potentially acting as a survival response mechanism for escaping apoptotic cell death induced by endostatin [29]. Besides affecting endothelial cells, endostatin can also, for example, directly inhibit the invasion of tumour cells and block the activation of MMPs, which are needed for matrix degradation during angiogenesis and tumour cell migration [72]. It has also been shown that endostatin exhibits anti-fibrotic activity and ameliorates transforming growth factor beta (TGF-β) and bleomycin-induced dermal and pulmonary fibrosis in animals [31,32]. In the kidney, however, endostatin appears to induce tissue fibrosis [33,34].

Frizzled (Fz)

Proteolytic processing of the long variant of collagen XVIII has been shown to occur *in vitro* in human embryonic kidney epithelial cells, and also *in vivo* in human liver cancers, resulting in the release of an N-terminal glycoprotein containing the Fz motif [17,41]. The release of the Fz motif was inhibited by EDTA, suggesting that a metalloprotease is responsible for the proteolytic processing of the N-terminus [41]. The collagen XVIII-derived Fz motif functioned in a secreted frizzled-related protein–like manner, binding to Wnt3a and decreasing baseline and Wnt3a-induced β-catenin stabilisation in cultured human colon cancer cells and reducing also tumour cell growth *in vivo* by slowing down proliferation and cell cycle progression [41,73]. In addition, the expression of the Fz domain was shown to correlate negatively with the β-catenin activity *in vivo* in liver tumours [41]. Moreover, the soluble Fz motif reduced human embryonic kidney epithelial cell's sensitivity to Wnt3a by binding to the cysteine-rich sequences of the frizzled 1 and 8 receptors [74].

Further evidence regarding the potential role of collagen XVIII-derived Fz in Wnt/β-catenin signalling has been presented in our recent study, which demonstrated that a lack of P2-driven isoforms led to impaired adipocyte maturation and a subsequent reduction in the number of adipocytes in mice [37]. We demonstrated that the Fz domain of collagen XVIII was able to bind Wnt10b, which is a potent adipogenic inhibitor [75]. We also demonstrated that isoform-specific collagen XVIII transcription was regulated concurrently with changes in Wnt10b expression in the differentiating adipocytes [37].

Glycosylation in collagen XVIII

According to a recent classification of proteoglycan gene families, the multiplexin collagens are the only known proteoglycan collagens [76]. The sequencing of collagen XVIII in human, mouse, and chick has revealed the presence of three conserved serine-glycine consensus attachment sites for GAGs at the N-terminus of the short isoform [2,6,8,9,45] (Fig. 1B). In chicks, all three sites are known to carry GAGs, either heparan sulphate (HS) or mixed

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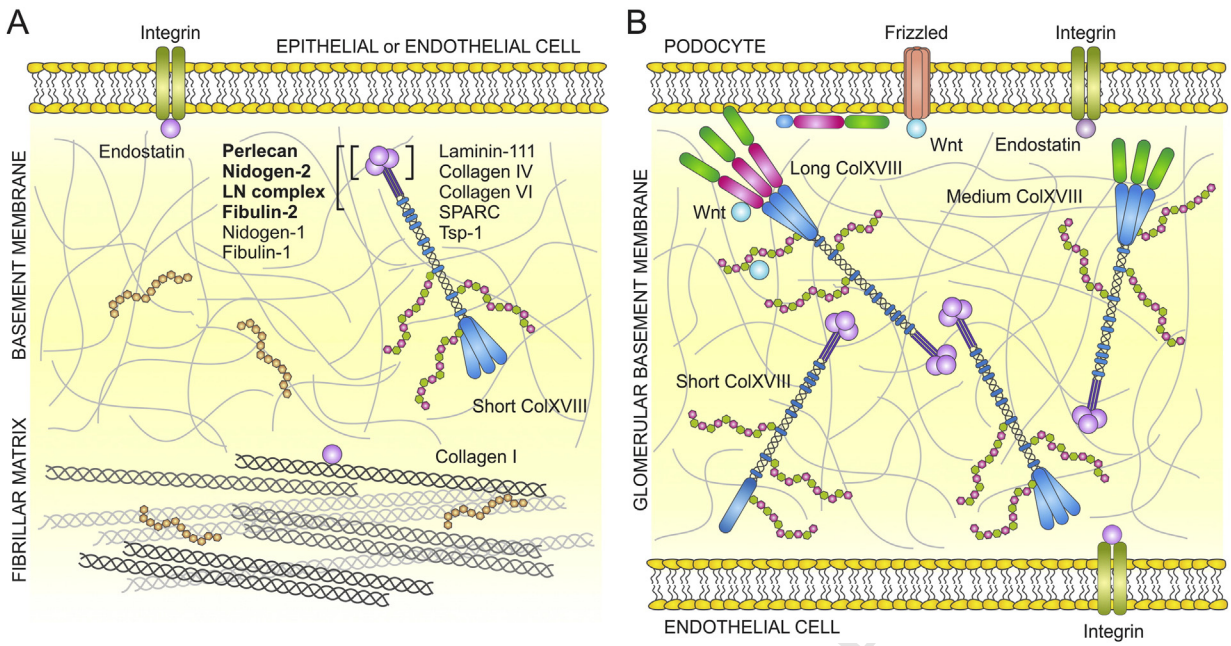

Fig. 2. **A)** Schematic illustration showing the polarised orientation of collagen XVIII in the basement membranes (BM). Based on the immunoelectron microscopy of several tissues (epidermal BM, kidney tubular BM, choroid plexus epithelial BM, heart valve endothelial BM, and the BM of the retinal pigment epithelium *i.e.* the Bruch's membrane), the C-terminal endostatin domain faces the plasma membrane and the N-terminus is orientated towards the fibrillary matrix. For simplicity's sake, only the short collagen XVIII isoform, the predominant form in endothelial and epithelial BMs, is presented. Reported binding activities with other ECM molecules are listed. The long square brackets indicate the binding partners for NC1 trimer, while the short square brackets indicate them for monomeric endostatin. The bold text denotes strong binding affinity (<1 nM), while the regular text denotes weaker (>5 nM) or undetermined binding affinity. LN complex, laminin-1-nidogen-1 complex. **B)** In the glomerular BM (GBM), the collagen XVIII isoforms show a different orientation. The N-terminus of medium/long isoforms resides on the podocyte site and the short isoform on the endothelial side, while C-terminal endostatin portions are within the GBM. All collagen XVIII isoforms are shown here as homotrimers, but, based on current evidence, their occurrence also as heterotrimers cannot be excluded. The frizzled (FZ) domain of long collagen XVIII, which shows structural homology with the Frizzled receptors, binds Wnt signalling molecules *in vitro*, and the N-terminus of the long collagen XVIII has been reported to associate with plasma membrane.

heparan and chondroitin sulphate (CS) chains, depending on the cell type where they are expressed [77]. In humans and mice, short collagen XVIII is also mainly an HS proteoglycan [1,36].

