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THE ROLES OF COLLAGEN XVIII AND ITS ENDOSTATIN DOMAIN IN WOUND HEALING, HAIR FOLLICLE CYCLING AND BONE DEVELOPMENT

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Abstract

Collagen XVIII is a basement membrane proteoglycan, which has three variant N-termini. These variants are coded by two promoters; promoter 1 directs the synthesis of a short variant and promoter 2 directs the synthesis of two longer variants, of which the middle variant is generated from the longest by splicing. The longest variant contains a cysteine-rich domain in its N-terminus, which shows homology to the frizzled receptors of the Wnt molecules and can inhibit Wnt/beta-catenin signalling *in vitro*. The C-terminal domain of collagen XVIII, endostatin, is an inhibitor of tumor growth and angiogenesis.

Lack of collagen XVIII accelerates cutanous wound healing and wound angiogenesis. Overexpression of endostatin leads to delayed wound healing and the presence of morphologically abnormal wound capillaries. Moreover, endostatin overexpression leads to delayed formation of the wound epidermal basement membrane and impaired maturation of hemidesmosomes.

Endostatin treatment decreases osteoblast proliferation *in vitro*. Moreover, osteoblast proliferation and mineralization of the matrix by osteoblasts are inhibited when cells are treated with endostatin together with VEGF. *In vivo*, lack of collagen XVIII leads to delayed formation of secondary ossification centers in mouse femurs, whereas overexpression of endostatin leads to a slower growth of bone length. However, both of these changes are transient and mild, suggesting that collagen XVIII/endostatin is not essential for skeletal development.

The growth of hair follicles is delayed in the mice overexpressing endostatin. This delay in growth is preceded by an impaired hair follicle associated angiogenesis. Lack of collagen XVIII causes an accelerated onset of the first hair cycle. A similar change can be seen in mice lacking the long variants of collagen XVIII. Lack of the short variant causes mild acceleration in the catagen of the first cycle, and anagen is also significantly accelerated in these mice. The long variants were located in the bulge region, which contains the hair follicle stem cells, and in the basement membrane surrounding the dermal papilla. As it is known that several Wnt-inhibitors are upregulated in the bulge, our results suggest that the longest variant of collagen XVIII may have a role as a regulator of Wnt-signalling in hair follicles.

Keywords: basement membrane, bone development, collagen type XVIII, endostatins, hair follicle, osteoblasts, wound healing

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Abbreviations

bFGF	basic fibroblast growth factor
BM	basement membrane
BMP	bone morphogenetic protein
bp	base pair
C-	carboxy-
ECM	extracellular matrix
eNOS	endothelial nitric oxide synthase
ERK1/2	extracellular signal-regulated kinase 1/2
ES-cell	embryonic stem cell
FACIT	fibril associated collagens with interrupted triple helices
GAG	glycosaminoglycan
HSPG	heparan sulphate proteoglycan
kb	kilobase
kDa	kilodalton
LDL	low density lipoprotein
MCP-1	monocyte chemotactic protein -1
MMP	matrix metalloproteinase
NC	noncollagenous, non-triple helical
N-	amino-
PCR	polymerase chain reaction
RhoA	Ras homolog gene family, member A
Shh	Sonic hedgehog
TEM	transmission electron microscopy
TGF-β	transforming growth factor – β
TNF	tumor necrosis factor
tPA	tissue plasminogen activator
uPA	urokinase plasminogen activator
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:

I   Seppinen L, Sormunen R, Soini Y, Elamaa H, Heljasvaara R & Pihlajaniemi T (2008) Lack of collagen XVIII accelerates cutaneous wound healing, while overexpression of its endostatin domain leads to delayed healing. Matrix Biol 27: 535–546.

II   Sipola A, Seppinen L, Pihlajaniemi T & Tuukkanen J (2009) Endostatin affects osteoblast behaviour *in vitro*, but collagen XVIII/endostatin is not essential for skeletal development *in vivo*. Calcif Tissue Int 85: 412–420.

III Seppinen L, Kinnunen A, Sormunen R, Eklund L, Heljasvaara R & Pihlajaniemi T (2009) Both lack of collagen XVIII and overexpression of its endostatin domain interfere with hair follicle growth and cycling. Manuscript.

Contents

1   Introduction

The extracellular matrix (ECM) consists of several collagenous and noncollagenous proteins. ECM is needed for providing a structural scaffold for the tissues, but also in regulating the behavior of cells. Basement membranes (BM) are specialized structures of the ECM separating epithelial and endothelial cells from the surrounding connective tissue. In addition to having a structural role, BMs are also involved in cell signalling as BM proteins can directly bind to cell surface receptors, or they can sequester growth factors and present them to their receptors. The proteolytic fragments of BM proteins have also been found to have antiangiogenic effects.

Collagen XVIII is a BM proteoglycan with three variant amino-termini (N-termini). These variants are coded by two different promoters, promoter 1 directs the synthesis of a short variant and promoter 2 directs the synthesis of two longer variants, of which the middle variant is generated from the longest by alternative splicing. The longest variant contains a cysteine-rich domain in its N-terminus, which shows homology to the frizzled-receptors of the Wnt-signalling molecules, and which can inhibit Wnt/beta-catenin signalling *in vitro*. The carboxy-terminal (C-terminal) domain of collagen XVIII, endostatin, has been found to be an inhibitor of tumor growth and angiogenesis.

The goal of this thesis work was to clarify the roles of collagen XVIII and its endostatin domain in wound healing, hair follicle growth and cycling, and bone development; processes which all require angiogenesis to proceed normally. Moreover, our aim was also to elucidate the functions of the different N-terminal variants of collagen XVIII in the hair cycle. For all these studies we have used genetically modified mouse lines that lack collagen XVIII or overexpress transgenic endostatin. For our studies on the specific functions of the N-terminal variants, we have generated promoter-specific knockout mice lacking either the short or the two longer variants of collagen XVIII. Our results show that lack of collagen XVIII can accelerate cutanous wound healing and wound angiogenesis, whereas overexpression of endostatin leads to delayed healing and the presence of morphologically abnormal wound capillaries. Endostatin can influence the behavior of osteoblasts *in vitro*, but lack of collagen XVIII or overexpression of endostatin do not have dramatic effects on skeletal development. Lack of collagen XVIII accelerates the hair cycle, and the same phenotype can be seen in mice lacking the long variants of collagen XVIII. Excess of endostatin impairs hair follicle associated angiogenesis and the growth of hair follicles.

2   Review of the literature

This review of the literature will briefly outline the roles of the extracellular matrix in biological organisms, the emphasis being on the collagen family of proteins, especially on collagen XVIII and its antiangiogenic endostatin domain. A homolog of collagen XVIII, namely collagen XV, will also be discussed in some detail. Moreover, an overview will be given regarding wound healing, hair follicle development and cycling, and bone development, all physiological processes which require angiogenesis to proceed normally.

2.1   Extracellular matrix

ECM is formed by several collagenous and noncollagenous proteins, which serve as a scaffold for maintaining tissue structure, but the ECM also influences the behavior of cells. ECM is being remodeled constantly as cells assemble and degrade it by producing new ECM proteins and secreting factors that proteolyse it. The remodeling of ECM is accelerated during developmental processes and tissue repair. (For reviews see Kolacna *et al.* 2007, Daley *et al.* 2008.)

   Basement membranes (BM) are specialized structures of the ECM produced by epithelial cells, endothelial cells and many mesenchymal cells. BMs are thin, sheet-like structures found below epithelial and endothelial cell layers, separating these cells from the collagenous matrix. BMs are also found surrounding other cells, such as skeletal muscle. The main components of BMs are laminins, collagen IV, nidogen/entactin and perlecan, but they contain also several other components such as collagen XVIII, collagen XV, agrin, fibulin and SPARC/osteonectin/BM40. The specific composition of the BM depends on the tissue and its physiological state. BMs have many important functions; they give structural support to cells, serve as growth factor reservoirs, provide binding sites for cell adhesion molecules, and binding of BMs to cell surface receptors can initiate several intracellular signalling cascades that influence for example proliferation, migration and differentiation of the cells. BMs are also needed in blood filtration to regulate which type of molecules can pass through the BM sieve. Proteolytic remodelling of BM molecules such as perlecan and collagens IV and XVIII can give rise to antiangiogenic polypeptides. These peptides may be released for example during neoplastic tissue remodelling to suppress tumor growth. (For reviews see Yurchenco & Schittny 1990, LeBleu *et al.* 2007.)

2.2   Collagen family of proteins

Collagens are the main proteins of the ECM, and the most abundant proteins in mammals. Collagens form trimeric molecules, whose main function is to maintain the integrity and architecture of tissues and to give them mechanical strength. However, collagens as well as other proteins of the ECM have been found to possess other important functions in regulation of cell signalling, proliferation, migration and apoptosis. (For reviews see Ortega & Werb 2002 Gelse *et al.* 2003.)

Common to all members of the collagen family is the presence of at least one collagenous domain characterized by the presence of several Gly-X-Y amino acid repeats, in which X positions are often occupied by proline residues and Y positions by hydroxyprolines. These Gly-X-Y repeats allow the formation of a triple-helical structure. Triple-helical collagen molecules consist of three polypeptide chains (called α-chains), which can be either identical (in homotrimers) or different (in heterotrimers). Before the formation of trimers, certain proline and lysine residues in collagen polypeptides are hydroxylated, and after this glycosylation of certain amino acid residues can occur. Besides the triple-helical collagenous domains, collagens contain also non-triple helical (NC for noncollagenous) domains. (For reviews see Myllyharju & Kivirikko 2004, Ricard-Blum & Ruggiero 2005.)

Collagens can be divided into several subgroups. The group of fibril-forming collagens includes collagens I, II, III, V, XI, XXIV and XXVII. Some collagens are located in association with the fibrillar collagens, but are not able to form fibrils by themselves. These collagens are called the FACITs (fibril-associated collagens with interrupted triple helices) and they consist of collagens XIV, XVI, XIX, XX, XXI, XXII and XXVI. The structurally related collagens IX and XII belong to the same subfamily. The family of hexagonal network forming collagens includes collagens VIII and X. One group is formed by the BM collagen IV, which consists of several isoforms. Collagen VI, which forms beaded filaments, makes one group, and another group consists of collagen VII, which is an anchoring fibril-forming collagen. Collagens XIII, XVII, XXIII and XXV form a group of transmembrane collagens. Collagens XV and XVIII form one subgroup of collagens, characterized by multiple triple helix domains and interruptions in their structure, and therefore also sometimes referred to as MULTPIPLEXINs (Oh *et al.* 1994b). (For a review see Myllyharju & Kivirikko 2004.)

Mutations in genes coding collagens can lead to a wide spectrum of disorders. Osteogenesis imperfecta, a disease characterized by brittle bones, is in most cases caused by mutations in the genes coding collagen I, but also mutations in proteins needed for prolyl hydroxylation of collagens can lead to a recessive form of osteogenesis imperfecta (Basel & Steiner 2009, Willaert *et al*. 2009). Mutations in the cartilage collagens, namely collagens II, IX, X and XI, can cause several types of chondrodysplasias, but mutations in these collagens have also been linked to intervertebral disc disease and osteoarthrosis. Ehlers-Danlos syndrome is characterized by joint hypermobility, skin hyperextensibility and thinness, skeletal deformities and changes in some internal organs, and it can be caused by mutations in collagens I, III and V. Also, mutations in lysyl hydroxylase, an enzyme needed in the biosynthesis of collagens, can lead to Ehlers-Danlos syndrome (Yeowell & Walker 2000). Mutations in the collagen IV α5 chain cause Alport syndrome, the clinical findings of it including renal failure, sensorineural deafness and eye lesions. Collagen IV α1 chain mutations have been found in patients suffering from porencephaly (Breedweld *et al*. 2006). Mutations in collagen VI are responsible for the development of a muscle disease called Bethlem myopathy. Epidermolysis bullosa, a skin blistering disease, is caused by mutations in collagen VII and XVII. Knobloch syndrome, characterized by ocular abnormalities and encephalocele, is caused by mutations in collagen XVIII. (For reviews see Kuivaniemi *et al*. 1991, Myllyharju & Kivirikko 2001.)

2.3 Collagen XVIII

2.3.1 Primary structure of collagen XVIII

The primary structure of collagen XVIII has been elucidated for human, mouse, chick, *C. elegans* and Xenopus laevis (Oh *et al*. 1994a, Rehn *et al*. 1994b, Rehn & Pihlajaniemi 1995, Saarela *et al*. 1998a, Ackley *et al*. 2001, Elamaa *et al*. 2002, Elamaa *et al*. 2003, Dong *et al*. 2003). Collagen XVIII has three variants coded by two promoters, the sizes of the variants being 1315, 1527, or 1774 amino acid residues in mouse and the sizes of their corresponding N-terminal noncollagenous domains being 301, 517 and 764 amino acid residues. The NC domains of collagen XVIII are usually numbered starting from the C-terminus, and this numbering will also be used here. The short variant has its own signal peptide and

two amino acids which are not found in the other two variants. The two longer variants share the same signal peptide and a 215-amino acid residue domain of unknown function (DUF959) in the N-terminus, followed by a 299-residue thrombospondin-1 like domain (TSP-1) which is also found in the NC11 of the short variant. The longest form, also called the frizzled variant, has an additional 247-residue long region located in the middle of its NC11 domain. This domain includes 10 cysteine residues and has a high homology to the extracellular part of the Wnt-binding frizzled receptors (Rehn & Pihlajaniemi 1994a, Rehn & Pihlajaniemi 1995, Rehn *et al*. 1996). Nine short noncollagenous domains (NC2-NC10) are found within a 654-residue collagenous domain, and the C-terminus of collagen XVIII contains a 315-residue NC1-domain (Rehn *et al*. 2004). (Fig. 1).

Collagen XVIII is also found in *C. elegans*, being one of the few collagen genes that are conserved in the nematodes. Cle-1 is the *C. elegans* homologue to the vertebrate collagen XV and XVIII genes, the homology being highest with collagen XVIII. Cle-1 has also three N-terminal variants, like vertebrate collagen XVIII, although the N-terminal parts of the Cle-1 molecules are not homologous with the collagen XVIII N-termini. The three Cle-1 variants are coded by three promoters, but in mouse and man only two promoters for collagen XVIII have been found to exist. As the N-terminus of the longest variant of collagen XVIII has a frizzled homologous domain, the equivalent region in Cle-1 is related to the netrin receptor unc-40. However, the Cle-1A and Cle-1B variants also have a thrombospondin-like domain in their N-terminus, which has 35% homology with the thrombospondin-like domain of vertebrate collagen XV/XVIII. (Ackley *et al*. 2001.) The presence of collagen XVIII homologs also in chick, Drosophila, *Xenopus laevis* and zebrafish suggests a fundamental role for these BM collagens (Halfter *et al*. 1998, Hynes & Zhao 2000, Elamaa *et al*. 2002, Haftek *et al*. 2003).
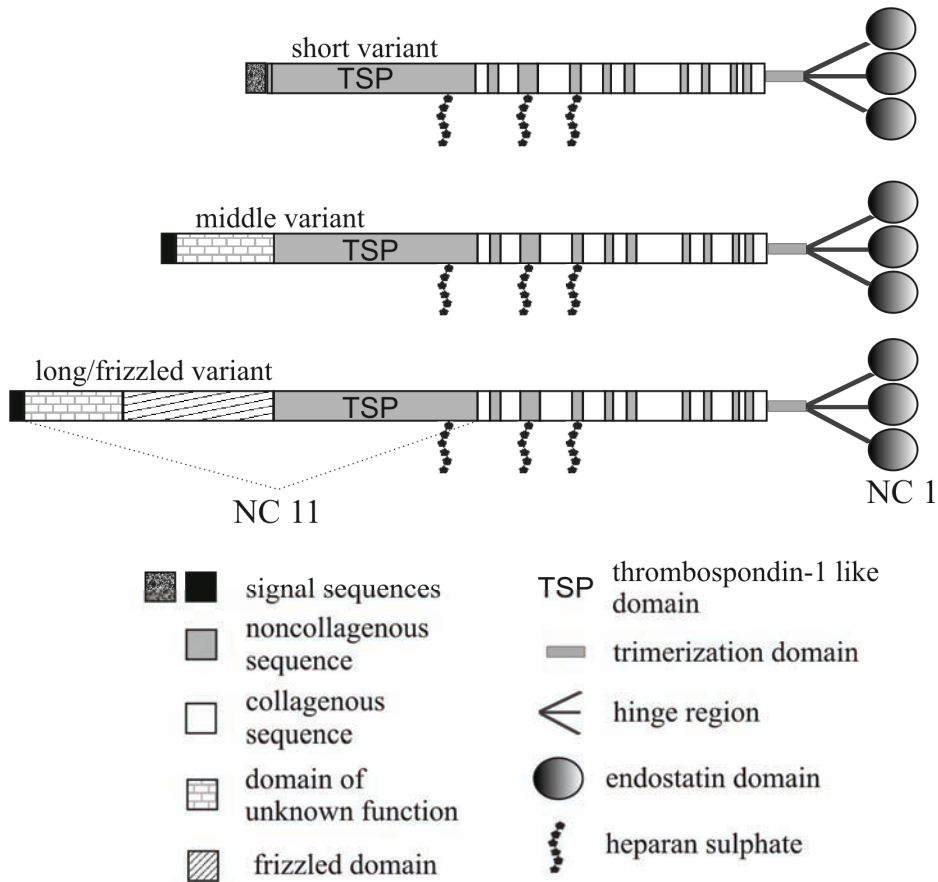
Fig. 1. Schematic picture of collagen XVIII. Putative signal sequence regions are shown in black; the short variant has its own signal sequence while the middle and long variant have the same signal sequence. All of the variants have a thrombospondin-1 like domain in their N-terminal noncollagenous part. The longest variant contains a cysteine-rich frizzled domain in its N-terminus. All three variants contain globular C-terminal endostatin domains and heparan sulphate side chains.