The GAG chains within the BMs mediate multiple biological roles by, for example, storing and presenting growth factors to their receptors or by generating morphogen gradients during development and in regenerative processes, and by binding several ECM proteins, contributing to the proper organisation and integrity of BMs [76]. Within this context, researchers have found that the HS chains of collagen XVIII interact with the cell adhesion protein L-selectin and chemokines to regulate renal inflammation [20,21,36,78], the cell-adhesion molecule-like receptor protein tyrosine phosphatase cPTPsigma to control retinal axon growth and growth cone morphology [79], and the apolipoprotein E (ApoE), which possibly affects the lipoprotein trapping function [37]. The studies with chicks have shown that HS chains mediate the binding of collagen XVIII to BMs [77], as well as to a sialyated vitreal ECM protein opticin, supposedly providing a link between vitreal collagen fibres and the inner limiting membrane (ILM) [80]. Atomic force microscopy measurements have suggested that HS chains of the BM proteoglycans collagen XVIII, perlecan and agrin, all expressed at an equal level in the ILM, bind large quantities of water to the BM and contribute to its thickness and stiffness [81]. Moreover, it has been proposed that CS/HS chains in the *Drosophila* collagen XV/XVIII orthologue Multiplexin (Mp) bind Wingless molecules and participate in the formation of a growth factor gradient, thereby regulating wing morphogenesis [82].

Besides GAG binding sites, there are several potential N-linked (asparagine) and O-linked glycosylation sites (serine, threonine or hydroxylysine) in mammalian collagen XVIII, which reside either in the N-terminal DUF959, Fz or Tsp-1 domains or in the NC8 and NC9 domains of the central collagenous region [2,5,6,8,9,17,45]. At least some of these sites seem to be occupied by glycans as Quelard et al. demonstrated both N-glycosylation and sialyation of

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the recombinant long NC11 fragment [41]. Additional studies have reported that plasma endostatin variants contain mucin-type O-glycosylations [83,84].

Collagen XVIII shows polarised orientation in BMs and regulates BM integrity

Collagen XVIII is a ubiquitous BM component and it is expressed by most endothelial, epithelial and mesenchymal cells throughout the mouse development [4,85,86]. The short form of collagen XVIII is the dominant form in vascular BMs and in most epithelial BMs [1,3,38,39]. The medium polypeptide is abundant in liver and localises in perisinusoidal spaces where fenestrated endothelium is present [1,3,37,38,47,87]. An antibody that recognises both the medium and long form associates with the glomerular BM (GBM) in between the discontinuous glomerular endothelium and podocytes [37,40,45]. Low levels of the longest Fz-containing transcript can be detected in most tissues [3,5,17]; however, visualising this form in tissues has proven to be challenging due to the limited amount of it, and a lack of antibodies specific to this particular variant. In humans, the long form of the collagen XVIII has been detected around the branching bronchioles in the developing foetal lung and in myotubes, especially at sites where myotendinous junctions occur [17]. Interestingly, this particular isoform, or the N-terminal portion of it, was detected also on the cell membrane [41]. More details on the expression and deposition of the differing collagen XVIII isoforms are provided in the following sections.

We and others have shown that collagen XVIII exhibits a polarised orientation in BMs (Fig. 2). This was first demonstrated in the Bruch's membrane underlying the retinal pigment epithelium (RPE) in the eye, where the C-terminal endostatin faces the RPE/endothelial cell and the N-terminus faces the collagenous layer of the membrane [56]. A similar polarised orientation, one with endostatin embedded within the *lamina densa* and the N-terminal portion facing towards the BM-fibrillar ECM interface, was observed in the skin epidermal BM, kidney tubular BM, brain ventricle choroid plexus epithelial BM and heart valve endothelial BM [40,56,88,89] (Fig. 2A). However, in the highly specialised, three-layered structure of the GBM, where the matrix resides between the endothelial cells and podocytes, the deposition and orientation of collagen XVIII is different (Fig. 2B). The short form of collagen XVIII is deposited on the endothelial side of the GBM and the long form(s) on the podocyte side, both oriented in such a way that endostatin is within the BM and the N-terminus at the BM-cell interface [40].

Collagen XVIII, or mainly its C-terminal endostatin and NC1 domains, has been shown to interact with various BM components (Fig. 2A). In a solid-phase binding assay, recombinant endostatin binds to heparin and HS chains, indicating its potential to interact with ECM proteoglycans [90–92]. The trimeric NC1 domain binds strongly to perlecan, nidogen-2, fibulin-2 and the laminin-1-nidogen-1 complex, and with a lower affinity to nidogen-1 and fibulin-1, while monomeric endostatin binds with a low affinity to most of these proteins [90,92]. Endostatin also co-immunoprecipitates with laminin-1 [93]. A surface plasmon resonance (SPR) array containing key ECM proteins further proved these interactions, and likewise identified other binding partners for endostatin, such as the matricellular proteins Tsp-1 and SPARC and the collagens I, IV and VI [94]. Immunogold labelling showed co-localisation of the endostatin domain — but not the N-terminal NC11 domain — with perlecan in the epidermal BMs of humans and mice and in the kidney proximal tubules of adult mice [4,95]. In contrast to the data from ligand-binding studies with isolated BM proteins, endostatin did not co-localise with nidogen-1 in the murine kidney tubular BMs [4]. Moreover, in isolated BM preparations of human skin, researchers found collagen XVIII in the *lamina densa* fractions containing perlecan [56]. These investigations indicate that collagen XVIII is anchored to BM networks containing perlecan *via* its C-terminus. At the moment, no data is available on potential binding partners for the variant N-termini of collagen XVIII, but their polarised orientation is suggestive of associations with components of the fibrillar matrix in most tissues, while the N-terminus may also bind BM components in the glomeruli.

Considering its universal expression, polarised orientation and binding activities in the BMs, it is obvious that collagen XVIII has important structural role in BMs. This is evident due to the loss of BM integrity in several tissues of the collagen XVIII-deficient *Col18a1*$^{-/-}$ mice that lack all three collagen XVIII variants [96]. For example, the endothelial BMs of capillaries in the iris, masseter muscle and atrioventricular valves of the heart, as well as the epithelial BMs at the dermal-epidermal junction, the choroid plexuses of the brain ventricles and kidney proximal tubules, are significantly broadened in the null mice in comparison with the wild type controls [88,89,97,98]. Interestingly, transgenic overexpression of the monomeric endostatin in the mouse skin results in a similar widening of the epidermal BM [88]. Taken together, the ultrastructural data from the knockout and endostatin-overexpressing mouse models suggests that trimeric endostatin within full-length collagen XVIII binds to perlecan and other BM components and ensures the compact structure of the BM, while excess of monomeric endostatin in the transgenic keratin 14-endostatin mice may compete with trimeric endostatin, leading to the displacement of the endogenous collagen XVIII from the BM.

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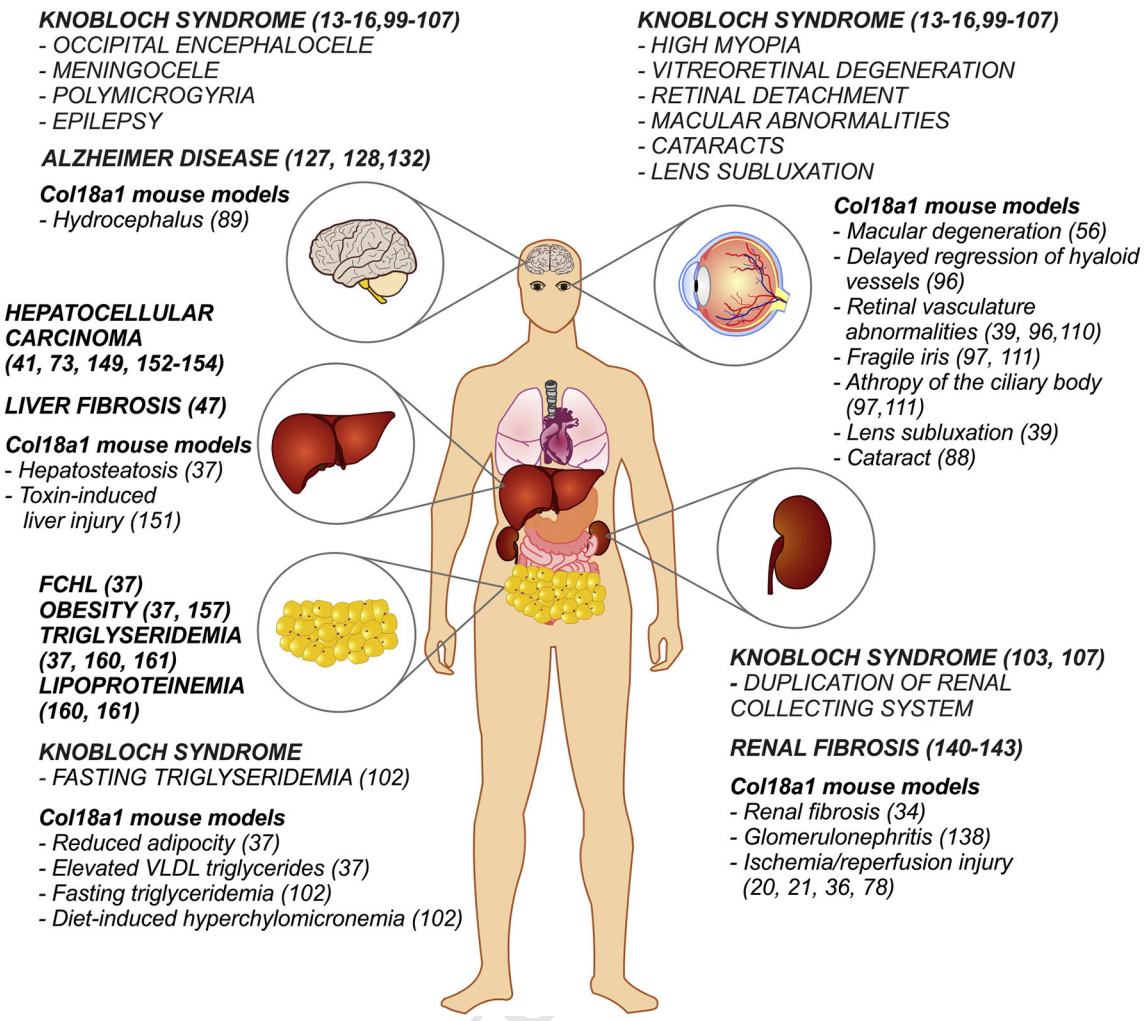

Fig. 3. Schematic illustration of selected tissues that are defective in Knobloch syndrome patients with mutations in the *COL18A1* gene, or in the *Col18a1* mutant mouse lines (knockout or transgenic), along with their key phenotypic alterations and the key references. Also, some other human diseases associated with *COL18A1* are listed. Human, *UPPER CASE*; mouse, *lower case*. FCHL, familial combined hyperlipidemia; VLDL, very low-density lipoprotein.

Knobloch syndrome

In humans, mutations in the *COL18A1* gene result in Knobloch syndrome, a rare, autosomal recessive development disorder characterised by stereotyped ocular abnormalities (high myopia, lens subluxation, vitreoretinal degeneration with retinal detachment, macular abnormalities and early-onset cataracts), which regularly lead to bilateral blindness at a young age. Besides eye defects, occipital midline skull deformities with encephalocele or meningocele and cutis aplasia are also major clinical features in Knobloch cases [13,14,99] (Fig. 3). A spectrum of other manifestations, including distinct central nervous system (CNS) anomalies (for instance, polymicrogyria of the frontal cortex, and dilation of ventricles), mental retardation and epilepsy, facial bone defects, renal abnormalities, persistent vasculature in the eye, acute lymphoplastic leukaemia and fasting hypertriglyceridemia, all figure in isolated cases [13,14,100–107] (Fig. 3). This wide range of indications in Knobloch patients highlights the importance of collagen XVIII in the normal organ development of and maintenance of tissue homeostasis.

A linkage study of a consanguineous Brazilian Knobloch family assigned the gene for this syndrome to 21q22.3, which is the *COL18A1* locus [100]. Subsequently, a homozygous mutation in the first intron of *COL18A1* was identified in this family, leading to truncation of the short isoform [101]. To date, scientist have described at least 90 cases of Knobloch syndrome in almost 50 families, with varying degrees of clinical heterogeneity, and more than 20 different mutations in the *COL18A1* gene [14]. The mutations mainly accumulate in exons 30–42, affecting the

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regions common to all three isoforms, and they are predicted to create premature stop codons and lead to a lack of collagen XVIII protein, even though a complete lack of collagen XVIII has only been confirmed in two patients [15]. In addition, SPR was used to demonstrate that a missense mutation at exon 41 reduces the binding affinity of the endostatin domain to the laminin-1-nidogen-1 complex and to fibulin-1 [15].