2.3.2 Genomic structure of collagen XVIII

The collagen XVIII coding gene has been mapped to chromosome 21q22.3 in human and chromosome 10 in mouse (Oh *et al.* 1994a). The murine gene for the α1 chain of collagen XVIII (*Col18a1*) is over 102 kb long and contains 43 exons (Rehn *et al.* 1996). The α1 chain of murine collagen XVIII contains ten triple-

helical collagenous domains separated by non-triple-helical regions (Oh *et al*. 1994b, Rehn *et al*. 1994). The C-terminal domain of collagen XVIII bears high homology to the C-terminal domain of collagen XV (Rehn & Pihlajaniemi 1994). The three variants of collagen XVIII are coded by two different promoters, and the middle variant is generated by alternative splicing of exon 3. The splicing leads to a removal of the frizzled domain from the transcript. (Rehn & Pihlajaniemi 1995, Elamaa *et al*. 2003.) The distance between the two alternative transcription start sites is about 50 kb due to the presence of a large second intron. Exons 1 and 2 code the sequence found in the short variant only, and exon 3 directs the synthesis of two longer variants, whereas exons 4–9 encode the N-terminal noncollagenous portion which is common to all variants, and exons 10–43 encode the common collagenous and C-terminal noncollagenous sequences. (Rehn *et al*. 1996.) The structure of the human *COL18A1* gene is highly similar with the mouse gene (Saarela *et al*. 1998a).

2.3.3 Posttranslational glycosylation of collagen XVIII

Collagen XVIII is a heparan sulphate proteoglycan (HSPG) (Halfter *et al*. 1998, Saarela *et al*. 1998b). Proteoglycans are proteins that have glycosaminoglycan (GAG) chains attached to them. GAG side chains are composed of repeating disaccharide units, the structure of them varying in different GAGs. (Hardingham & Fosang 1992.) Heparan sulphate GAG chains consist of alternating N-acetylated or N-sulphated glucosamine units and uronic acids. HSPGs may function as growth factor reservoirs, they can present chemokines to their cell membrane receptors, cells may use them to adhere to the ECM, and they also function as physiological barriers for example in glomeruli. (Bishop *et al*. 2007.)

In Western blotting, collagen XVIII isolated from human kidney and placenta or chick vitreous body has been found to occur as a smear with a molecular weight of approximately 300 kDa. By treatment with heparinase, the size of the molecule is reduced to a protein core of 180 kDa (Halfter *et al*. 1998, Saarela *et al*. 1998b). Collagen XVIII has several potential GAG attachment sites, three of them being conserved between mouse, human, Xenopus and chick (Fig. 1). All of these three sites located in the NC11, NC9 and NC8 domains were also found to be glycosylated in recombinant chick collagen XVIII produced in chick meningeal cells (Dong *et al*. 2003).

Collagen XVIII can interact with L-selectin, a molecule involved in leukocyte infiltration, and this interaction mediates adhesion of lymphoma cells. Collagen

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XVIII also interacts with monocyte chemotactic protein -1 (MCP-1), presenting it to monocytic cells and leading to enhanced binding of these cells to vascular adhesion molecule-1. Both of these interactions are dependent on the heparan sulphate chains of collagen XVIII (Kawashima *et al*. 2003). Only some of the collagen XVIII molecules bind to L-selectin, and this binding seems to be dependent on the O-sulfation and length of the GAG chains (Celie *et al*. 2005). Heparan sulphate chains also seem to be needed for the binding of collagen XVIII to BMs (Dong *et al*. 2003). Collagen XVIII has been shown to be a ligand for the avian ortholog of receptor protein tyrosine phosphatase sigma, which is needed for intraretinal axon growth *in vitro* and for the control of growth cone morphology. This interaction is dependent on the heparan sulphate side chains of collagen XVIII. (Aricescu *et al*. 2002.) The deletion of certain heparan sulphate chains of perlecan leads to degeneration of the eye lens, and this degeneration is accelerated when collagen XVIII is lacking, suggesting that the heparan sulphate chains of collagen XVIII can partly substitute for those of perlecan in maintaining the normal structure and function of the lens (Rossi *et al*. 2003).

2.3.4 Tissue distribution of collagen XVIII

Collagen XVIII is expressed ubiquitously in different vascular and epithelial BM structures throughout the body (Muragaki *et al*. 1995, Saarela *et al*. 1998b). The long variants of collagen XVIII are found especially in the liver, whereas the short variant is localized in most blood vessels and epithelial BMs and around muscular structures (Saarela *et al*. 1998a, Saarela *et al*. 1998b). The expression of the longest frizzled variant of collagen XVIII has been detected in fetal brain, liver, lung, kidney, skeletal muscle, retina and fibroblasts (Suzuki *et al*. 2002, Elamaa *et al*. 2003). Moreover, the long variants of collagen XVIII have been detected as 150 kDa soluble proteins in human plasma (Musso *et al*. 2001a). The longest frizzled variant of collagen XVIII localizes at cell membranes *in vitro* and *in vivo* (Quélard *et al*. 2008).

In epidermal and endothelial BMs, collagen XVIII shows a polarized orientation, with the C-terminal endostatin domain localized in the lamina densa and the N-terminus residing on the matrix side (Marneros *et al*. 2004, Elamaa *et al*. 2005). The endostatin domain of collagen XVIII has been found to be localized across the elastic fibers of large arteries, where it colocalizes with fibulin-1, fibulin-2 and nidogen-2. Consistent with this, endostatin is able to bind fibulins and nidogen-2 (Miosge *et al*. 1999). In adult mouse kidney, the endostatin

domain has been shown to colocalize with perlecan, but not with nidogen-1 (Miosge *et al*. 2003). The endostatin containing NC1 domain of collagen XVIII can also bind to nidogen-1 and perlecan (Sasaki *et al*. 2000).

Collagen XVIII expression can be found in many tissues already during development. Collagen XVIII is expressed in the epithelium of developing mouse lung and kidney, and antibody treatment against collagen XVIII can interfere with the development of lung epithelial tips or ureter bud cultured within lung mesenchyme *ex vivo* (Lin *et al*. 2001). During mouse cardiac development, collagen XVIII expression can be found in the connective tissue core of the endocardial cushions and forming atrioventricular valve leaflets (Carvalhaes *et al*. 2006). Collagen XVIII expression has also been detected surrounding the developing teeth (Väänänen *et al*. 2004).

In the human eye collagen XVIII is localized to almost all structures including different BMs, the epithelial layers of iris, the internal wall of Schlemm's canal and trabeculae, and muscle cells of the ciliary body and iris (Ylikärppä *et al*. 2003, Määttä *et al*. 2007). In liver, collagen XVIII is found in perisinusoidal spaces and BM zones. In cirrhotic liver, collagen XVIII forms a thick deposit along capillarized sinusoids. Hepatocytes and activated stellate cells are the most important sources of collagen XVIII in liver. (Musso *et al*. 1998.) Promoter 2 of the *Col18a1* gene, which is responsible for the production of the two longer variants, has been found to contain liver-specific regulatory elements (Liétard *et al*. 2000). Sp1, Sp3 and YY1 regulate the transcription of collagen XVIII by binding to promoter 2 (Armelin-Correa *et al*. 2005). Collagen XVIII is also expressed in fat tissue, with the expression level being increased during adipocyte differentiation (Inoue-Murayama *et al*. 2000, Errera *et al*. 2008). Using an anti-endostatin antibody, collagen XVIII/endostatin has been found to be localized in the articular cartilage and fibrocartilage (Pufe *et al*. 2004).

Increased amounts of collagen XVIII have been observed in many pathological conditions. Immunohistochemistry with an antibody directed against the long variants of collagen XVIII shows accumulation of the protein in amyloid-laden vessels and senile plaques in Alzheimer's disease brains (van Horssen *et al*. 2002, Deininger *et al*. 2002a). An antibody against the endostatin domain of collagen XVIII has revealed accumulation of collagen XVIII in the brains of patients who died with cerebral malaria, an infectious parasitic disease characterized by dysfunction of the blood-brain barrier (Deininger *et al*. 2002b). Accumulation of collagen XVIII/endostatin has also been observed in the brains of patients suffering from traumatic brain injury, where the injured area can

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expand for a long time after the initial trauma because of hypoperfusion (Deininger *et al*. 2006, Mueller *et al*. 2007). These results suggest a possible role for collagen XVIII in the control of neurodegenerative diseases. Moreover, increased expression of collagen XVIII has been observed in bullous scleroderma skin (Santos *et al*. 2005).

All three variants of collagen XVIII are also expressed at early and late stages of Xenopus laevis development at different levels (Elamaa *et al*. 2002). In *C. elegans*, the expression of Cle-1, the collagen XVIII homolog, has been found to be enriched near synaptic contacts, and mutation in the molecule leads to a defect in the organization of neuromuscular junctions (Ackley *et al*. 2003). Collagen XVIII is also needed for the migration of motor growth cones from the spinal cord into the periphery in zebrafish (Schneider & Granato 2006).

2.3.5 Biological function of collagen XVIII

Mutations in the *COL18A1* gene lead to Knobloch syndrome, which is an autosomal recessively inherited disease (Sertié *et al*. 2000). Most of the cases are caused by nonsense mutations in the *COL18A1* gene leading to premature stop codons, but a missense mutation has also been described. In most of the cases all the variants of collagen XVIII are affected, but one known mutation affects only the short isoform. (Suzuki *et al*. 2009.) Knobloch syndrome is characterized by the occurrence of high myopia, vitreoretinal degeneration with retinal detachment, macular abnormalities and occipital encephalocele, which is a neural tube closure defect (Passos-Bueno *et al*. 2006). Moreover, there is some evidence that Knobloch syndrome may predispose patients to epilepsy (Suzuki *et al*. 2002). Persistence of fetal vasculature of the eye has also been reported in one Knobloch syndrome patient (Duh *et al*. 2004).

Mice lacking collagen XVIII show several ocular abnormalities including delayed regression of the hyaloid vessels of the eye, abnormal outgrowth of retinal vessels, and fragile iris leading to separation of the posterior iris pigment epithelial cell layer, which adheres with its BM to the posterior ciliary body and lens. There is also flattened morphology of the ciliary body and atrophy of its pigmented epithelial cells, age-dependent thickening of the anterior iris BM, abnormal migration of the iris pigment cells to the retina and age-dependent loss of vision due to abnormal vitamin A metabolism in the retinal pigment epithelium. (Fukai *et al*. 2002, Marneros & Olsen 2003, Ylikärppä *et al*. 2003a, Marneros *et al*. 2004.) An increased number of astrocytes and reduced

susceptibility to high oxygen-induced retinal neovascularization have also been observed in the eyes of collagen XVIII-deficient mice (Hurskainen *et al*. 2005). Besides the eye abnormalities, *Col18a1*^{-/-} mice also show an increased susceptibility to hydrocephalus in a certain genetic background, and they have broadened epidermal BMs, suggesting a structural role for collagen XVIII in maintaining the integrity of BMs (Utriainen *et al*. 2004). Furthermore, lack of collagen XVIII enhances atherosclerosis and leads to increased intimal neovascularization in atherosclerotic plaques and increased vascular permeability (Moulton *et al*. 2004).

The frizzled domain of collagen XVIII can inhibit Wnt/beta-catenin signalling in cancer cells *in vitro* by binding to Wnt-molecules and presumably regulating their accessibility to their receptors. Frizzled domain-containing polypeptides are produced *in vivo*, and only these shorter polypeptides, not the full-length frizzled variant of collagen XVIII, can inhibit Wnt-signalling. The frizzled variant transfected to hepatoma cells has been shown to localize in cell membranes, suggesting that this variant, contrary to the two others, may not be a basement membrane protein. (Quélard *et al*. 2008.) Proteolytical processing of the full-length frizzled variant has been shown to occur both *in vitro*, and in cancerous liver tissues also *in vivo*, producing N-terminal peptides which contain the frizzled domain (Elamaa *et al*. 2003, Quélard *et al*. 2008). Moreover, polymorphism in the frizzled domain of *COL18A1* gene has been shown to associate with a higher risk for obesity in type 2 diabetic patients (Errera *et al*. 2008).

2.3.6 *Endostatin*

Endostatin is a C-terminal fragment of collagen XVIII, and it was first identified from murine hemagioendothelioma cell culture medium (O'Reilly *et al*. 1997). Endostatin is able to inhibit angiogenesis and tumor growth by inhibiting endothelial cell proliferation (O'Reilly *et al*. 1997) and migration (Yamaguchi *et al*. 1999), and by inducing endothelial cell apoptosis (Dhanabal *et al*. 1999).

Biosynthesis

Endostatin can be cleaved from collagen XVIII by different proteinases, such as MMP-3, -7, -9, -13, -14 and -20, elastase and cathepsin L (Wen *et al*. 1999, Felbor *et al*. 2000, Ferreras *et al*. 2000, Chang *et al*. 2005, Heljasvaara *et al*. 2005). The

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size of the C-terminal NC1 domain of collagen XVIII is 38 kDa, while the size of the endostatin domain is 22 kDa. There are several endogenous cleavage sites in the NC1 domain, and endostatin-containing fragments of varying size (ranging from 22 kDa to 38 kDa) can be found in mouse serum and tissues. (Sasaki *et al.* 1998.) The full-length NC1 domain contains an N-terminal association domain, which leads to trimerization of the peptides, whereas the 22 kDa endostatin fragments are monomers. Only the monomeric endostatin is efficient in inhibiting angiogenesis (Sasaki *et al.* 2000), whereas the trimerized NC1 domain actually induces migration of several cell lines, including endothelial cells (Kuo *et al.* 2001). The trimerized NC1 domain binds strongly to laminin-111, and the interaction of this domain with laminin seems to be important for its motogenic effects (Javaherian *et al.* 2002).