Collagen XVIII is indispensable for the eye

Collagen XVIII is present in almost all ocular structures of the human eye, and thus it is easy to understand why Knobloch patients are characterised by several eye defects (Fig. 3). Immunohistological and proteomic analyses have shown that collagen XVIII is present in the majority of BMs within the human eye, in particular in the Bruch's membrane and the lens capsule. It was also detected in the epithelial layers of the iris, in the internal wall of Schlemm's canal and trabeculae, and in the muscle cells of the ciliary body and iris. In addition, ocular fluid samples (tear fluid, aqueous humour and vitreous gel) contained endostatin fragments [97,108–111].

As in humans, collagen XVIII is localised to the various ocular BMs in the eyes of mice, such as the Bruch's membrane, the outer plexiform layer of the retina and the lens capsule. In the developing eye of a mouse, collagen XVIII is present in the *vasa hyloidea proprea* and *tunica vasculosa lentis* [96,97,110–112]. The studies with antibodies against the Tsp-1 and DUF959 domains, and immunostainings of mutant mice lacking exclusively the P1-driven short form of collagen XVIII, or alternatively the two P2-driven isoforms, has made possible a more detailed characterisation of the expression patterns of the collagen XVIII isoforms in the eyes of mice. Thus, both the P1- and P2-driven forms were found to be present in the BM zones of the ciliary body, iris epithelia, and Bruch's membrane in, while only the short form was present in the ILM of the retina and lens epithelia [39].

As with the studies on Knobloch patients, it has been found that mice deficient in collagen XVIII suffer from diverse eye abnormalities, and thus the characterisation of collagen XVIII-deficient mice has provided insights into the pathogenic mechanisms of this rare human disease (Fig. 3). *Col18a1*$^{-/-}$ mice exhibit delayed regression of hyaloid vasculature in the vitreous body, possibly resembling the persistence of the foetal eye vasculature in one Knobloch patient [104], and abnormal retinal vascularisation [96,97,110,111]. *Col18a1*$^{-/-}$ mice also show an overproliferation of astrocytes in the retina of mice and reduced susceptibility to oxygen-induced neovascularisation [96,110]. Characterisation of the eyes of the isoform-specific mutant mice demonstrated that the absence of the short isoform is sufficient to cause aberrant vascularisation of the retina, as previously reported for mice lacking all isoforms of collagen XVIII [39].

Besides defects associated with the blood vessels, collagen XVIII deficiency also results in anterior ocular defects, such as atrophy of the ciliary body and fragile iris [97,111] (Fig. 3). It has been found that electroretinograms that provide information about the function of the retina are normal in young *Col18a1*$^{-/-}$ mice. However, experiments with older null mice revealed a reduction in visual function, and this loss was associated with impaired RPE function as well as the age-dependent accumulation of abnormal basal laminar-like sub-RPE deposits [56]. Iris atrophy, synechiae, the accumulation of iris pigment on the lens capsule and RPE abnormalities have also been reported in Knobloch patients [105,113,114]. Recently, it was found that *Col18a1*$^{-/-}$ mice display a dysfunctional autophagy flux and disturbed RPE proteostasis during the ageing process [115].

Collagen XVIII deficiency in mice led to the separation of the vitreal matrix from the ILM [56,96], and early posterior vitreous detachment has also been reported in Knobloch patients [104] (Fig. 3). The N-terminus of collagen XVIII localised in particular to areas where fibrils inserted into the ILM. The number of these fibrils was reduced in the *Col18a1*$^{-/-}$ mice, suggesting that the detachment of vitreous from the retina is due to the loss of adhesion between the N-terminus of collagen XVIII and vitreal collagenous fibrils, potentially mediated by the interaction between HS-chains of the N-terminus and opticin [77,80,95,96]. It has been suggested that the HS chains of collagen XVIII also play a role in maintaining the normal structure and function of the lens. The deletion of certain HS chains of perlecan, another HS proteoglycan (HSPG) in the lens capsule, leads to lens degeneration, which is accelerated when collagen XVIII is also lacking [116].

Several studies have shown that endostatin administration prevents retinal detachment as well as retinal and choroidal neovascularisation in the eyes of mice [117–119]. However, when endostatin is overexpressed in the lens, mice develop lens opacity at the age of four months [88]. Compared to endostatin overexpression, an excess amount of the N-terminal collagen XVIII Tsp-1 domain produced in the cornea and lens resulted in increased axial length and substantial incidences of cataracts, lens subluxation, phthisis, retinal ablation, corneal vascularisation and intraocular haemorrhages [39] (Fig. 3). These distinct eye phenotypes in the endostatin and Tsp-1 overexpressing mice likely reflect different roles for the C- and N-terminal domains in various BMs of the eye, but in both cases they interfere with the normal functions of the full-length collagen XVIII, and thus lead to deleterious outcomes.

Please cite this article as: R. Heljasvaara, et al., Collagen XVIII in tissue homeostasis and dysregulation — Lessons learned from model organisms and human patients, Matrix Biol (2016), http://dx.doi.org/10.1016/j.matbio.2016.10.002

Collagen XVIII in the nervous system

Encephalocele is one hallmark of Knobloch syndrome, but also other CNS malformations have been infrequently associated with this condition [13,14] (Fig. 3), which supports the view of an important role for *COL18A1* in the development of the human brain. During early embryonic development of the mouse and *Xenopus*, collagen XVIII is expressed in the neuroectoderm [4,12]. At later stages of brain development, and in the adult brain, it can be found in the pial BM, vascular BM and epithelial BM of the choroid plexuses both in mice and in humans [14,89].

Xebrafish LH3, or Diwinka, an enzyme with lysyl hydroxylase and glycosyltransferase activities, has been shown to control hydroxylysine glycosylation in collagen XVIII [120], and both LH3 and Collagen18A1 mutant embryos show a similar neural crest cell migration defect, although the phenotype is somewhat stronger in LH3 mutants [121]. These observations suggest that a lack of proper post-translational modifications in collagen XVIII may also contribute the development of neural tube closure disorders, such as encephalocele in Knobloch patients. The *Col18a1*$^{-/-}$ mice do not show marks of encephalocele or other occipital defects, whereas another congenital CNS disorder, hydrocephalus, has been reported for a specific C57BL/6J substrain [89] (Fig. 3). Magnetic resonance imaging showed that dilation of the brain's ventricular system is fully penetrant in the null mice, even without external signs of hydrocephalus. Hydrocephalus is characterised by abnormalities in the production, flow or resorption of cerebrospinal fluid (CSF), resulting in ventricular dilatation in the brain [122]. CSF is produced by choroid plexuses which showed several changes in the mice lacking collagen XVIII, including abnormal epithelial cell morphology and tight junctions, apical microvilli with vacuoles and broadened BMs of the choroid plexuses, suggesting disturbances in the production of CSF [89].

ECM proteins are implicated in the pathogenesis of neurodegenerative disorders, including Alzheimer's disease (AD) [123–125]. HSPGs co-localise in senile plaques and neurofibrillary tangles, which are characteristics of AD brains and also contribute to the formation and persistence of deposits of Amyloid-beta (Aβ) peptide and ApoE in these lesions. HS, and its highly 6-O-sulphated glucosamine residues in particular [126], support fibrillogenesis by interacting with the Aβ precursor and induce the conformational change required for fibril assembly. HS also remains associated with the fibrils and improves their stability [124]. In addition, perivascular Aβ, ApoE and HSPG accumulation leads to cerebral amyloid angiopathy (CAA), which compromises blood vessel function [123–125].

Several studies have suggested a link between AD and collagen XVIII/endostatin (Fig. 3). Using isoform-specific antibodies to test such an assertion, we first demonstrated that the short form of collagen XVIII localises in all types of cerebral blood vessels, CAA-affected vessels and classic senile plaques, while long forms of it appear in large cortical and leptomeningeal vessels, and especially in all amyloid-laden vessels and senile plaques [127]. Endostatin was shown to accumulate in the neurons and extracellular space in AD brains [128]. Neurofibrillary tangles did not contain full-length collagen XVIII [127,128].

SPR studies have demonstrated that endostatin directly interacts with Aβ [94]. It has also been reported that, like Aβ, endostatin alone can form amyloid-like structures that bind to neuronal cells and compromise their survival and that the fibrillogenesis of endostatin may account for its interaction with Aβ [129,130]. The reduction of disulphide bonds within endostatin may also facilitate the formation of amyloid [131]. Recently, endostatin concentration was shown to increase in the CSF of AD patients even more than Aβ, and its ratio to established AD markers was suggested as a novel biomarker that can distinguish AD from other dementia cases [132]. Whether collagen XVIII/endostatin, like many other chromosome 21 genes including Aβ precursor [133], accounts for the early-onset AD associated with Down syndrome remains to be seen. Likewise, it remains to be shown whether collagen XVIII that binds ApoE *via* its HS chains [37], affects the clearance of soluble Aβ by neurons and glia in brains, a process that is known to depend on the interactions between ApoE, HSPGs and lipoprotein receptors [134].

In the peripheral nervous system, ECM molecules and their proteolytically released fragments are needed for the formation and maintenance of motor nerve terminals [35]. The *C. elegans* collagen XV/XVIII orthologue, CLE-1, is highly expressed in the nervous system, where it concentrates near the synapse-rich regions [10,135]. CLE-1 regulates cell motility and axon guidance *via* its NC1/endostatin domains [10,136], and it is needed for the proper organisation of presynaptic zones and for synapse function at the neuromuscular junction (NMJ) [135]. Motor axon pathfinding defects have been reported also for *Drosophila* Multiplexin, and these defects could be rescued by overexpressing either full-length Mp or monomeric endostatin in flies [137]. In zebrafish, LH3 controls the glycosylation of myotomal collagen XVIII, enabling its interactions with receptor tyrosine phosphatases that guide motor axon migration from the spinal cord to the periphery [120].

While collagen XVIII appears to be dispensable for NMJ formation in mice, it regulates synaptogenesis in their cerebellum [35]. All three collagen XVIII isoforms are expressed in Purkinje neurons of mouse cerebellum, and their expression coincides with postnatal synaptogenesis. When this collagen

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is lacking, Purkinje cell morphology is normal, but the number of synapses forming between the climbing fibre axon terminals on the Purkinje cell dendrites is compromised. Moreover, monomeric endostatin induces climbing fibre-specific presynaptic differentiation *in vitro via* integrin α3β1 signalling [35].

Collagen XVIII regulates kidney development as well as inflammatory response and fibrosis in the kidneys

The kidneys contain a repertoire of BMs with different properties and permeabilities that are crucial for maintaining proper electrolyte levels and filtering, excreting and re-absorbing metabolites. In the mature kidneys of mammals, collagen XVIII is expressed in the Bowman's capsule, in the GBM and tubular BM, and in the mesangial matrix [1,3,4,38]. Studies with isoform-specific mutant mice and N-terminal antibodies have led to the conclusion that the short collagen XVIII isoform is mainly located in Bowman's capsule and tubular BMs. The longer variants prevail in the glomeruli and are deposited on the podocyte side, while short form is present on the endothelial side of the GBM [37,40]. *Col18a1*$^{-/-}$ mice show structural abnormalities in tubular and glomerular BMs [89], and more specifically, the lack of the P1-driven short form of collagen XVIII leads to abnormal loosening of the proximal tubular BMs, while the loss of the P2-driven medium/long isoforms results in podocyte foot process effacement in the glomeruli of *Col18a1*$^{P2/P2}$ kidneys [40]. Despite these ultrastructural changes, mutant mice have a normal lifespan without obvious signs of severe kidney malfunction. However, the serum creatinine levels elevated slightly in untreated null mice, and significantly in nephritic null mice, indicating alterations in kidney filtration capacity when collagen XVIII was lacking [89,138].

In the kidneys of developing mice, collagen XVIII is expressed throughout the epithelial ureter bud at the early stage of kidney organogenesis but is lost from the ureter tips and confined to the stalk region during before ureter branching. The opposite expression pattern has been observed in the branching lung epithelium, thus suggesting that locally expressed collagen XVIII may participate in the control of inductive signals, which are involved in epithelial branching morphogenesis [85]. Endostatin was also shown to inhibit ureteric bud outgrowth and branching by binding to cell surface glypicans, potentially after local degradation of collagen XVIII at the ureteric tip and accumulation of endostatin in this region [65,139]. The importance of collagen XVIII in kidney development seems to be clinically relevant also in humans, as congenital uni- or bilateral duplication of the renal collecting system have been reported in Knobloch patients [103,107] (Fig. 3).