Structure – function considerations

Endostatin contains a zinc-binding site in its N-terminal part (Ding *et al.* 1998). There is some controversy on whether the zinc-binding site has more a structural or a functional role, as there are results which imply that it is not needed for endostatin's ability to inhibit endothelial cell migration and tumor growth (Yamaguchi *et al.* 1999, Hohenester *et al.* 2000), but the latest results suggest that the zinc-binding is needed for endostatin's antiangiogenic and tumor growth suppressing effects (Tjin Tham Sjin *et al.* 2005).

Endostatin also has a heparin-binding domain (Hohenester *et al.* 1998). Binding of endostatin to blood vessels is not dependent on the presence of heparan sulphates (Chang *et al.* 1999, Rychkova *et al.* 2005), although heparan sulphate binding ability seems to increase the binding of endostatin to endothelial cells (Gaetzner *et al.* 2005). Moreover, endostatin's inhibitory effect on angiogenesis seems to be dependent on its ability to bind to heparin (Dixelius *et al.* 2000, Olsson *et al.* 2004, Gaetzner *et al.* 2005). The structure and size of the heparan sulphate saccharides is important for binding to endostatin, as the saccharides need to be able to interact with endostatin's two separate arginine clusters (Kreuger *et al.* 2002, Blackhall *et al.* 2003). Both of these arginine clusters are needed to inhibit FGF-induced angiogenesis, but for the inhibition of VEGF-induced angiogenesis, only one of the arginine-containing heparin-binding sites is needed (Olsson *et al.* 2004). Endostatin has been shown to compete with bFGF for binding to heparin, which may explain its FGF antagonizing effects (Reis *et al.* 2005). An endostatin-derived synthetic arginine-rich peptide can

promote the motility of endothelial cells in a β1 integrin- and heparin-dependent manner when applied in an immobilized form, but in a soluble form it inhibits endothelial cell migration and tubule formation (Wickström *et al*. 2004). Binding of endostatin to heparin and heparan sulphates also requires the presence of divalent cations (Ricard-Blum *et al*. 2004). The motogenic effects of the oligomeric NC1 domain of collagen XVIII on endothelial cells can be inhibited with the addition of exogenous glycosaminoglycans, and morphological changes induced by the NC1 domain on epithelial cells are dependent on heparan sulphates (Clamp *et al*. 2006). Endostatin contains two disulphide bonds, which are important for its proper structure and biological function (Zhou *et al*. 2005). Endostatin can also be glycosylated, as O-glycosylated endostatin fragments have been found in human plasma (John *et al*. 1999, John *et al*. 2005).

Effects on tumor growth and angiogenesis

Endostatin has been very extensively studied after its discovery, and the main reason for this is its tumor growth suppressing effect. The emergence of tumor angiogenesis has been reported to result from a shift in balance between angiogenic and antiangiogenic molecules (Rastinejad *et al*. 1989). Endostatin has been shown to inhibit the growth of several different tumors of both mouse and human origin. Moreover, in clinical studies it does not show any toxicity or develop drug resistance even in treatments lasting over 3.5 years. (Folkman 2006.) In some clinical trials the efficacy of endostatin has been tested. In one phase II study, endostatin failed to show an antitumorigenic effect on hypervascular neuroendocrine tumors (Kulke *et al*. 2006). No antitumor response, as judged by standard response criteria, was observed in the treatment of melanoma with endostatin alone or with interferon-α2b (Moschos *et al*. 2007). However, in China endostatin has been approved as a treatment for non-small-cell lung cancer (Folkman 2006). The removal of a primary tumor has been shown to lead to a decrease in circulating endostatin concentration and increase in the metabolic activity and growth of the tumor's distant metastases (Wu *et al*. 2004, Peeters *et al*. 2005, Sun *et al*. 2005). Down syndrome, trisomy of chromosome 21, is known to correlate with a decreased incidence of solid tumors (Hasel *et al*. 2000). Increased serum endostatin levels have been measured in Down syndrome patients, suggesting a role for the extra copy of collagen XVIII as a tumor suppressor (Zorick *et al*. 2001). Interestingly, transgenic mice overexpressing endostatin specifically in their endothelial cells show a 1.6-fold increase in

circulating endostatin levels, which is sufficient to inhibit tumor vascularization and slow down the growth of transplanted tumors (Sund *et al*. 2005). Endostatin's efficacy on endothelial cell migration *in vitro* and tumor growth *in vivo* follows an U-shaped dose-response curve, the efficacy being the best between the very low and very high concentrations and depending also on the type of cancer treated (Celik *et al*. 2005, Tjin Tham Sjin *et al*. 2006). Endostatin can also have inhibitory effects directly on cancer cells (Nyberg *et al*. 2003, Wilson *et al*. 2003).

As already mentioned earlier, endostatin can inhibit the migration and proliferation of endothelial cells (O'Reilly *et al*. 1997, Yamaguchi *et al*. 1999) and to induce their apoptosis (Dhanabal *et al*. 1999). *In vivo*, endostatin has been shown to inhibit the recruitment of pericytes into capillaries, thus reducing the number of mature vessels (Skovseth *et al*. 2005). Endostatin is able to inhibit the growth factor-induced mobilization of endothelial progenitor cells into circulation, and also thereby suppress angiogenesis (Schuh *et al*. 2003). Even though endostatin inhibits the growth factor-induced endothelial tube formation *in vitro*, the endothelial tubes that have formed in the presence of endostatin had shown a longer time of survival in one study (Ergün *et al*. 2001). In an aortic explant assay, lack of collagen XVIII/endostatin results in increased outgrowth of microvessels, affirming the assumption of a role for endogenous endostatin as a negative regulator of vessel growth (Li & Olsen 2004). The effect of endostatin on angiogenesis seems to be dependent on the cell type, as different endothelial cell lines show different responses to endostatin treatment and the response to endostatin seems to be also dependent on the time of culture and the maturity of the cells (Schmidt *et al*. 2004, Khan *et al*. 2006). Moreover, it has been shown that the effects of endostatin on endothelial cells are modulated by the ECM proteins on which the cells are plated (Delaney *et al*. 2006). Endostatin also inhibits lymphatic endothelial cell migration and proliferation in a dose-dependent manner (Shao & Xie 2005). Mice overexpressing endostatin show a decreased amount of dermal lymphatic vessels in the skin as well as in skin papillomas and carcinomas induced by chemical treatment, and also a reduced number of lymph node metastases (Brideau *et al*. 2007).

Other biological functions

In the eye, endostatin prevents choroidal neovascularization in a model where neovascularization was induced by rupturing the Bruch's membrane of the mouse eye with a laser (Mori *et al*. 2001), and it also inhibits retinal neovascularization,

retinal detachment and retinal vasopermeability induced by VEGF (Takahashi *et al*. 2003, Campbell *et al*. 2006). Overexpression of endostatin in lens and skin leads to broadening of epidermal BMs and the development of cataract (Elamaa *et al*. 2005).

Endostatin also has effects on bone as endostatin treatment reduces bone formation in a mouse ectopic ossification model *in vivo* (Sipola *et al*. 2007). Endostatin can inhibit the VEGF-mediated osteoclastic bone resorption and differentiation of osteoclasts (Sipola *et al*. 2006), and it inhibits the fluid shear stress -induced primary osteoblast proliferation and the upregulation of β-catenin transcription normally associated with it (Lau *et al*. 2006). However, in articular chondrocyte culture endostatin has been shown to induce cell proliferation, spreading and adhesion (Feng *et al*. 2005).

Endostatin has been reported to have beneficial effects on the development of inflammatory diseases. In a TNF-induced inflammatory arthritis model, endostatin leads to the inhibition of angiogenesis in the synovial tissue (Yin *et al*. 2002), and when using human rheumatoid arthritis synovial grafts implanted in immunodeficient mice, recombinant endostatin was also shown to decrease inflammation and angiogenesis (Matsuno *et al*. 2002). Endostatin can also inhibit inflammation and angiogenesis in cyclophosphamide induced cystitis (Beecken *et al*. 2004) and prevent the development of allergen-induced acute asthma in a mouse model (Suzaki *et al*. 2005).

Moreover, endostatin treatment has been observed to inhibit the growth of endometriotic lesions in mice (Becker *et al*. 2005). Endostatin can also inhibit the invasion of trophoblasts *in vitro*, suggesting a regulatory role for endostatin in controlling the invasiveness of these cells (Pollheimer *et al*. 2005). Endostatin has been shown to inhibit the wound repairing ability of distal lung epithelial cells as well as to reduce the viability of these cells (Richter *et al*. 2009). Increased endostatin serum levels, associated with impaired healing of gastric ulcers, have been measured after ticlopidine treatment (Ma *et al*. 2001). Endostatin has been observed to inhibit the progression of peritoneal sclerosis (Tanabe *et al*. 2007) and streptozotocin-induced type 1 diabetes (Ichinose *et al*. 2005) in mouse models. Endostatin can protect apolipoprotein E-deficient mice from atherosclerosis by inhibiting intimal neovascularization and growth of atherosclerotic plaques (Moulton *et al*. 1999) and it can also prevent the retention of LDL in the subendothelial matrix (Zeng *et al*. 2005).

Lack of the C-terminal endostatin containing NC domain of *C. elegans* collagen XVIII leads to defects in cell migration and axon guidance, and similar

changes are seen in animals that overexpress endostatin, indicating that endostatin inhibits the promigratory effects of the full-length C-terminal NC domain (Ackley *et al*. 2001).

Mechanisms of action

No uniform picture has emerged of endostatin's molecular mechanisms of action. Instead, several different mechanisms have been proposed, and it seems that endostatin can affect the behavior of cells via many different pathways. Endostatin has been shown to interact with many different receptors on the cell membrane, with the most evidence involving its interaction with α5β1 integrin. However, endostatin can also affect the activation of different proteases and thereby affect angiogenesis and other biological processes. Below I will provide a more detailed introduction of these mechanisms.

In endothelial cells, endostatin has been shown to bind α5β1 integrin, and then move to endothelial cell membrane lipid rafts in a heparan sulphate dependent manner to associate with caveolin-1, these interactions leading to activation of Src. Src phosphorylation leads to inactivation of RhoA, followed by disassembly of actin stress fibers and focal adhesions, which leads to decreased migratory capacity of the cells. (Wickström *et al*. 2002, Wickström *et al*. 2003.) In addition to integrin α5β1, endostatin also binds integrin αv, and interestingly, immobilized endostatin has been shown to enhance endothelial cell migration, this effect being blocked by the addition of soluble integrin α5 or αvβ3 antibodies (Rehn *et al*. 2001, Sudhakar *et al*. 2003). Endostatin can inhibit the adhesion of endothelial cells to collagen I via a collagen receptor α2β1 integrin (Furumatsu *et al*. 2002). Other cell surface receptors of endostatin are glypicans, the GAG chains of glypicans being needed for this interaction. Glypicans seem to be needed for the antimigratory effects of endostatin on endothelial cells, and endostatin can also inhibit branching morphogenesis of renal tubular cells in a glypican-dependent manner (Karumanchi *et al*. 2001) as well as modulate ureteric bud branching *ex vivo* (Karihaloo *et al*. 2001). Endostatin can also directly bind to VEGFR-1, VEGFR-2 and VEGFR-3, and prevent the association of VEGF molecules with their receptors (Kim *et al*. 2002, Kojima *et al*. 2008). Endostatin has been shown to bind also to the cell surface receptor nucleolin; this complex is internalized and translocated to the nucleus, where endostatin can inhibit the phosphorylation of nucleolin and thus inhibit cell proliferation. Blocking nucleolin function with antibodies has been shown to lead to abrogation of

endostatin's inhibitory effects on angiogenesis and tumor growth *in vivo*. (Shi *et al*. 2007.)

In vitro, endostatin can modulate plasminogen activation in endothelial cells by affecting the uPA (urokinase plasminogen activator) system, leading to disassembly of the focal adhesions and dissociation of the actin fiber network (Wickström *et al*. 2001). In another study, treatment of endothelial cells with endostatin or FGF-2 alone led to increased actin stress fiber formation and number of focal adhesions, but treatment of cells with endostatin and FGF-2 together had the opposite effect (Dixelius *et al*. 2002). Endostatin can bind to tropomyosin, which is a stabilizer of actin filaments, and it has been suggested that via this interaction endostatin may disrupt the integrity of the actin cytoskeleton and lead to inhibition of angiogenesis (MacDonald *et al*. 2001). Treatment of endothelial cells with insoluble amyloid endostatin stimulates tPA (tissue plasminogen activator) –induced plasmin formation, associated with the detachment of endothelial cells and degradation of the ECM (Reijerkerk *et al*. 2003). Binding of endostatin to phosphatidylserine-containing cell membranes, which are found in many tumor cells and tumor vascular endothelial cells, has been shown to trigger the formation of amyloid fibers (Zhao *et al*. 2005). Lack of endostatin's disulphide bonds has been associated with an increased tendency for amyloid fibril formation (He *et al*. 2006). Vitronectin and fibronectin seem to be important for the antiangiogenic effects of endostatin, as the lack of vitronectin or plasma fibronectin in mice leads to impaired antiangiogenic response after endostatin treatment in a matrigel plug model (Yi *et al*. 2003), and it has been suggested that the plasma fibronectin and vitronectin are needed as cofactors for several antiangiogenic peptides to form integrin-binding complexes (Akerman *et al*. 2005). E-selectin is also needed for the effects of endostatin, as mice lacking E-selectin fail to show an antiangiogenic response to endostatin treatment, and endothelial cells overexpressing E-selectin have an increased sensitivity to endostatin (Yu *et al*. 2004). Endostatin can prevent the VEGF-induced activation of eNOS, endothelial nitric oxide synthase, and hence also inhibit the production of angiogenic nitric oxide (Urbich *et al*. 2002). Endostatin also downregulates the downstream target of nitric oxide, soluble guanylate cyclase, *in vivo* and *in vitro* (Schmidt *et al*. 2005). However, without the addition of VEGF and using short-term endostatin treatment, endostatin has been shown to induce eNOS activation and nitric oxide production (Li *et al*. 2005) and induce vasorelaxation (Wenzel *et al*. 2006). Moreover, peptides derived from the endostatin-containing NC11 domain of collagen XVIII have been shown to inhibit angiotensin I converting

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enzyme, providing another possible mechanism to regulate blood pressure (Farias *et al*. 2006). Endostatin can promote β-catenin degradation and hence inhibit Wnt/beta-catenin signaling, and this arrests the cell cycle by inhibition of the cyclin-D1 promoter (Hanai *et al*. 2002a, Hanai *et al*. 2002b). Moreover, endostatin inhibits the activation of proMMP-2, proMMP-9 and proMMP-13, as well as the catalytic activity of MMP-2 and membrane type-1 MMP (Kim *et al*. 2000, Nyberg *et al*. 2003). Endostatin-induced endothelial cell apoptosis involves the opening of the mitochondrial permeability transition pore via voltage-dependent anion channel 1 (Yuan *et al*. 2008). Overexpression of ornithine decarboxylase, a molecule involved in the biosynthesis of polyamines, is seen in many cancers, and it is associated with promoted tumor growth and angiogenesis. Suppressed levels of collagen XVIII and endostatin are found in tumor cells and samples overexpressing ornithine decarboxylase (Nemoto *et al*. 2002).

Endostatin has been shown to downregulate 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, adhesion molecules and cell structure components (Shichiri & Hirata 2001). In a genome-wide microarray analysis of cultured human endothelial cells, endostatin treatment was found to significantly affect the expression levels of approximately 12% of the genes analyzed, downregulating the angiogenic genes and upregulating the antiangiogenic genes, thereby reinforcing the overall antiangiogenic effect (Abdollahi *et al*. 2004). Hypoxia, a lack of oxygen, leads to strong induction of angiogenesis. Downregulation of endostatin expression has been observed in endothelial cells, pericytes and endometrial stromal cells as a result of hypoxia, suggesting that hypoxia not only leads to an upregulation of angiogenic genes, but also to a downregulation of the antiangiogenic genes (Wu *et al*. 2001, Nasu *et al*. 2004). p53 is an important tumor suppressor, and it has been shown to be able to bind to the promoter of *COL18A1* gene and lead to its activation (Miled *et al*. 2005). p53 also increases the transcription of α(II) collagen prolyl-4-hydroxylase, which leads to increased synthesis of collagen XVIII and collagen IV, and p53 then accelerates the proteolytical processing of C-terminal antiangiogenic molecules from these collagens (Teodoro *et al*. 2006).

2.4   Collagen XV

The gene coding the α1 chain of collagen XV is found in human chromosome 9 and in mouse chromosome 4, and shows high similarity to collagen XVIII as these molecules have very similar N-terminal and C-terminal noncollagenous domains. They also contain a triple-helical collagenous domain separated by several noncollagenous domains. (Huebner *et al*. 1992, Muragaki *et al*. 1994, Kivirikko *et al*. 1994, Rehn & Pihlajaniemi 1994, Rehn *et al*. 1994, Hägg *et al*. 1997a.) In human tissues and cultured cells, collagen XV is a chondroitin sulphate proteoglycan (Li *et al*. 2000). Northern blot analysis shows collagen XV expression in marked amounts in skeletal tissue and heart, and in smaller amounts in organs such as kidney and pancreas. *In situ* hybridization has revealed expression of collagen XV in myoblasts, fibroblasts and endothelial cells. (Kivirikko *et al*. 1995.) Immunohistochemical analyses show that collagen XV is localized in vascular, neuronal, mesenchymal and some epithelial BM zones (Myers *et al*. 1996). Immuno-electron microscopy suggests that collagen XV may link banded collagen fibers in very close proximity to the BMs (Amenta *et al*. 2005). Moreover, collagen XV can also be detected in the cartilage perichondrium (Muona *et al*. 2002) and in the fibrillar collagen matrix for example in dermis, and an increased amount of collagen XV has been observed in kidney fibrosis, suggesting a role for collagen XV in fibrotic processes (Hägg *et al*. 1997b). On the other hand, downregulation of type XV collagen has been reported in fibroblasts isolated from patients affected by another fibroproliferative disease, Dupuytren's contracture, characterized by the appearance fibrotic nodules and contracted cords within the palmar fascia (Satish *et al*. 2008).

Lack of collagen XV in mice leads to skeletal myopathy, and these mice show cardiac injury and a diminished heart inotropic response after exercise (Eklund *et al*. 2001). Despite the high structural similarity of collagens XV and XVIII, double knockout mice lacking both of these collagens reveal that there is no major functional compensation between them, suggesting entirely different biological roles for these two collagens (Ylikärppä *et al*. 2003b). Collagen XV has been suggested to be a negative regulator of glial cell recruitment around vessels of the central nervous system, as the persistent hyaloid vessels that are seen in the *Col18a1*^{-/-} mice which are normally devoid of gial cells, are covered with astrocytes in *Col18a1*^{-/-};*Col15a1*^{-/-} double knock-out mice (Hurskainen *et al*. 2005). Restin, the C-terminal noncollagenous domain of collagen XV which shows high homology to endostatin, is able to inhibit migration of endothelial

cells (Ramchandran *et al*. 1999). Restin also inhibits chorioallantoic angiogenesis induced by FGF-2 or VEGF (Sasaki *et al*. 2000).

2.5   Cutaneous wound healing

Wound healing is a complex process which aims to rapidly and efficiently repair the break in the protective barrier. At first, a blood clot is formed to give a temporary protection to the injured area. The clot releases growth factors and cytokines which are needed to initiate the healing process. After the formation of the clot, inflammatory cells, fibroblasts and endothelial cells start to invade to the wound region. Inflammatory cells are needed to clean the wound from foreign material, but they also serve as a source of growth factors and cytokines. Wound fibroblasts produce collagen-rich matrix in the wound area, but some fibroblasts are also transformed into myofibroblasts, which have a role in contraction of the wound. Numerous capillaries are needed to nourish the healing wound tissue, which is called granulation tissue. Meanwhile, epidermal cells from the wound edge start to proliferate and migrate to cover the injured area. (For a review see Martin 1997.)

In skin, keratinocytes are attached to the underlying BM by hemidesmosomal structures, the main constituents of which are α6β4 integrins and collagen XVII (Uitto & Pulkkinen 1996). For the reepithelialization to occur in the wound, the keratinocytes have to lose their contacts with the underlying BM, and the leading edge keratinocytes must start to express new integrins and relocalize other integrins in order to be able to migrate on the wound matrix (Cavani *et al*. 1993). Keratinocytes also need to express proteases to dissolve the fibrin and be able to invade between the wound clot and the dermis. Important proteases in this process are plasmin (Grøndahl-Hansen *et al*. 1988, Romer *et al*. 1996) and several MMPs (Gills & Parks 2008). Once the wounded area has been covered with keratinocytes, new hemidesmosomal adhesions are assembled (Compton *et al*. 1989).

Myofibroblasts play a key role in the remodelling of wound connective tissue. Their role is to contract the wound to bring the wound margins closer to each other. Myofibroblasts originate from the wound fibroblasts, this transformation being triggered by TGF-β1 and mechanical stress. Myofibroblasts express α-smooth muscle actin, which makes it possible for the cells to produce contractile forces. Myofibroblasts can also express uPA (Sieuwerts *et al*. 1998) and some MMPs (Soini *et al*. 2001, McKaig *et al*. 2003, Lee *et al*. 2003), and in

this way participate in the degradation of the wound ECM. When wound healing is completed, myofibroblasts die in an apoptotic manner. (For reviews see Martin 1997, Desmoulière *et al.* 2005, Hinz 2007.)

The formation of new blood vessels, angiogenesis, is an essential step in the wound healing process. Several capillaries are needed to bring enough nutrients and oxygen to the wound tissue, which is going through rapid remodelling. The growth factors inducing wound angiogenesis include VEGF, FGF, angiopoietin-1 and TGF-β, VEGF being the key mediator of wound angiogenesis. (Martin 1997, Corral *et al.* 1999, Li *et al.* 2003, Cho *et al.* 2006, Bitto *et al.* 2008).
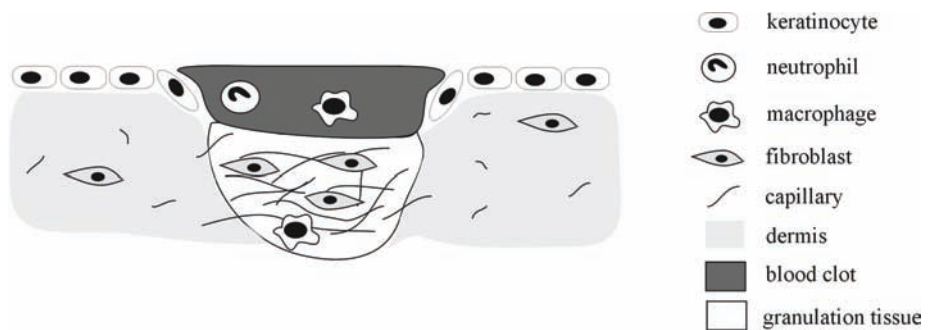
Fig. 2. Cutaneous wound healing. The injured area is covered with a blood clot. Inflammatory cells within the clot release growth factors and cytokines which are needed to initiate re-epithelialization, connective tissue contraction, recruitment of inflammatory cells from circulation, and angiogenesis. Keratinocytes begin to migrate to cover the injured area. Fibroblasts invade to the wound dermis, called granulation tissue, and they are responsible for the production of new collagen matrix. Some fibroblasts differentiate into myofibroblasts, which are needed for wound contraction. Capillaries invade the wound tissue from the surrounding dermis to nourish the rapidly remodeling tissue. Inflammatory cells of the granulation tissue are needed to clear the wound from pathogenic organisms and to produce cytokines.

2.5.1 The role of ECM molecules and their receptors in wound healing

Integrins

Several ECM molecules have important roles in the wound healing process. Keratinocyte attachment and migration require the expression of different integrin receptors on the keratinocyte surface, as well as the presence of ECM molecules

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such as fibronectin, vitronectin and collagens in the wound region (O'Toole 2001). Problems in the interaction between cells' integrin receptors and the surrounding matrix can interfere with the wound healing process. The loss of β1 integrin in keratinocytes leads to severely delayed wound reepithelialization due to the inhibition of keratinocyte migration (Grose *et al*. 2002). Integrin α5β1 has also been reported to be important for the wound repair of airway epithelium *in vitro* (Hérard *et al*. 1996). The lack of αvβ6 integrin leads to a delay in wound healing in aged mice (AlDahlawi *et al*. 2006). Conditional ablation of α9 integrin in keratinocytes leads to decreased keratinocyte proliferation and impaired reepithelialization (Singh *et al*. 2009). There are controversial results on the role of the integrin α3 subunit in wound healing, as lack of integrin α3 has been reported to lead to both retarded (Reynolds *et al*. 2008) and accelerated reepithelialization of wounds (Margadant *et al*. 2009). *In vitro*, lack of integrin α6β4 leads to improper assembly of laminin-332 which interferes with the motility of the keratinocytes (Sehgal *et al*. 2006). *In vivo*, lack of integrin β4 leads to a decrease in keratinocyte proliferation and delayed wound reepithelialization (Nikolopoulos *et al*. 2005). Keratinocytes from mice lacking the integrin β5 subunit show a decreased migration capacity, but the wound healing process in the integrin β5 knockout mice is not affected (Huang *et al*. 2000). Signalling via integrin receptors can also be significant for regulating other signalling pathways important in the wound healing process. Deficiency of β3 integrin leads to an induction of TGF-β1 signalling and accelerated wound reepithelialization (Reynolds *et al*. 2005). Increased expression of β6 in keratinocytes has been shown to lead to spontaneous formation of chronic fibrotic ulcers, accompanied by strongly increased TGF-β1 levels in the lesions (Häkkinen *et al*. 2004). In cell culture, integrin αVβ6 has been shown to induce expression of MMP-9 and to promote oral keratinocyte migration (Thomas *et al*. 2001).

Integrins can also affect wound angiogenesis as lack of integrin α2β1 leads to enhanced wound angiogenesis, possibly due to upregulation of MMPs needed for the invasion of endothelial cells (Grenache *et al*. 2007, Zweers *et al*. 2007). Interaction of integrin α5β1 with its fibronectin ligand induces endothelial cell invasion, the invasiveness of the cells being negatively regulated by integrin α4β1 (Zeng *et al*. 2009). The collagen receptor integrin α1β1 has been suggested to have a role in wound contraction, as mice deficient in integrin α1β1 show a reduced number of myofibroblasts as well as reduced angiogenesis in a matrigel plug assay (Rodriguez *et al*. 2008). Also, blocking of integrin α1 with antibodies leads to inhibition of collagen contraction by liver myofibroblasts *in vitro*

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(Racine-Samson *et al*. 1997). αV integrins have also been suggested to have a role in the transformation of fibroblasts into myofibroblasts as inhibition of αV integrins with function-blocking antibodies suppresses myofibroblast differentiation *in vitro* (Lygoe *et al*. 2004) and wound angiogenesis *in vivo* (Christofidou-Solomidou *et al*. 1997, Jang *et al*. 1999). β2 integrins are leukocyte adhesion molecules. Lack of integrin β2 leads to delayed healing of wounds, and is associated with decreased TGF-β1 levels and impaired myofibroblast differentiation (Peters *et al*. 2005). Lack of a leukocyte receptor αMβ2 integrin has also been shown to lead to delayed wound healing (Sisco *et al*. 2007).

Basement membrane proteoglycans

Perlecan is a BM heparan sulphate proteoglycan, necessary for the structural integrity of BMs (Costell *et al*. 1999), but the heparan sulphate chains of perlecan are also needed for regulating the accessibility of growth factors to target cells (Iozzo 1998). Mice that lack heparan sulphate side chains from their perlecan molecules display delayed wound healing associated with impaired angiogenesis *in vivo* and reduced binding of FGF-2 to their fibroblasts *in vitro* (Zhou *et al*. 2004).

The role of collagen XVIII in wound healing has not been studied before, but there are some reports on the effects of its endostatin domain on the wound healing process. Treatment of mice with recombinant endostatin has been shown to inhibit the healing of excisional cutaneous wound in one study (Michaels *et al*. 2005), but not in another where a lower dose of endostatin (0.3 mg/kg vs. 20 mg/kg) was used (Bloch *et al*. 2000). Endostatin injections led to a decreased number of wound capillaries in the study where a high dose of endostatin was administered (Michaels *et al*. 2005), but not when a lower dose was given (Bloch *et al*. 2000), or when the wounds were incisional (Berger *et al*. 2000). In one study, an increased number of capillaries was seen after endostatin treatment with a dose of 0.3 mg/kg (Schmidt *et al*. 2006). Morphological changes of newly formed wound capillaries were detected in some of the studies (Bloch *et al*. 2000, Schmidt *et al*. 2006), and there is some evidence that these changes are due to the dephosphorylation of ERK1/2, a downstream target of VEGF signalling (Schmidt *et al*. 2006). However, as there are a few reports on the effects of endostatin on cutaneous wound healing, their results are conflicting in some respects.

Other basement membrane proteins

Lack of the α3 chain of collagen IV, which is the source of an antiangiogenic tumstatin fragment, does not have an effect on cutaneous wound healing even though accelerated tumor growth and tumor angiogenesis are observed in these mice (Hamano *et al*. 2003). Arresten, the C-terminal fragment of the α1 chain of collagen IV, is also antiangiogenic as it is capable of inhibiting endothelial cell proliferation and migration *in vitro* (Colorado *et al*. 2000) and tumor angiogenesis *in vivo* (Sudhakar *et al*. 2005), and inducing endothelial cell apoptosis *in vitro* (Nyberg *et al*. 2008). The C-terminal fragment of the α2 chain of collagen IV, canstatin, also inhibits endothelial cell migration and proliferation and induces the apoptosis of endothelial cells *in vitro*, and is able to inhibit tumor angiogenesis *in vivo* (Kamphaus *et al*. 2000). However, the roles of the α1 and α2 chains of collagen IV, or their antiangiogenic C-terminal fragments, in wound healing have not been studied.

Laminin-332 has been shown to be important for the migratory capacity of keratinocytes *in vitro* (Zhang *et al*. 1996, Hartwig *et al*. 2007), and it can induce epithelial cell motility even in a soluble form (Kariya & Miyazaki 2004). Deficiency of laminin-332 due to genetic mutations leads to a disease called junctional epidermolysis bullosa, which is characterized by blistering of the skin due to a fragile epidermal-dermal junction, but also wound healing is impaired in these patients (Schneider *et al*. 2007). Laminin-332 expression is reduced in diabetic wounds, but treatment of diabetic wounds with soluble laminin-332 does not improve the closure of wounds (Sullivan *et al*. 2007). Also, the laminin α5 chain (a constituent of laminins -511 and -521) has been shown to be an inductor of keratinocyte proliferation and migration (Pouliot *et al*. 2002). Fragments of laminin α1 and γ1 chains, responsible for binding to integrins αvβ3 and α5β1, have been shown to accelerate re-epithelialization and contraction of rat cutaneous wounds (Malinda *et al*. 2008). Endothelial overexpression of laminin-411, a constituent of vascular BMs, accelerates endothelial cell migration and tubule formation *in vitro* (Li *et al*. 2006).

Deficiency of nidogen-1 leads to impaired cutaneous wound healing characterized by delayed differentiation of the newly formed epidermis. Surprisingly, no alteration in the formation of new BM was observed in these mice. (Baranowsky *et al*. 2009.)