Several studies have linked collagen XVIII to renal fibrosis, which accompanies all chronic renal diseases. Gradually increasing plasma endostatin levels were detected in humans suffering from chronic kidney disease [140–143] (Fig. 3). Collagen XVIII expression also increased during progression of the disease in experimental renal disease models [138,144–146]. In ageing mice, renal expression of endostatin was significantly elevated in parallel with microvascular rarefaction, which plays a key role in the induction of tubulointerstitial fibrosis and glomerular sclerosis [33,34]. Also, high plasma endostatin level in elderly people has been associated with renal injury and dysfunction as well as with the duration of hypertension [142,147]. Finally, a quite recent study has identified high circulating endostatin as an independent predictor of kidney disease and mortality in patients with type 2 diabetes [148].

In murine anti-GBM glomerulonephritis, a model of GBM autoimmune disorder, collagen XVIII expression was elevated in the Bowman's capsule and GBM, while collagen XVIII deficiency augmented the typical responses of the model. *Col18a1*$^{-/-}$ mice showed a more severe inflammatory response, capillary rarefaction, vascular endothelial cell damage, matrix accumulation and glomerular and tubulointestitial injury than the control mice (Fig. 3). Treatment of *Col18a1*$^{-/-}$ mice with recombinant endostatin did not affect the progression of the disease, suggesting that an intact collagen XVIII molecule, or other functional domains of collagen XVIII, is needed to preserve the integrity of the ECM and capillaries in the kidneys, and thus protecting from progressive glomerulonephritis [138].

The short collagen XVIII isoform has been identified as one of the L-selectin binding HSPGs in the tubular BMs of the outer medulla, and it mediates leukocyte infiltration into inflamed kidneys [20,21,36,78]. Besides binding L-selectin, the HS GAGs within the Tsp-1 domain of short collagen XVIII isoforms bind the monocyte chemoattractant protein-1 (MCP-1), the dominant chemokine involved in monocyte/macrophage recruitment in renal inflammation. The length and O-sulphation of the HS chains appears to be an important structural determinant for MCP-1 and L-selectin binding to collagen XVIII [20,36]. In the renal ischemia/reperfusion (I/R) model, mice lacking collagen XVIII showed reductions in early inflammatory cell influx and tubular damage, which decreased further when *Col15a1* was also deleted [20,36,78] (Fig. 3). I/R injury always occurs during acute kidney failure or after renal transplantation, and thus multiplexins might represent a potential intervention target for the reduction of inflammation under these conditions [36].

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Long forms of collagen XVIII prevail in the liver but also short forms appear in pathological situations

Already the first studies on collagen XVIII identified high levels of medium and long isoform transcripts in the liver of humans and mice [3,5,6]. These transcripts are produced by hepatocytes and deposited into the perisinusoidal space. In contrast, the short form of collagen XVIII is expressed by the bile duct epithelial, endothelial and vascular smooth muscle cells, and it is deposited in vascular, biliary epithelial, muscle fibre and peripheral nerve BMs [1,38,47,87].

In fibrotic human livers, collagen XVIII forms thick deposits along the capillarised sinusoids, and the short isoform becomes highly expressed by activated hepatic stellate cells/myofibroblasts and proliferating bile ducts (Fig. 3). The production of the medium/long form by hepatocytes also increases, but to a lesser extent [47,149]. In rat, hepatocytes and biliary epithelia appear to be the major source of collagen XVIII both in normal and fibrotic liver, and its expression remains constant in acute carbon tetrachloride-induced fibrosis and is slightly upregulated in a bile duct ligation model [150]. These findings indicate that in pathological situations liver collagen XVIII, and particularly the short form, produced by myofibroblasts and bile duct epithelia, is associated with BM remodelling during angiogenesis and ductular reactions around the portal tracts [47,151].

In human hepatocellular carcinoma (HCC), low medium/long collagen XVIII expression by tumour hepatocytes is associated with large tumours, tumour progression and high recurrence rates as well as with high micro-vessel density, possibly suggesting the anti-angiogenic and anti-tumourigenic actions of endostatin [47,149,152] (Fig. 3). In another type of liver cancer, cholangiocarcinoma, or bile duct cancer, the short form of collagen XVIII becomes upregulated in the tumour cells. This variant is also highly expressed in the tumour stroma by myofibroblasts and endothelial cells and is deposited in the ECM and BMs of the tumour in primary and metastatic liver cancers [149]. The Fz-containing long collagen XVIII transcript was found to be upregulated in fibrogenesis and in small, well-differentiated liver tumours, but downregulated in advanced human liver cancers [41]. High tissue and circulating endostatin levels have been observed in human HCC, and associated with long-term survival in HCC [153]. In another study, researchers suggest that high collagen XVIII/endostatin expression in adjacent non-tumour cells predicts a poor prognosis and short disease-free and overall survival rates in HCC [154]. This discrepancy likely reflects the challenge in accurately measure the expression of different collagen isoforms in tissues and in circulation where both cleaved endostatin and full-length medium/long forms have been detected [17,149].

As mentioned above, collagen XVIII is largely absent in tumour hepatocytes when the disease progresses to malignancy. An antibody that specifically recognises the Fz domain in the long collagen XVIII only weakly stained liver cancer nodules, and the staining was negatively associated with Wnt/β-catenin pathway activity *in vivo* [41]. The proteolytically released Fz module is able to bind to Wnts and inhibit the Wnt/β-catenin activity *in vitro,* possibly by sequestering Wnts through its cysteine-rich sequence or by forming an inactive complex with Fz receptors [37,41,73,74]. This inhibition leads to reduced tumour cell proliferation and cell cycle arrest in cancers that are driven by Wnt/β-catenin signalling [41,73].

To ensure hepatocyte function and viability, the ECM undergoes changes during the regenerative response to drug- and toxin-induced liver injury. A recent study demonstrated that collagen XVIII is crucial for hepatocyte survival [151]. In contrast to the wild type mice, recovery from acute toxin-mediated liver injury was severely compromised in the *Col18a1*$^{-/-}$ mice, leading to rapid death of the null animals (Fig. 3). Hepatocyte survival was shown to be dependent on their adhesion to collagen XVIII, mediated by the collagen-binding integrin α1β1, while α5β1, another integrin highly expressed in hepatocytes and a known endostatin receptor, was not involved in the adhesion process. Consistently, endostatin administration did not improve the survival of the *Col18a1*$^{-/-}$ mice in this model. The interaction between collagen XVIII and integrin α1β1 appeared to provide survival cues through integrin-linked kinase and the Akt pathway. The study also showed that TGFβ, which is highly expressed upon liver injury, induced the expression of long collagen XVIII through the transcription factor FoxA2 (aka hepatocyte nuclear factor 3B), which regulates the expression of medium/long collagen XVIII [155]. Moreover, the deposition of collagen XVIII on the surface of cells increased after TGFβ treatment [151]. These findings demonstrate that collagen XVIII is an important functional component of the liver matrix and is crucial for hepatocyte survival during injury and stress.