2.5.2 The role of VEGF in wound healing

The expression of VEGF is markedly increased during epithelial wound healing (Brown *et al.* 1992). It has been shown to be an important mediator of wound angiogenesis during the proliferative phase of wound healing (Nissen *et al.* 1998). Diabetic mice, which show impaired healing of wounds, fail to show this elevation in the VEGF levels during wound healing (Frank *et al.* 1995). In chronic wounds VEGF expression is increased, but the VEGF molecules are rapidly degraded because of enhanced proteolytic activity (Lauer *et al.* 2000). Overexpression of VEGF-A in skin leads to a highly increased skin vasculature in mice (Larcher *et al.* 1998), and surprisingly, also to enhanced lymphangiogenesis in association with wound healing (Hong *et al.* 2004). When skin substitutes overexpressing VEGF are grafted onto full-thickness wounds of athymic mice, there is accelerated vascularization of the wound areas (Supp *et al.* 2000, Supp & Boyse 2002). Neutralization of VEGF-A with antibodies leads to a dramatic inhibition of wound angiogenesis and granulation tissue formation in surgically created swine ventrical hernias (Howdieshell *et al.* 2001). Delivery of a truncated, inactive VEGF receptor 2 (VEGFR2/KDR/flk-1) to murine wounds also leads to impaired wound angiogenesis and granulation tissue formation (Tsou *et al.* 2002), and loss of VEGF from keratinocytes causes a substantial delay in wound healing (Rossiter *et al.* 2004). VEGF treatment enhances wound healing in healthy mice (Deodato *et al.* 2002, Romano Di Peppe *et al.* 2002), diabetic mice (Kirchner *et al.* 2003, Galeano *et al.* 2003a, Galiano *et al.* 2004) and in mice with burn injuries (Galeano *et al.* 2003b) or ischemic wounds (Zhang *et al.* 2003). Also, VEGF-C treatment has been shown to accelerate wound healing in diabetic mice, enhancing both wound angiogenesis and lymphangiogenesis, whereas inhibition of lymphangiogenic VEGF-C and –D molecules leads to delayed wound healing (Saaristo *et al.* 2006). Clinical studies are now ongoing to establish the medical usability of VEGF in treating wounds, and in a phase I trial studying the safety of topical administration of recombinant VEGF the treatment proved to be well tolerated (Hanft *et al.* 2008). Bevacizumab is a humanized monoclonal antibody against VEGF used for treatment of cancer. This treatment is associated with impaired wound healing, which complicates its use in cancer patients undergoing surgery. (Gordon *et al.* 2009.)

2.6 Hair follicle development and cycling

The most obvious role of the hair follicles is to produce hair shafts. Hair of mammals is needed for a variety of functions, e.g. insulation, physical protection, camouflage, sensory functions and for social communication. The development of hair follicles in mice occurs in three waves which give rise to different types of hair follicles; the first one starts around E14, and the development of the last follicles starts around E18-P0. Interaction between the epidermis and underlying dermis is crucial for the formation of hair follicles, as well as other ectodermal appendages. Key signalling molecules in the epidermal-mesenchymal interaction are the Wnt/wingless family, the hedgehog family, and members of the TGF-β/BMP (transforming growth factor-β/bone morphogenetic protein), FGF and TNF (tumor necrosis factor) families (Sellheyer *et al*. 1993, St-Jacques *et al*. 1998, Foitzik *et al*. 1999, Headon & Overbeek 1999, Huelsken *et al*. 2001, Laurikkala *et al*. 2002, Petiot *et al*. 2003). Inductive signals from the dermis lead to a local increase in the hair follicle activating signals, which then override the hair follicle inhibiting signals. This sets the scene for the formation of thickenings of the epithelium, called placodes, which then start to grow deeper into the dermis to form hair follicles. The hair placode epithelium induces the formation of a mesenchymal cell condensate below it, which then gets wrapped by the epithelial cells of the growing hair follicle; from this point onwards it is called the dermal papilla. Later during development, hair follicle epithelial cell proliferation leads to the production of the hair shaft as well as different layers of the hair follicle itself. (For reviews see Stenn & Paus 2001, Mikkola 2007, Schneider *et al*. 2009.)

After their development, or morphogenesis, hair follicles start to undergo a continuous cycle of regression, rest and growth periods. The reasons for this cycling behavior probably are the possibilities that it gives for the animal to e.g. control the length of the hair uniquely at different sites of the body and in response to changing environmental conditions, shed the fur to allow cleansing of the skin surface and protect it from the improper formation of the hair follicles. In mice the cycling of the follicles starts around P17, as the follicles enter the regression stage of the cycle, called catagen. In catagen, the cells in the lower part of the hair follicle undergo rapid apoptosis, which leads to a reduction in the length of follicles. This brings the dermal papilla, which resides below the lowest part of the follicle epithelium, close to the vicinity of the hair follicle bulge region, which is the site where the hair follicle stem cells reside. Molecular interaction between the dermal papilla and the stem cells of the bulge is needed

for the growth of follicles. After regression the hair follicles enter telogen, which is the rest stage of hair follicles in terms of growth. When sufficient concentration of follicle growth-inducing signals is reached, the hair follicles start to grow again, the growth stage being called anagen. Many signalling molecules that are needed for hair follicle morphogenesis are also needed for the induction of hair follicle growth during the hair cycle. Wnt/β-catenin and Sonic hedgehog (Shh) signalling are needed for the growth of hair follicles also during anagen (Sato *et al*. 1999, Huelsken *et al*. 2001, Van Mater *et al*. 2003). Inhibition of BMP by noggin also seems to be needed for the induction of anagen (Botchkarev *et al*. 2001). FGF-5 and EGF are regulators of catagen induction (Murillas *et al*. 1995, Hansen *et al*. 1997, Suzuki *et al*. 2000), and TGF-β1 and 17β-estradiol are also known to induce catagen (Foitzik *et al*. 2000, Ohnemus *et al*. 2005). (For reviews see Stenn & Paus 2001, Krause & Foitzik 2006, Schneider *et al*. 2009.)
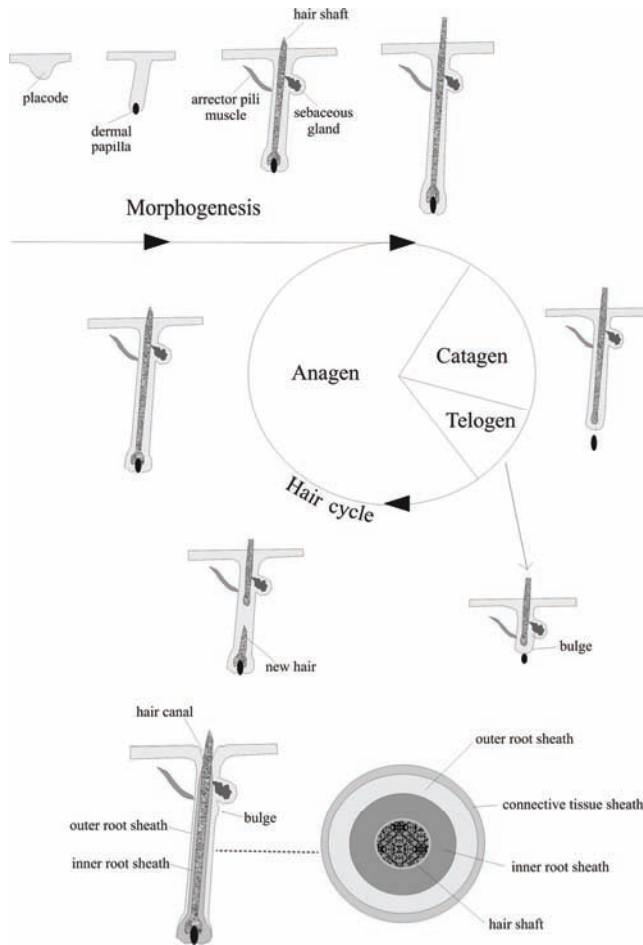
Fig. 3. Hair follicle morphogenesis and cycling. Hair follicle morphogenesis starts as a result of epidermal-mesenchymal interaction with a formation of an epithelial thickening called the placode. The placode grows deeper into the dermis and induces the formation of a mesenchymal condensation below it, called the dermal papilla. The hair follicles start to produce the hair shaft during their growth. Once the hair follicles have reached their maximal length during morphogenesis, they enter the hair cycle. The hair follicles start to regress in a stage called catagen. Hair follicle cells are rapidly dying in an apoptotic manner from the lowest part of the follicle. As a result of this, the dermal papilla reaches the vicinity of the bulge. Eventually, growth-inducing signals derived from the dermal papilla towards the bulge cells override the telogen rest period, the follicle starts to grow again, and a new hair shaft is produced in the anagen growth stage.

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2.6.1 Wnt/β-catenin signalling in hair follicle development and cycling

Wnt/β-catenin signalling is obligatory for the development of hair follicles. During morphogenesis, inhibition of Wnt-signalling by ectopical expression of Dickkopf-1 leads to the failure of hair placode formation (Andl *et al*. 2002). Introduction of a conditional mutation of β-catenin in the epidermis during embryogenesis blocks the formation of hair follicle placodes. The reason for these findings is that in the absence of β-catenin, epidermal cells adopt an epidermal rather than follicular fate (Huelsken *et al*. 2001). Expression of constitutively active β-catenin in keratinocytes, or mutating endogenous β-catenin to a dominant-active form leads to an extensive switch of keratinocytes from the epidermal to hair follicle fate (Zhang *et al*. 2008, Suzuki *et al*. 2009). However, sustained β-catenin activity suppresses the down-growth of newly-formed hair follicles (Närhi *et al*. 2008). Expression of Wnt-10b and -10a is upregulated in the placodes at the onset of hair follicle morphogenesis, and Wnt-5a is expressed in the developing dermal condensate under the control of Shh (Reddy *et al*. 2001).

Wnt/β-catenin signalling is also required for proper hair follicle cycling. Introduction of a conditional mutation in β-catenin in the epidermis after the hair follicles have formed results in a complete loss of hair after the first hair cycle (Huelsken *et al*. 2001). Usually the number of hair follicles is permanent, i.e. after morphogenesis new follicles are not formed, but in certain situations, for example in wounded skin, epidermal stem cells can adopt a hair follicle stem cell phenotype and produce new follicles. This phenomenon is dependent on Wnt-signalling as inhibition of Wnt-signalling after wound closure completely blocks this follicle neogenesis, whereas overexpression of Wnt-7a leads to an increased number of new follicles (Ito *et al*. 2007). Transient expression of β-catenin in the epidermis of transgenic mice is sufficient to induce follicle neogenesis from the interfollicular epidermis (Lo Celso *et al*. 2004). Expressing constitutively active β-catenin under the keratin 14 promoter leads to *de novo* formation of hair follicles in postnatal mice (Gat *et al*. 1998). Wnt-10b and -10a expression is upregulated in postnatal hair follicles beginning a new anagen (Reddy *et al*. 2001). Wnt-signalling is also needed to maintain the dermal papilla cells in a hair growth-inducing state (Shimizu & Morgan 2004). The hairless gene product, which is essential for hair follicle regeneration, represses the expression of the Wnt-modulator Wise, thus triggering Wnt-signalling (Beaudoin *et al*. 2005).

In addition to being needed for hair placode formation and hair follicle cycling, Wnt/ β-catenin signalling also has a role in controlling the growth of the hair shaft. Wnt-10b promotes the growth of the hair shaft in organ culture (Ouji *et al*. 2007) and sustained β-catenin activity suppresses the production of a hair shaft (Närhi *et al*. 2008).

2.6.2 Hair follicle stem cells

Stem cells provide a possibility for the dynamic cycling behaviour of hair follicles by offering a reservoir of cells able to undergo proliferation and produce cells which account for the rapid self-renewal of the hair follicle during anagen. Stem cells themselves divide rarely, but when they divide, they can give rise to so-called transiently amplifying cells, which can proliferate very rapidly and generate the cycling portion (around two-thirds of the hair follicle) in each cycle. Hair follicle stem cells reside in an area called the bulge, which is located in the lowest permanent part of the hair follicle. (Cotsarelis *et al*. 1990.) During hair follicle growth, bulge stem cells and/or their progeny migrate down from the bulge to the lowermost part of the hair follicle, the bulb, and from there up again to form the hair shaft. In addition to being able to form all the cells of the hair follicles and sebaceous gland (Claudinot *et al*. 2005), bulge stem cells can also differentiate to form cells of interfollicular epidermis for example during cutaneous wound healing (Taylor *et al*. 2000, Ito *et al*. 2005). Bulge stem cells have also been reported to be capable of forming blood vessels and neural tissue (Amoh *et al*. 2004, Amoh *et al*. 2005). There are a few studies that have looked at the molecular signature of the bulge stem cells. These studies show an upregulation of genes regulating cell cycling as well as upregulation of inhibitors of Wnt- and BMP-signalling pathways. Interestingly, the transcription of the *Col18a1* gene is also upregulated in the bulge cells, both in catagen and anagen follicles. (Tumbar *et al*. 2004, Blanpain *et al*. 2004.) There are also several markers that have been used to identify and isolate the slow-cycling bulge stem cells. The most commonly used markers include keratin 15 and the hematopoietic stem cell marker CD34. However, human hair follicles do not express CD34, which restricts the use of this marker to mice. (For reviews see Ohyama 2007, Waters *et al*. 2007, Tiede *et al*. 2007, Fuchs 2009.)

2.6.3 Hair follicle-associated angiogenesis

Because hair follicles are growing very rapidly during anagen, they need a substantial amount of oxygen and nutrients. It has been observed that angiogenesis is a physiological phenomenon associated with hair follicle growth, with inhibition of it retarding the anagen stage of follicles. The anagen bulb of the hair follicle has a capability to induce the growth of blood vessels. (Stenn *et al.* 1988, Mecklenburg *et al.* 2000.) VEGF has an important role also in hair follicle-associated angiogenesis as inhibition of VEGF leads to retarded hair growth, whereas accelerated hair growth has been observed in transgenic mice overexpressing VEGF (Yano *et al.* 2001). Moreover, controlled release of VEGF from a biodegradable collagen hydrogel implant can promote the growth of murine hair follicles (Ozeki & Tabata 2002). Overexpression of an angiogenesis inhibitor thrombospondin-1 leads to delayed growth of hair follicles, and lack of thrombospondin-1 causes a prolonged follicle growth phase (Yano *et al.* 2003). There is evidence that certain bulge stem cells, which express nestin (a marker for neural stem cells), also form dermal blood vessels interconnecting hair follicles, suggesting a role for hair follicle stem cells also in hair follicle-associated angiogenesis (Amoh *et al.* 2004). Doxorubicin, a cancer chemotherapy drug, disrupts the hair follicle-associated vessel network without affecting the hair follicle stem cells. This suggests that the hair follicle dystrophy associated with chemotherapy-induced alopecia may be at least partly due to inhibition of hair follicle associated angiogenesis. (Amoh *et al.* 2007.)