Collagen XVIII regulates adipogenesis and fat deposition

Increasing evidence suggests that collagen XVIII plays an important role in adipocyte differentiation and in the maintenance and function of adipose tissue depots. This was first proposed due to its upregulation during bovine adipocyte differentiation and high levels of it in bovine adipose tissue [156].

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Later, researchers discovered that collagen XVIII is highly expressed during human adipocyte differentiation and that a single nucleotide polymorphism (SNP) in the exon 3 within *COL18A1* was associated with obesity in patients with type 2 diabetes [157] (Fig. 3). This region contains the Fz domain, suggesting that the identified SNP may result in disturbances in Wnt signalling, which is known to play a major role in adipogenesis [75,158,159]. Moreover, genetic linkage studies have provided evidence of a linkage between the chromosome 21 interval housing *COL18A1* and the familial combined hyperlipidemia-triglyceride trait [37] as well as between this particular gene locus and increased serum triglyceride and low-density lipoprotein (LDL) in hypertensive pedigrees [160] (Fig. 3). Also, a positive correlation between the expression of medium/long isoforms in visceral fat and serum-free fatty acid levels has been found, suggesting that *COL18A1* expression contributes to the regulation of adipose tissue metabolism in visceral obesity [37]. Another report stated that the low-frequency *COL18A1* variant has a significant effect on serum triglyceride levels and a smaller effect on high-density lipoprotein (HDL) levels [161]. In addition, Knobloch syndrome patients with null mutations in the short variant exhibited lowered plasma lipoprotein lipase (Lpl) mass and activity as well as fasting hypertriglyceridemia [102] (Fig. 3). Reduced plasma levels and activity of the Lpl as well as mild fasting hypertriglyceridemia and diet-induced hyperchylomicronemia were reported in *Col18a1*$^{-/-}$ mice, too [102].

We recently showed that a specific lack of the medium/long isoforms of collagen XVIII in mice leads to reduced adiposity, increased fat deposition in the liver and elevated serum levels of very low-density lipoprotein (VLDL) triglycerides [37] (Fig. 3). These abnormalities were not seen in mice lacking the short isoform only. The size of the adipocytes was not altered in adult *Col18a1*$^{-/-}$ or *Col18a1*$^{P2/P2}$ mice, indicating that committed preadipocytes in the wild type and mutant mice exhibit the same capacity to accumulate lipids. Instead, the white adipose tissue of the mice lacking all or medium/long isoforms contains more early adipocyte progenitors and less committed preadipocytes, suggesting that the N-terminal sequences of medium/long isoforms may facilitate the conversion of the early progenitor cells into preadipocytes, or support the differentiation of precursors to mature adipocytes and also their maintenance. In support of this finding, embryonic fibroblasts isolated from the *Col18a1*$^{-/-}$ or *Col18a1*$^{P2/P2}$ mice showed a significantly reduced adipocyte differentiation potential relative to the wild type or *Col18a1*$^{P1/P1}$ mice. Interestingly, studies with *Drosophila* have suggested that Multiplexin is involved in the formation or maintenance of the fat-body BMs and, by extension, in regulating lipid metabolism [82].

Wnts are key mediators of adipogenesis, activating the commitment of progenitor cells to the preadipocyte lineage in early differentiation and inhibiting terminal differentiation in the late adipogenic programme [75,159]. The N-terminus of the long collagen XVIII isoform harbours an Fz motif, and it is thus endowed with the potential to modulate Wnt/β-catenin signalling. Wnt10b is the main Wnt ligand expressed by preadipocytes and a potent adipogenic inhibitor whose expression decreases along with terminal differentiation. We found high amounts of the short collagen XVIII isoform and only low amounts of the medium/long forms in the undifferentiated mouse embryonic fibroblasts and 3T3-L1 preadipocytes, while opposite expression patterns were noted after their induction to terminal differentiation *ex vivo*. These changes in the isoform-specific expression occurred concomitantly with changes in the Wnt10b expression, and they may be of physiological significance since the Fz domain of collagen XVIII was able to interact with Wnt10b *in vitro,* suggesting that the Fz-containing long isoform needs to be downregulated in preadipocytes to prevent its potential inhibitory effect on Wnt10b [37]. In summary, our results for collagen XVIII describe a novel ECM-directed mechanism, which contributes of the multistep adipogenic programme that determines the number of precursors committed to adipocyte differentiation and the maintenance of the differentiated state. The downstream consequences of reduced adiposity in the *Co18a1* mutant mice include increased fat deposition in the liver and high circulating VLDL triglycerides [37].

Collagen XVIII in cancer

The endostatin domain of collagen XVIII has been widely studied within the context of cancer and tumour angiogenesis, and several reviews tackle this issue in exemplary fashion [25–28]. The published data convincingly demonstrates that recombinant endostatin exerts an efficient inhibitory effect on tumour angiogenesis and growth in various animal models. It has been proven safe and is well tolerated by humans, and promising responses in phase II clinical trials have been obtained, for example, for non-small cell lung cancer [162,163], breast cancer [164], melanoma [165] and head and neck cancer [166]. However, contradictory data is also available for some of these types of cancer [167,168], and thus the use of endostatin as a therapeutic anti-angiogenic and anti-tumourigenic agent, either alone or in combination with other therapies, is still uncertain or warrants further investigation.

Collagen XVIII expression is upregulated in many solid tumours, either in tumour cells, as shown, for example, for cutaneous squamous cell carcinoma (SCC) [169], oral SCC [170], non-small

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cell lung cancer [171] and invasive breast cancer [172], or in stromal cells in liver cancer [149,154], pancreatic cancer [173], colorectal cancer [174] and ovarian cancer [174,175], just to name a few. In addition, increased serum endostatin levels have been reported for many cancer types [26,28]. On the other hand, the downregulation or even absence of collagen XVIII has been associated with certain types of cancer, such as HCC as already discussed earlier in this review [41,73,149,152], and leukaemia, in which elevated serum endostatin levels were associated with a favourable prognosis [106,176].