2.7 Skeletal development

Skeletal development starts during embryogenesis with the formation of mesenchymal condensations. The formation of bones can occur directly from these condensations in a process called intramembranous ossification, as occurs for example for the flat bones of the skull. Most bones, however, are formed in a process called endochondral ossification, in which a cartilage anlage is formed at the site of mesenchymal condensations. The mesenchymal cells in the cartilage anlage differentiate into chondrocytes, which start to proliferate and produce extracellular matrix molecules typical for cartilagineous tissue, such as collagens II, IX and XI, and aggrecan. The transcription factor Sox-9 has been shown to be important in regulating the formation of these chondrocytes (Bi *et al.* 1999). Later the chondrocytes exit the cell cycle, differentiate to hypertrophic chondrocytes,

and start to express proteins such as collagen X and VEGF, with transcription factors Runx-2 and Runx-3 being needed for the formation of hypertrophic chondrocytes. At this point the mineralization of the cartilage matrix begins. VEGF induces angiogenesis in the central part of the cartilage anlage, and the vessels bring hematopoietic cells to this region. These cells first differentiate into osteoclasts, which degrade the hypertrophic cartilage matrix. This triggers the formation of osteoblasts from mesenchymal progenitor cells, which start to produce bone-specific ECM using the degraded cartilage as a scaffold. The central area of the cartilage anlage, where the angiogenesis and bone formation starts, is called the primary ossification center. From this primary ossification center, the ossification spreads to replace other parts of the anlage with bone as well. However, near the epiphyses (the ends of the bones) there are chondrocytes which are still in the non-hypertrophic stage. These cells proliferate, producing new cartilagenous matrix, and thus allowing bones to grow. The area where the proliferating non-hypertrophic chondrocytes differentiate into hypertrophic chondrocytes forms the growth plate. The growth plate separates the growing bone from the epiphyseal cartilage. The ossification of the epiphyses occurs from secondary ossification centers, which form and expand in a similar manner as the primary ossification centers, but they are formed at a later stage of development. (For reviews see Cancedda *et al*. 2000, Horton 2003, Aguila & Rowe 2005, Colnot 2005, Karsenty 2008, Blair *et al*. 2008.)
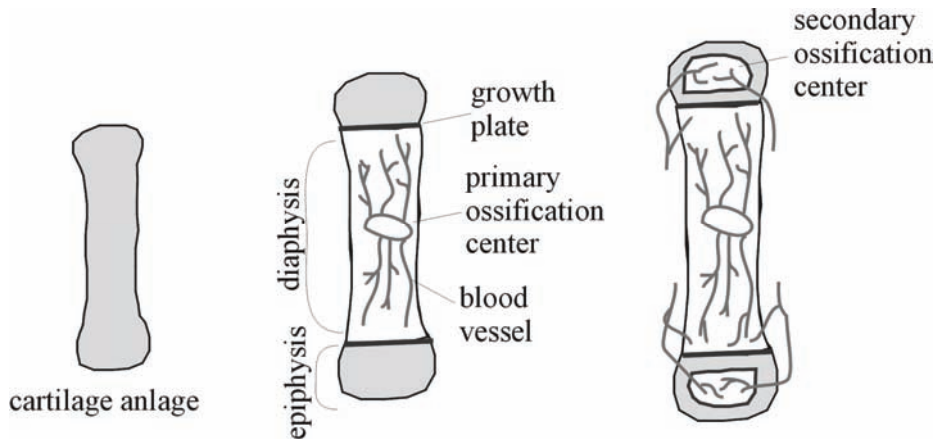
Fig. 4. Bone development. The development of bones starts with the formation of a mesenchymal condensation, which serves as a template for the formation of a cartilage anlage. Blood vessels start to invade the central part of the bone, osteoclasts degrade the cartilage matrix, and osteoblasts start to produce bone-specific matrix at this site, now called the primary ossification center. The growth of the bone continues in the growth plate, which separates the epiphyseal and diaphyseal parts of the bone. Next, the epiphyses become ossified and vascularized in a similar manner as the primary ossification centers, these ossified centers being called the secondary ossification centers.

2.7.1 The role of VEGF in skeletal development

VEGF is essential for endochondral ossification as inactivation of VEGF suppresses blood vessel invasion, cartilage resorption and formation of new bone, and leads to an expansion of the hypertrophic chondrocyte zone. This suggests that VEGF-mediated bone angiogenesis is an important signal in the regulation of cartilage remodelling. (Gerber *et al.* 1999.) In addition to affecting angiogenesis, VEGF enhances osteoclastic bone resorption and the survival of osteoclasts (Nakagawa *et al.* 2000). VEGF also induces osteoblastic activity and hence stimulates bone mineralization (Zelzer *et al.* 2002). Adenoviral VEGF-A delivery has been shown to be efficient in promoting bone formation *in vivo* (Hiltunen *et al.* 2003, Tarkka *et al.* 2003). VEGF has also been shown to be needed for chondrocyte survival during skeletal development (Zelzer *et al.* 2004). The expression of VEGF can be detected in the hypertrophic chondrocytes of growth plates (Horner *et al.* 1999, Carlevaro *et al.* 2000). Runx-2 upregulates VEGF

production, and mice deficient in Runx-2 lack upregulated VEGF production in hypertrophic chondrocytes (Zelzer *et al*. 2001). VEGF produced by the hypertrophic chondrocytes is bound to the ECM, and MMP-9 is needed to release it and make it available to attract endothelial cells and osteoclasts (Engsig *et al*. 2000). VEGF is expressed as three splice variants; soluble VEGF120, matrix-bound VEGF188, and VEGF164, which can exist both as soluble and ECM-bound forms. VEGF120 and VEGF164 mRNAs have been detected in growth plate cartilage samples (Petersen *et al*. 2002). Mice lacking the two longer isoforms and only expressing the soluble VEGF120 isoform show reduced bone growth associated with delayed calcification of the metaphyseal hypertrophic cartilage, impaired angiogenesis and osteoclast invasion, expansion of the hypertrophic chondrocyte zone and decreased expression of MMP-9 (Maes *et al*. 2002). However, mice lacking the two shorter isoforms and only expressing the matrix-bound VEGF188 show disrupted development of the growth plates and secondary ossification centers. Mice only expressing VEGF164 show no obvious defects in skeletal development. This suggests that soluble VEGF is needed for epiphyseal ossification, whereas matrix-bound VEGF is essential for proper vascularization and development of the metaphyseal bone. (Maes *et al*. 2004.) Interestingly, VEGF-D has also been shown to induce differentiation of osteoblasts and matrix mineralization via VEGFR3 (Orlandini *et al*. 2006). Mice lacking the tyrosine kinase domain of VEGFR1 show a decreased volume of trabecular bone, suggesting a role for this receptor in osteoblast function (Otomo *et al*. 2007).

3   Outlines of the present study

When this thesis work was started, the endostatin domain of collagen XVIII was known to reduce tumor angiogenesis, but not much was known about its effects on physiological angiogenesis. Moreover, there was relatively little data available on the roles of endogenous collagen XVIII in processes involving physiological angiogenesis, or about the specific biological roles of the different N-terminal variants of collagen XVIII. Previously, very little has been known about the biological functions of the specific N-terminal variants, and only their gene structure and studies of the localizations of the variants have provided some clues of their possible roles. Many of the studies about endostatin's effects on angiogenesis have been done using cell culture models, but the great variation in the *in vitro* results support the necessity for *in vivo* experiments to fully understand the biological function of endostatin.

We have used genetically modified mouse lines to study the biological roles of collagen XVIII/endostatin. Wound healing, skeletal development and hair follicle cycling are all processes which require angiogenesis to proceed normally. Wnt-signalling also has a crucial role in hair follicle cycling, and a recent study demonstrates that the N-terminus of the longest variant of collagen XVIII can bind Wnt molecules and affect Wnt/β-catenin signalling *in vitro* (Quélard *et al*. 2008). Therefore, in addition to exploring the role of the C-terminal endostatin part of collagen XVIII, we were interested to study roles of the N-terminal variants of this collagen type. The mouse lines used in this study were *Col18a1*$^{-/-}$ (lacking all three variants of collagen XVIII), ES-tg (overexpressing endostatin in the skin under the keratin 14 promoter), *Col18a1*$^{\Delta3/\Delta3}$ (lacking the two longer variants of collagen XVIII), and *Col18a1*$^{\Delta1-2/\Delta1-2}$ (lacking the short variant of collagen XVIII) line, which was generated as part of this thesis project. The specific aims were:

1. to study the cutaneous wound healing process in mice deficient in collagen XVIII and mice overexpressing endostatin;
2. to study the skeletal development in mice deficient in collagen XVIII and mice overexpressing endostatin, and to investigate the effects of endostatin on osteoblasts *in vitro*; and
3. to generate a mouse line deficient in the short variant of collagen XVIII, and to study the hair follicle formation and cycling in mice deficient in collagen XVIII or some of its variants, and in mice overexpressing endostatin.

4   Materials and methods

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

4.1   Wound healing experiment (I)

For the wound healing experiments, collagen XVIII knockout mice (*Col18a1*$^{-/-}$) with C57BL/6J wild-type controls, and transgenic mice overexpressing monomeric endostatin in the keratinocytes (ES-tg) with FVB/N wild type controls were used. The *Col18a1*$^{-/-}$;ES-tg mice were generated by mating *Col18a1*$^{-/-}$ mice with the ES-tg mice. The generation of collagen XVIII knockout, ES-tg (also called J4) and *Col18a1*$^{-/-}$;ES-tg mice has been described in previous reports (Fukai *et al*. 2002, Elamaa *et al*. 2005). The animals were 8–12 weeks old, and both females and males were used. The animals were obtained from the Laboratory Animal Center at the University of Oulu. All the experimentation was approved by the Animal Care and Use Committee of the University of Oulu, and in the case of the *Col18a1*$^{-/-}$ mice also by the State Provincial Office in Oulu. The wounding and analysis of the wound area was performed as described previously (Salonurmi *et al*. 2004). The mice were anaesthetized with an intraperitoneal injection of ketamine and medetomidine and two excisional full-thickness wounds of 3 mm in diameter were made on their shaved dorsal skin using a biopsy punch. Mice received buprenorphine injections for pain control at 12 hour intervals after the anaesthesia. The wound outlines were traced on object glasses 0, 3 and 6 days after wounding, isoflurane inhalation being used to anesthetize the mice for this at 3 and 6 days. The wound areas were scanned and measured using Quantity One 4.4.1. software. The mice were sacrificed 3, 6 or 14 days after wounding and the wound areas were collected for further analyses.

4.2   Histological analysis of the wounds, bones and hair follicles (I, II, III)

The wound samples were fixed overnight in 4% paraformaldehyde (PFA) or 10% phosphate buffered formalin and embedded in paraffin, and 5 μm sections were stained with haematoxylin/eosin. Sections from the central wound area (the part where the wound area is longest) were used for analyses. The length of the wound area was measured in order to evaluate wound contraction, and re-

epithelialization of the 3-day-old wounds was measured with the following formula: degree of re-epithelialization = length of the wound area covered by epithelium/total length of wound area.

Bone specimens from 14- and 30-day old mice were fixed in 70% ethanol, decalcified and embedded in paraffin. For the histological analyses, 5 µm bone sections were used. For the analysis of embryonic bones, femurs from E15.5 and E18.5 embryos were dissected under a microscope, blotted in TissueTek and stored in -70 ºC. The whole tissue block was cut into 5 µm cryosections, which were stained with haematoxylin-eosin.

For the analysis of hair follicles, skin samples were collected from ES-tg mice and their FVB/N controls at postnatal days 1, 6, 8, 12, 17, 21, 25 and 28, and from $Col18a1^{-/-}$, $Col18a1^{\Delta3/\Delta3}$, $Col18a1^{\Delta1-2/\Delta1-2}$ and their C57BL/6J controls at postnatal days 1, 6, 8, 11, 17, 21 and 25 after depilation. Samples were collected parallel to the paravertebral line from the dorsal skin near the neck region. Samples were either fixed in 4% paraformaldehyde and embedded in paraffin, or snap frozen and embedded in TissueTek using a technique described by Paus *et al*. (1999). Sections were cut 5 µm thick and stained with haematoxylin/eosin. Hair follicle lengths were measured from a minimum of three mice from each timepoint and genotype, and the length of a minimum of ten hair follicles was measured from each individual. The length of the hair follicle was measured as the distance from the bottom of the hair bulb to the epidermis. The images were taken with an Olympus DP 50 microscope digital camera system and the measurements were performed using the Analysis application.

4.3   Immunohistochemical and immunofluorescence stainings (I, II, III)

The PFA- or formalin-fixed and paraffin-embedded 5 µm wound sections were dewaxed, washed in PBS and stained with CD31, α-smooth muscle actin, fragmented caspase 3, collagen XVIII anti-all or collagen XVIII anti-long antibodies using a tyramide signal amplification (TSA) kit (PerkinElmer). Before incubation with the primary antibody, epitope retrieval was performed either using trypsin, or by heating in Tris-EDTA or citrate buffer.

For the immunofluorescence stainings, 5 µm cryosections were fixed in ethanol or acetone. The sections were blocked with 1% BSA-PBS for 1 h at room temperature followed by an overnight incubation in +4 ºC with collagen XVIII anti-all, collagen XVIII anti-long, RES (endostatin), cytokeratin 15 or CD 31

antibodies. This was followed by a 60-minute incubation at room temperature with a Cy3- or Cy2-conjugated secondary antibody.

4.4 Transmission electron microscopy (TEM) (I, III)

Skin samples were fixed in a 1% glutaraldehyde 4% formaldehyde mixture in 0.1 M phosphate buffer, postfixed in 1% osmiumtetroxide, dehydrated in acetone and embedded in Epon Embed 812 or LX 112. Thin sections were cut with a Leica Ultracut UCT ultramicrotome and examined in a Philips CM100 transmission electron microscope. Images were captured with a CCD camera. For the wound capillary measurements, 5–12 capillaries were measured from each sample, and the means of these values were used in the statistical analyses. The wound capillaries measured were within 20–40 μm distance from the epidermis, and the thickness of the capillary was measured from the outer wall of the endothelial cell to the opposite outer endothelial wall, at the site in which this distance was the shortest. The wound epidermal BM measurements included both lamina lucida and lamina densa. Twenty-five BM measurements were made from each sample, and the means of these measurements were used in the statistical analyses for each individual.

4.5 Determination of wound and hair follicle-associated angiogenesis, myofibroblast density, and apoptosis of cells (I, III)

Wound angiogenesis was determined from CD31-stained sections. One to five photographs were taken of the granulation tissue of each of the 6- and 14-day-old wound samples. The number of photographs taken from each wound depended on the length of the wound area, as pictures from central and peripheral wound areas were always taken from each wound to cover the whole wound area. The number of capillaries was determined from each picture, and the mean number was calculated for each wound. For the analysis of hair follicle associated angiogenesis, images were taken from the dermis at the site where the hair follicle bulbs reside, with an Olympus DP 50 microscope digital camera system and the Analysis application with 20x magnification and 1500 ms exposure time. The relative immunopositive area was measured from images using the Analysis application. The timepoints analyzed were postnatal days 1, 17, 21 and 25. Three images were taken from each individual mouse from different sites of dermis, and

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the average of these three measurements was used in statistical analyses for this individual.

Myofibroblast density was determined from α-SMA-stained wound sections. One photograph was taken from the hot spot areas of each 6-day-old wound sample. The number of stained cells (excluding the cells surrounding vascular structures) was determined from each picture.

The number of apoptotic endothelial cells in the wound granulation tissue was counted for the 6-day-old wound sections, the number of apoptotic fibroblasts for hot spot areas in one microscopic field (40x) in the 6 and 14-day-old wound samples and apoptotic keratinocytes for the whole wound area, dividing this number by the length of the wound area so that the analysis would not be affected by differences in wound length. Counting of apoptotic cells was done from fragmented caspase 3-stained samples by a specialist of pathology. The differentiation between fibroblasts and endothelial cells was based on the localization of endothelial cells in the walls of capillary structures.

4.6   X-ray imaging and pQCT densitometry (II)

Radiographs of the dissected bones were taken in anteroposterior (AP) and lateral projection on a Faxitron Specimen Radiography System with a high-resolution algorithm. The imaging parameters were identical for all the specimens and the following settings were used: 6 s, 30 kV for the 30-day-old mice and 20 kV for the 14-day-old mice, pixel matrix 1024 × 1054; resolution 200 pixels mm^{-1}. The images were transferred to a personal computer for analysis. Lengths of tibias and femurs were measured from radiographs.

pQCT analyses were performed on femurs and tibias of 14- and 30-day old mice. Bones were scanned with a Stratec XCT 960 A peripheral quantitative computed tomography (pQCT) system. Voxel size of 0.148 × 0.148 × 1.25 mm^3 (for transgenic mice overexpressing endostatin and FVB/N control mice) and 0.092 × 0.092 × 1.25 mm^3 (for mice deficient in collagen XVIII and C57BL/6J control mice) were used for the measurements. The scout view of the pQCT system determined the middle point of each bone, from which one cross-sectional slice was scanned and the total mineral content and density were measured. As the weight of the mice varied from litter to litter in both mutant and control mice, the bone density data was normalized by the bodyweight.

4.7 MC3T3-E1 osteoblast culture (II)

MC3T3-E1 cells are pre-osteoblasts derived from murine calvarias. They were maintained in α-MEM supplemented with 10% FCS, 2nM L-glutamine, 100 IU/ml penicillin and 10 µg/ml streptomycin (α -MEM-FCS), and passaged every 2–3 days using standard techniques. After pre-culturing the cells were seeded at a density of 10 000 cells/cm^2 in the presence of α -MEM with 50 µg/ml ascorbic acid and 10 mM β-glycerophosphate (mineralization medium). The cells were cultured in six groups and the medium was supplemented as follows: 1) control with no added substances, 2) VEGF-A 100 ng/ml (Calbiochem), 3) endostatin 2 µg/ml (Calbiochem), 4) endostatin 20 µg/ml, 5) VEGF-A + endostatin 2 µg/ml and 6) VEGF-A + endostatin 20 µg/ml. Each experiment was repeated at least two times.

4.8 Determination of osteoblast proliferation (II)

The proliferation of MC3T3-E1 cells was measured by a WST-1 assay, which quantifies the reduction of the tetrazolium salt WST-1 in viable cells. Analyses were performed 3, 7, 10 and 14 days after the cells were plated. WST-1 reagent and α-MEM with ascorbic and β-glyserophosphate were added to the cells and the culture was incubated at +37°C (5% CO_2, 95% air) for 1 hour. The absorbance of the reaction product was measured at a wavelength of 450 nm.

4.9 Alizarin red S staining and quantification of calcium deposition (II)

The mineralization of matrix and bone nodules was determined using Alizarin red S staining. Briefly, MC3T3-E1 cell cultures were fixed at culture day 14 with 3% PFA/ 2% sucrose in PBS for 10 minutes at room temperature, washed with PBS and stained for 30 min with a 1% solution of Alizarin red S pH 4.2 at room temperature. The cultures were washed with deionized water and 70% ethanol before the addition of 100 nM cetylpyridinium chloride for 1 hour to solubilize and release calcium-bound Alizarin red into the solution. The solution was collected and the Alizarin red S concentration was determined by absorbance measurements at 570 nm.

4.10 Generation of the knockout mouse line deficient in the short variant of collagen XVIII (*Col18a1*^{Δ1-2/Δ1-2}) (III)

The collagen XVIII gene (*Col18a1*) was targeted in embryonic stem (ES) cells by standard methods. A LacZ-Neo cassette was inserted in-frame into the first exon of the *Col18a1* gene with homologous recombination. The linearized targeting construct was electroporated into ES cells by the Biocenter Oulu Transgenic Mouse Core Facility services. The ES cell clones resistant to neomycin were screened for homologous recombination by PCR and Southern blotting. For PCR the primers used were the forward primer 5'-GGA TCC GGC CTT TGA AGC CT-3' from the first promoter region of the *Col18a1* gene, and the reverse primers 5'-GAG CCG CTT AAG TCT GGC CA-3' from the second intron of the *Col18a1* gene which amplify a 1820 bp fragment in the wild type mice, or 5'-TTC CCA GTC ACG ACG TTG TA-3' from the 5' end of the LacZ-gene which amplify a 1410 bp fragment in the knock-out mice. The PCR-conditions were 94 ºC for 2 min, 5 cycles of 94 ºC for 10 seconds, 58 ºC for 30 seconds and 94 ºC for 2 min, 5 cycles of 94 ºC for 10 seconds, 58 ºC for 30 seconds and 94 ºC for 3 min, 5 cycles of 94 ºC for 10 seconds, 58 ºC for 30 seconds and 94 ºC for 4 min, 5 cycles of 58 ºC for 30 seconds and 94 ºC for 5 min, 5 cycles of 58 ºC for 30 seconds and 94 ºC for 6 min, and 94 ºC for 5 min. The correct targeting was also confirmed by Southern blotting; the genomic DNA was digested with EcoRI and EcoRV, and a 0.5 kb probe from the first promoter area of the *Col18a1* gene was used to visualize a fragment of 7 kb in the allele with the Neo-LacZ cassette, and a 10.5 kb fragment in the wild type allele. The copy number was also confirmed with Southern blotting by using a 0.7 kb probe from the LacZ gene. Cells from a correctly targeted clone were injected into Sv129 blastocysts by the Biocenter Oulu Transgenic Mouse Core Facility services, and a chimeric mouse line was produced. These mice lacking the exons 1 and 2 of collagen XVIII gene (*Col18a1*^{Δ1-2/Δ1-2}) were backcrossed to the C57BL/6J mouse line 10 times. For genotyping of the mice, the same primers and PCR conditions were used as for the screening of the ES-clones (described above). The experiments were approved by the Animal Care and Use Committee of the University of Oulu.

4.11 Statistical analyses (I, II, III)

Wound closure from day 0 to day 6 was analyzed with ANOVA for repeated measurements. The length of the wound (in histological sections), wound re-

epithelialization, wound myofibroblast density, wound capillary density, number of apoptotic cells in wounds, hair follicle length and the hair follicle associated relative capillary area were analyzed with the Student's t-test for independent samples, p-values being given as two-tailed significances. Cell proliferation, Alizarin red concentration, bone length and bone density were analyzed using one-way analysis of variance (ANOVA) followed by the Student's t-test. P-values below 0.05 were considered statistically significant.

5   Results

5.1   Wound healing rate in mice overexpressing endostatin or deficient in collagen XVIII (I)

The healing of the cutaneous wounds was delayed in ES-tg mice, seen as a slower rate of reduction in the wound area. Also the relative length of the 6-day-old wounds in the histological sections was slightly larger for the ES-tg mice than for the wild-type mice, although this difference was not statistically significant. The healing of the wounds in the *Col18a1*$^{-/-}$ mice, measured as the rate of reduction in wound area from day 0 until day 6, did not differ significantly from that seen in the control C57BL/6J mice, although there seemed to be a tendency for faster healing in the knockout mice. However, the relative length of the 6-day-old wounds in the histological sections was smaller in mice lacking collagen XVIII. These results indicate delayed wound healing in ES-tg mice and a faster rate of wound healing in mice deficient in collagen XVIII. No differences in wound lengths or degree of re-epithelialization between the *Col18a1*$^{-/-}$ or ES-tg mice and their wild-type controls were seen in the 3-day-old wounds. This implies that the healing rate differences might be due to changes in wound contraction. Indeed, the density of myofibroblasts was significantly higher in the 6-day-old wounds of the *Col18a1*$^{-/-}$ mice compared to their controls. In ES-tg wounds the myofibroblast densities within the wound area were not significantly altered in comparison to the wild type mice.

5.2   Wound angiogenesis in mice overexpressing endostatin or deficient in collagen XVIII (I)

Capillary density within the wound granulation tissue of the 6-day-old wounds was higher in the *Col18a1*$^{-/-}$ mice compared to their wild-type controls. This was due to a faster ingrowth of capillaries into the central regions of the wounds. In ES-tg mice, no significant changes in the wound capillary densities could be observed. However, transmission electron microscopy revealed morphologically abnormal capillaries in the granulation tissue of 6-day-old wounds in the ES-tg mice. The endothelial cells in these capillaries were swollen and the lumens of these vessels were narrowed. The pericytes of these capillaries were thicker and their endoplasmic reticulum was more prominent than in the capillaries of control

mice, possibly indicating a less differentiated phenotype. These changes were seen in some of the small capillaries but not all, while the larger vessels in the wound were normal in appearance. The capillary BM also seemed more disorganized in the ES-tg wounds than in the wild-type mice. The abnormal capillaries were found within a 20–40 μm distance from the epidermis. In this region, the size of the capillaries was also smaller in ES-tg wounds compared to the control mice. No changes in the morphology of the wound capillaries or their BMs were observed in the *Col18a1$^{-/-}$* mice. To see if the abnormal morphology of the ES-tg capillaries is due to endothelial cell apoptosis, we stained wound sections with an antibody against cleaved caspase 3. We could not, however, detect any changes in the numbers of apoptotic endothelial cells. Moreover, there were no changes in the keratinocyte or fibroblast apoptosis between the ES-tg and FVB/N wounds.

5.3 Changes in the wound basement membrane zone in mice overexpressing endostatin (I)

Electron microscopy revealed that the formation of a new epidermal BM was delayed in the ES-tg mice, the BM of the ES-tg wounds appeared to be morphologically less well organized, and there were more regions where it was totally lacking. Furthermore, we observed a detachment of the epidermis from the underlying granulation tissue in 50% of the histological sections of 6-day-old wounds in the ES-tg mice, suggesting greater fragility of the epidermal-dermal junction. No detachment was observed in the FVB/N controls. To see if the epidermal detachment in ES-tg mice was due to an alteration in hemidesmosomes, we looked at the structure of the hemidesmosomes from TEM samples. In both control and mutant mice normal hemidesmosomes were seen in the wound region. In the areas where these mature hemidesmosomes were seen, the BM was already formed. However, in the regions where the BM was absent or amorphous, only immature forms of hemidesmosomes were observed. In the ES-tg mice there were more regions in which the BM was not well developed, and hence we could also see more regions with immature hemidesmosomes in these. In one (n=3) of the 6-day-old TEM samples of the ES-tg mice we could see detachment of the epidermis from the granulation tissue. At the site of detachment the BM was not yet developed, and only non-organized amorphous material (possibly BM molecules) was seen under the epidermis. The detachment had occurred beneath this electron-dense material.

The new epidermal BM in *Col18a1*^{-/-} mice was equivalent in morphology to that in the wild type mice. The only difference was in its width as that of the 14-day-old wounds in the *Col18a1*^{-/-} mice was significantly broader than in the wild type mice. Broadening of the epidermal BM was also seen in the ES-tg mice. These observations are consistent with the previous findings of a broader epithelial BM in the normal skin of *Col18a1*^{-/-} and ES-tg mice (Utriainen *et al*. 2004, Elamaa *et al*. 2005).

5.4 Localization of endogenous and transgenic endostatin in wounds (I)

The localization of endogenous collagen XVIII/endostatin was analyzed from 6-day-old wounds of wild-type mice using an endostatin antibody. Endostatin staining was seen in the epidermal-dermal zone and in the capillaries. In the knockout mice serving as negative controls, very faint unspecific staining was seen in the epidermis but not in the epidermal-dermal zone, capillaries or the granulation tissue. The localization of transgenic endostatin was studied from *Col18a1*^{-/-};ES-tg mice lacking endogenous collagen XVIII and expressing transgenic endostatin. In these mice, endostatin expression could be seen in the epithelial cells of the wounds, in the epidermal-dermal zone, and as a diffuse staining in the granulation tissue. In the wound epidermal-dermal zone, the endostatin staining was discontinuous, unlike in normal skin where the staining was seen as a continuous line.

5.5 Bone development in mice overexpressing endostatin or deficient in collagen XVIII (II)

The bone mineral content and density of the 30-day old mice were analyzed with pQCT. However, no alterations were observed in ES-tg or *Col18a1*^{-/-} mice as compared to their FVB/N or C57BL/6J controls. Moreover, there were no significant differences in bone sizes between the *Col18a1*^{-/-} mice and their controls. However, in the ES-tg mice, the length of femurs and tibias was smaller at the age of 14 days compared to their FVB/N controls. This delay of growth was transient as no difference was seen at 30 days of age. In the femurs of 14-day-old *Col18a1*^{-/-} mice, we detected a delay in the formation of secondary ossification centers. At the age of one month, no clear differences could be observed between the knockout and control mice: in both the ossification of the epiphyses had

occurred. In ES-tg mice we could see no difference in the ossification of the epiphyseal area as compared to their FVB/N controls. We studied the formation of primary ossification centers from E15.5 and E18.5 mouse femurs to see whether the ossification of diaphyses is delayed in $Col18a1^{-/-}$ mice, but at these embryonic stages no differences could be seen between the $Col18a1^{-/-}$ mice and their C57BL/6J controls.

5.6 Localization of collagen XVIII in bone (II)

Immunohistochemistry showed localization of collagen XVIII in some of the blood vessels of bone marrow and periosteum, and at the skeletal muscle attachment site. The staining was seen with the antibody against all three variants. The short variant of collagen XVIII was responsible for this immunosignal, as the antibody that stains the two longer variants of collagen XVIII did not stain the samples. Staining with collagen XVIII antibodies also revealed signals in the epiphyseal cartilage, surrounding the chondrocytes, but this signal proved to be unspecific as similar staining could be seen in cartilage of mice lacking collagen XVIII.

5.7 Effects of endostatin on osteoblasts *in vitro* (II)

The proliferation of cultured MC3T3-E1 osteoblasts was measured using the WST-1 assay. Endostatin at 20 µg/ml was found to decrease osteoblast proliferation after 14 days of culture. Also, endostatin at 2 µg/ml slightly decreased osteoblast proliferation, but this difference was not statistically significant. Interestingly, 2 µg/ml of endostatin together with VEGF-A further decreased osteoblast proliferation, while a higher dose of endostatin (20 µg/ml) together with VEGF-A did not significantly affect proliferation.

The MC3T3-E1 cells produce mineralized nodules when they are undergoing the developmental stages associated with differentiation. The mineralization can be measured using Alizarin red staining. Consistent with the results obtained in the proliferation assay, a low dose of endostatin (2 µg/ml) together with VEGF-A decreased the mineralization. Endostatin at 20 µg/ml together with VEGF-A did not significantly affect mineralization when compared to the basal control level, but when compared to treatment with VEGF-A alone, a decrease in matrix mineralization could be observed.

5.8 Generation of mice lacking the short variant of collagen XVIII (III)

Collagen XVIII has three variants which differ by their N-terminus. Promoter 1 directs the synthesis of the short variant in conjunction with exons 1 and 2, whereas promoter 2 directs the synthesis of the two longer variants in conjunction with exon 3. Exons 4–9 encode the N-terminal noncollagenous (NC) portion which is common to all variants, and exons 10–43 encode the common collagenous and C-terminal noncollagenous sequences. To achieve specific depletion of the promoter-1-specific short variant, we cloned a targeting construct by inserting a LacZ-Neo cassette in-frame into the first exon of the *Col18a1* gene. The construct was electroporated into ES-cells. Correctly targeted ES-cells were used to generate mice lacking the short variant of collagen XVIII (*Col18a1*$^{\Delta 1-2/\Delta 1-2}$ mice). The mice were born at Mendelian ratios, and they were viable and fertile. The lack of the short isoform was confirmed by immunohistochemistry from tissues known normally to express only the short variant of collagen XVIII using the anti-all antibody. The presence of the long variants of collagen XVIII was confirmed from skin, liver and kidney using the anti-long antibody. In the tissue distribution, no compensation of the short variant with the long variants was detected with the anti-all antibody.

5.9 Hair follicle growth in mice deficient in collagen XVIII and mice overexpressing endostatin (III)

The analyses of hair follicle lengths revealed that the catagen of the first hair cycle occurs faster in mice completely lacking collagen XVIII. Also, the first real anagen begins faster in the mutant mice. Interestingly, the phenotype in the *Col18a1*$^{\Delta 3/\Delta 3}$ mice is similar as in the *Col18a1*$^{-/-}$ mice, suggesting that this finding is due to the lack of the long variants of collagen XVIII. In the *Col18a1*$^{\Delta 1-2/\Delta 1-2}$ mice, the regression of the follicles is also slightly accelerated, and the growth of the follicles begins faster also in these mice compared to the controls.

The growth of hair follicles is delayed in the ES-tg mice. A slight delay in the growth can be seen already during the morphogenesis, and a more distinguishable difference is observed during the first anagen, as the hair follicles of ES-tg mice are significantly shorter at postnatal days 21 and 25 compared to the controls. The follicles in the 28-day-old ES-tg mice are also slightly shorter than in the controls, but this difference is not statistically significant. These results suggest that the

growth of the hair follicles is delayed at the beginning of anagen, but the follicles in the ES-tg mice do eventually reach the same length as the follicles in the controls.