Remodelling of the vascular and epithelial BMs during carcinoma progression is a natural source of plasma endostatin, but the extent to which the increased expression of collagen XVIII by tumour cells contributes to circulating endostatin, or to other collagen XVIII fragments found in circulation [17,149], is not entirely clear. The causes and consequences of elevated collagen XVIII expression in tumour cells are not well characterised either, though transcriptional or epigenetic activation of *COL18A1* has been shown to occur in some types of cancer [177,178]. We have observed that overexpression of endostatin in mouse skin keratinocytes causes relatively minor changes in tumour growth and angiogenesis, but it significantly reduces lymphangiogenesis and lymph node metastasis in a chemical skin carcinogenesis model [179]. On the other hand, other studies have found that even a relatively minor (~1.5-fold) endothelial-specific overexpression of endostatin significantly decreased angiogenesis and the growth of tumour xenografts, possibly also explaining the observed low numbers of solid tumours among Down syndrome individuals with an extra copy of *COL18A1* [180–182].

While a general epithelial and vascular BM deposition of collagen XVIII can be observed in normal tissues, it is gradually lost from the epithelial BM during tumourigenesis in skin and pancreatic cancer [169,173]. In pancreatic cancer endostatin persists in tumour vasculature, and it is also liberated into the circulation system, likely due to increased amounts of MMPs in the tumours [173]. In advanced human cutaneous SCCs, however, collagen XVIII was selectively reduced in the tumour vasculature, while other BM components were present, and this decrease in collagen XVIII deposition was also associated with cancer progression [169]. The murine chemical skin carcinogenesis model that mimics the development of human SCC also emphasises the loss of collagen XVIII from tumour vasculature [169]. Whether the selective reduction of collagen XVIII in skin tumour's vasculature is due to regulated proteolysis, or to transcriptional or epigenetic downregulation in the tumour's endothelial cells, or both, still needs to be studied further.

Conclusions and perspectives

Collagen XVIII-derived endostatin has attracted a great deal of interest during the last few decades because of its potent anti-angiogenic and anti-tumourigenic functions and, more recently, also because of its emerging roles in other biological processes, such as autophagic cell death or tissue fibrosis [26,28]. We and others have proceeded to unravel the significance of the three collagen XVIII isoforms and have demonstrated, for example, that the P2-directed long isoforms are critical for determining the number of adipocyte precursors committing to terminal differentiation [37]. It is likely that such a failure in adipose tissue development has disadvantageous effects for the whole-body energy balance and metabolic processes as well.

Moreover, it has been established that the short form is critical for retinal vascularisation and that the overexpression of the Tsp-1 or endostatin domains in the eyes of mice interferes with the normal functions of collagen XVIII in the various ocular BMs, thereby resulting in severe phenotypic alterations in eye structure and function [39]. An excess amount of these fragments in the BMs may disrupt the interactions of collagen XVIII with other BM components or cellular receptors, leading to structural defects in the BM and perturbations in the extracellular signalling cues. The observed alterations in the BM integrity in several tissues of *Col18a1*$^{-/-}$ mice [40,88,89] are likely to compromise the signalling function of the BM.

In addition to adipose tissue, collagen XVIII may also contribute to the maintenance and differentiation of stem and progenitor cells in other tissues. In this context, it is worth mentioning that collagen XVIII belongs to a group of approximately 50 genes whose expression is upregulated in several types of stem cells, including haematopoietic stem cells within the bone marrow [183] and epidermal stem cells in the hair follicle bulge [184] as well as breast cancer stem cells [185]. It is intriguing to speculate that the neural tube closure defects observed in Knobloch patients could arise from imperfections in neural stem cell niches due to lack of collagen XVIII.

Recent studies with various model organisms have made important advances in our understanding of the functions of collagen XVIII in normal and pathological situations. However, many aspects of the biochemical and biophysical properties of collagen XVIII isoforms, such as the binding activities of the variant N-termini in the ECM, or on the plasma membrane, their mechanisms of action as well as details of their proteolytic processing, remain largely unexplored. In addition, the complex post-translational glycosylations of collagen XVIII isoforms, and their relevance in various biological processes have not yet been fully clarified. Importantly, significant associations between collagen XVIII and common human diseases, such as

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metabolic disorders, cancer and AD, have been recently observed, in addition to the association of the Knobloch syndrome with null mutations in *COL18A1*. The modern tools of biomedical research, including "omics" and bioinformatics will facilitate and accelerate the future studies on the multiple roles of collagen XVIII in tissue homeostasis and dysregulation.

Financial support

This study was supported by the Health Science Council of the Academy of Finland (Centre of Excellence 2012–2017 Grant 251314), and by Sigrid Jusélius Foundation.

Acknowledgements

We thank Anne Heikkinen, Valerio Izzi, Raman Devarajan and Aino Kinnunen for help in preparing the manuscript.

Received 25 May 2016;
Received in revised form 12 September 2016;
Accepted 10 October 2016
Available online xxxx

Keywords:
Basement membrane;
Knobloch syndrome;
Multiplexin;
Heparan sulphate proteoglycan

Abbreviations used:
Aβ, amyloid beta peptide; AD, Alzheimer disease; ApoE, apolipoprotein E; BM, basement membrane; CLE-1, *C. elegans* collagen XV/XVIII orthologue; CNS, central nervous system; CS, chondroitin sulphate; CSF, cerebrospinal fluid; DUF, domain of unknown function; ECM, extracellular matrix; Fz, frizzled; GAG, glycosaminoglycan; GBM, glomerular basement membrane; HCC, hepatocellular carcinoma; HS, heparan sulphate; HSPG, heparan sulphate proteoglycan; ILM, inner limiting membrane; LH, lysyl hydroxylase; LM, laminin; MMP, matrix metalloprotease; MW, molecular weight; NC, non-collagenous; NMJ, neuromuscular junction; RPE, retinal pigment epithelium; SNP, single nucleotide polymorphism; SPR, surface plasmon resonance; Tsp-1, thrombospondin-1.

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Please cite this article as: R. Heljasvaara, et al., Collagen XVIII in tissue homeostasis and dysregulation — Lessons learned from model organisms and human patients, Matrix Biol (2016), http://dx.doi.org/10.1016/j.matbio.2016.10.002

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Please cite this article as: R. Heljasvaara, et al., Collagen XVIII in tissue homeostasis and dysregulation — Lessons learned from model organisms and human patients, Matrix Biol (2016), http://dx.doi.org/10.1016/j.matbio.2016.10.002

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