5.10 Impaired hair follicle-associated angiogenesis, and ultrastructural changes in the capillary BMs and hair follicle hemidesmosomes in endostatin overexpressing mice (III)

Angiogenesis is known to be important for the proper growth of hair follicles. Therefore, we studied whether the delay in hair follicle growth in ES-tg mice could be due to impaired angiogenesis. At postnatal days 1 and 17 we could see that near the hair follicle bulbs, the area covered by capillaries was significantly smaller in the ES-tg mice compared to the controls. This suggests that angiogenesis is impaired just before or at the time when the hair follicles are beginning to grow, but not at later stages of anagen, as at postnatal days 21 and 25 no significant differences could be detected between the mouse lines. In TEM analyses, the number of capillaries in the 17-day-old ES-tg skin samples was also clearly lower than in the control samples.

As we observed in the wound healing study that overexpression of endostatin can lead to morphological alterations in capillaries during wound angiogenesis, we wished to study whether there were similar morphological changes in the capillary structure during hair follicle-associated angiogenesis. We could not detect any swollen capillaries with narrowed lumens in the 17-day-old ES-tg mice, but similarly as in the wound capillaries, the capillary BMs in the 17-day-old mice were more disorganized in structure in these mice. Moreover, we wanted to look if there were any changes in the hair follicle BM region in the ES-tg mice. We did not detect any morphological changes in the BM of the ES-tg mice, but we could see substantially more immature hemidesmosomes in the hair follicles of these mice. Immature hemidesmosomes are classified as electron-dense thickenings of the plasma membrane with no obvious attachment plaque or tonofilaments (Gipson *et al*. 1983).

5.11 Localization of endogenous collagen XVIII and transgenic endostatin in hair follicles (III)

With an antibody staining all three variants of collagen XVIII we could detect the localization of endogenous collagen XVIII in the basement membranes of

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interfollicular epidermis and hair follicles, and in the skin capillaries. Moreover, we could see cellular collagen XVIII staining in the middle part of the anagen hair follicles in 8-day-old mice. Staining with an antibody against the long variants of collagen XVIII showed a faint signal in the BM of interfollicular epidermis and hair follicle BM, suggesting that both short and long variants are located in these BMs. A strong signal with the anti-all antibody was seen in hair follicle BM surrounding the dermal papilla in the bulb, and in the cells of hair follicles in the middle part of the follicles. This cellular staining of collagen XVIII colocalized with the staining of the bulge marker keratin 15, revealing that the long variants of collagen XVIII are localized in the bulge. Some signal with the anti-endostatin antibody could also be detected in the middle region of the anagen hair follicles, and as expected, also in the BM regions of skin, hair follicles and capillaries. This suggests that the long variants of collagen XVIII are found in the bulge at least to some extent as full-length molecules instead of proteolysed forms.

To see the localization of transgenic endostatin we stained skin samples of *Col18a1*^{-/-};ES-tg mice lacking endogenous collagen XVIII and overexpressing endostatin with an endostatin antibody. The staining was seen in the interfollicular and hair follicle epithelial cells and BM zones, revealing that the transgenic endostatin is also expressed in hair follicles.

6   Discussion

This thesis work has focused on evaluating the effects of collagen XVIII and its endostatin domain on wound healing, hair follicle formation and cycling, and bone development. All these are processes which require angiogenesis to proceed normally. Endostatin, the C-terminal domain of collagen XVIII, was first described to inhibit angiogenesis in 1997 by O'Reilly *et al*. Since then, a large number of studies have been performed to evaluate the effects of endostatin on angiogenesis. However, most of these studies have been done using tumor models in mice, and much less is known about the effects of endostatin on physiological angiogenesis. Our studies show that transgenic endostatin produced in skin can delay the healing of cutaneous wounds and growth of hair follicles, and also affect the angiogenesis related to these processes either by inhibiting the vessel growth or causing the formation of morphologically abnormal capillaries. Bone development was only very mildly affected in our study by excess of endostatin. The reason for this may be that the endostatin overexpression took place in skin, and even though this led to an increased amount of endostatin also in the circulation of the transgenic mice, it is possible that the local endostatin concentration in bone was not high enough to cause any drastic changes.

Lack of endogenous collagen XVIII leads to accelerated healing of wounds accompanied with accelerated wound angiogenesis, providing further evidence for the role of the endostatin domain as a regulator of physiological angiogenesis in wound healing. In bone development lack of collagen XVIII leads to a mild delay in epiphyseal ossification, but otherwise bone development is normal, suggesting that collagen XVIII does not have a crucial role in bone development. Attempts to understand the physiological roles of collagen XVIII using the *Col18a1*$^{-/-}$ mice have highlighted the essential role of this collagen in the eye (Fukai *et al*. 2002, Marneros & Olsen 2003, Ylikärppä *et al*. 2003a, Marneros *et al*. 2004). Moreover, its lack leads to broadened BMs (Utriainen *et al*. 2004), but otherwise no major phenotypical changes have been observed in the null mice previously. Lack of collagen XVIII also leads to susceptibility to hydrocephalus – but only in a certain genetic background, suggesting that also other mutations are needed for this predisposition (Utriainen *et al*. 2004). Thus, in unchallenged situations lack of collagen XVIII in laboratory mice does not have major effects in organs other than the eye. The eye is also the most severely affected organ in Knobloch syndrome, a human condition resulting from mutations in the *COL18A1* gene. (Sertie *et al*. 2000.) However, it is likely that when there are

some challenges to the organism from the environment or in combination with some other genetic factors, mutations in the gene coding collagen XVIII can have additional effects in other organs and tissues. Therefore, it is possible that even though collagen XVIII is not a key player in the bone development, it may be needed for e.g. proper healing of bone fractures or other injuries.

As angiogenesis is known to be important for hair follicle growth, we wanted to study the effects of endostatin on hair follicle morphogenesis and cycling. The indentification of abnormalities in the growth of hair follicles of endostatin overexpressing mice triggered further studies with mice lacking collagen XVIII/endostatin. Since the longest variant of collagen XVIII may inhibit Wnt/β-catenin signalling (Quélard *et al*. 2008), we considered it possible that collagen XVIII may affect hair follicle cycling not only through its endostatin domain, but also through its N-terminal frizzled domain. Therefore we used not only the *Col18a1*$^{-/-}$ mice, but also mice lacking either the short variant or the two longer variants of collagen XVIII to evaluate the role of different variants of collagen XVIII.

Interestingly, the lack of collagen XVIII led to a faster start of the first hair cycle, and the same was seen in mice lacking the two longer variants. As the short variant is the only collagen XVIII in skin capillaries, it seemed unlikely that the phenomenon observed in the knockout mice is caused by an effect on angiogenesis. Immunostainings revealed the presence of the long variants of collagen XVIII in the bulge region, suggesting a role for collagen XVIII in regulating the behaviour of skin stem cells.

Based on our results it can be said that the endostatin domain of collagen XVIII also affects angiogenesis in physiological processes, namely wound healing and hair follicle cycling. Moreover, our results show that collagen XVIII should not only be considered as a precursor for endostatin, but it also has other domains of biological interest especially in its N-terminal NC domain. Below I will provide a more detailed discussion on the results of each individual study.

6.1   Collagen XVIII/endostatin in cutaneous wound healing

We evaluated the effects of collagen XVIII deficiency, and excess of endostatin on cutaneous wound healing. There are some earlier experiments on the effects of endostatin on wound healing using injectable endostatin, but these results are somewhat conflicting. The effects of collagen XVIII on wound healing have never been studied before. Clinical trials are ongoing to evaluate the applicability

of endostatin as a chemotherapeutic agent in treating cancer. However, as most cancer patients also require surgical treatment, it is important to know whether endostatin affects the wound healing process.

Our results show that lack of collagen XVIII leads to accelerated wound healing, while overexpression of endostatin leads to delayed healing of wounds. Endostatin has been shown to delay wound healing in one previous study (Michaels *et al*. 2005) but not in another, where a lower dose of endostatin was used (Bloch *et al*. 2000). Hence it is possible that the effects of endostatin in wound healing are dose-dependent. In ES-tg mice, the concentration of circulating endostatin is about seven-fold compared to the controls (Brideau *et al*. 2007), but the local concentrations in the skin are probably much higher.

Morphologically abnormal capillaries with narrowed lumens were found in the wound tissue of endostatin overexpressing mice. Similar capillaries were observed in the wounds of endostatin treated mice in the study of Bloch *et al*. (2000). There is some evidence that these changes are caused by dephosphorylation of ERK1/2 kinase in response to endostatin (Schmidt *et al*. 2006). Interestingly, the wound capillary density was not altered in the ES-tg mice, suggesting that the endostatin concentration in these mice is not adequate to affect wound angiogenesis. Endostatin injections led to decreased wound capillary density in the study of Michaels *et al*. (2005), where the healing rate was also affected, but no changes in the capillary densities were seen when a lower concentration of endostatin was used (Bloch *et al*. 2000). These studies were done using excisional wounds. When the wounds were incisional, no change in capillary density was observed even when the endostatin concentration was high (Berger *et al*. 2000). Thus, it seems that the effects of endostatin on wound angiogenesis may also be dose-dependent. Moreover, it is known that endostatin's efficacy on endothelial cell migration *in vitro* and tumor growth *in vivo* follows an U-shaped dose-response curve with the efficacy being the best between the very low and very high concentrations (Celik *et al*. 2005, Tjin Tham Sjin *et al*. 2006). So, it is possible that a similar dose-response curve could be found for wound healing effects.

Delayed formation of epidermal BMs and hemidesmosomes was observed in ES-tg mice. We also detected detachment of epidermis from the underlying granulation tissue in half of the 6-day-old wounds of the ES-tg mice. No such detachment has been observed in unwounded ES-tg skin (Elamaa *et al*. 2005), indicating that the endostatin overexpression in itself is not sufficient to cause it. Because in the healing wounds the BM is not yet fully developed, the mild

structural abnormalities observed in the epidermal BM of the ES-tg mouse line may render the mutant mice susceptible to the formation of ruptures in the newly formed BM areas.

While excess endostatin did not affect the amount of vessels in the wound, lack of collagen XVIII led to accelerated vascularization of the wounds. Six-day-old wounds of the control mice were not yet fully vascularized in the central region, whereas the wounds of the *Col18a1*$^{-/-}$ mice were already rich in capillaries at this time point. A decrease in collagen XVIII expression has been shown to correlate with increased vascular density in human hepatocellular carcinoma (Musso *et al*. 2001b). This observation supports the idea of endogenous collagen XVIII as a negative regulator of vessel growth either through its endostatin domain or other domains such as the N-terminal thrombospondin-1 like domain.

In conclusion, lack of collagen XVIII accelerates wound healing, evaluated in terms of wound size and angiogenesis. The mechanism behind this is still unclear, but it is possible that the lack of the endostatin domain of collagen XVIII is responsible for these changes, as excess of endostatin was shown to delay healing of wounds. Even though the effects of endostatin on wound healing were not very drastic, our findings nevertheless suggest that further evaluation is needed in the form of clinical trials.

6.2 Collagen XVIII/endostatin in bone development, and the effects of endostatin on cultured osteoblasts

We have evaluated the effects of endostatin on osteoblasts *in vitro* and collagen XVIII/endostatin on bone development *in vivo* using genetically modified mouse lines. These effects of collagen XVIII/endostatin have never before been studied. From earlier studies we know that endostatin can affect ectopic ossification (Sipola *et al*. 2007), and hence it was our interest to study whether endostatin affects the behavior of osteoblasts, which are the cells responsible for formation of new bone. Moreover, as VEGF signalling and angiogenesis are known to be crucial for skeletal development, we wished to see whether the angiogenesis inhibitor endostatin has effects on bone development.

Endostatin decreased osteoblast proliferation in cell culture compared to the non-treated cells. Interestingly, the low dose of endostatin, but not the high, together with VEGF-A decreased osteoblast proliferation compared to the non-treated cells. Moreover, matrix mineralization by osteoblasts was inhibited when VEGF-A and a low dose of endostatin were added together. As in the case of

osteoblast proliferation, the inhibitory effect of the endostatin + VEGF combination could not be detected when a higher concentration of endostatin was used. Endostatin has been shown to affect several signalling pathways, including Wnt/β-catenin (Hanai *et al*. 2002b), VEGF-mediated signalling (Kim *et al*. 2002), ERK1/2 signalling (Schmidt *et al*. 2006) and integrin α5β1-mediated signalling (Wickström *et al*. 2002). It has also been shown that endostatin's effects on angiogenesis and tumor growth are biphasic, operating over a U-shaped curve (Celik *et al*. 2005). Therefore, endostatin may affect the behavior of cells via many different signalling routes, and the concentration of endostatin may be crucial for obtaining a certain effect. It is unknown why the inhibitory effect of endostatin on osteoblast proliferation and mineralization is highest when used together with VEGF. There is some evidence that in endothelial cells VEGFR-1 might work as a trap for VEGF, thereby regulating its accessibility to VEGFR-2 (Olsson *et al*. 2006). Endostatin has been shown to bind VEGFR-1, -2 and -3 (Kim *et al*. 2002, Kojima *et al*. 2008), and VEGFR-2 and -3 have been implied to have a role in matrix mineralization by osteoblasts (Maeda *et al*. 2003, Orlandini *et al*. 2006). It is possible that binding of VEGF-A to VEGFR-1 may partly prevent the binding of endostatin to this receptor, and thus leave more endostatin peptides available for binding to other receptors with more biological relevance.

Endostatin overexpression was found to lead to a slight delay in the growth of bone length. This delay was temporary as it was only observed in the 14-day-old mice, but not in the 30-day-old mice. Mice lacking collagen XVIII showed a delay in the formation of secondary ossification centers. This was seen in 14-day-old mice. This delay was also temporary as in the 30-day-old mice the epiphyseal areas were well ossified also in the knockout mice. Possible reasons for this delay might be the effects of collagen XVIII on Wnt/β-catenin signalling, or the role of collagen XVIII in binding and presenting growth factors to the cells. The formation of secondary ossification centers was delayed in the cartilaginous skeletal anlagen of a chick embryo wing expressing constitutively active β-catenin (Tamamura *et al*. 2005). The frizzled domain of the long variant of collagen XVIII has been shown to be able to inhibit Wnt/ β-catenin signalling by binding Wnts (Quélard *et al*. 2008). Moreover, the heparan sulphate proteoglycan nature of collagen XVIII renders it likely to have a role in binding growth factors (Hurskainen *et al*. 2005, Quélard *et al*. 2008). Thus, it is possible that collagen XVIII has roles in binding and presenting Wnt or VEGF molecules to cells.

Collagen XVIII was found to be expressed in some of the blood vessels in bone marrow and periosteum and in the skeletal muscle attachment site. For the

sparse immunolabelling of bone marrow vessels, one explanation could be that vessels in the bone marrow are mainly sinusoids which do not have a continuous endothelium, and thus not a continuous, but fractioned BM.

Our results reveal that endostatin can affect the behavior of osteoblasts *in vitro*. These effects are, by a yet unknown mechanism, boosted with a simultaneous treatment of cells with VEGF and depend on concentration. *In vivo*, lack of collagen XVIII/endostatin leads to a delay in the formation of secondary ossification centers and excess of endostatin causes a temporary delay in the growth of bones, but otherwise bone development in collagen XVIII-deficient or endostatin overexpressing mice is comparable to controls. These results suggest that even though mild delays can be detected in the bone development process in both mutant mouse lines, all in all collagen XVIII/endostatin does not have an indispensable role in skeletal development.

6.3 Collagen XVIII/endostatin in hair follicle development and cycling

Endostatin is a known inhibitor of angiogenesis, and we wanted to test whether it can also affect hair follicle associated angiogenesis and hair follicle growth itself. Moreover, Wnt-signalling is crucial for hair follicle development and cycling (Huelsken *et al*. 2001, Andl *et al*. 2002). In view of the apparent *in vitro* roles of the longest collagen XVIII variant in Wnt-signalling by binding Wnt-molecules (Quélard *et al*. 2008), we studied the effects of collagen XVIII, its N-terminal variants, and its C-terminal endostatin domain on hair follicle development and cycling.

Our results demonstrate that excess of endostatin leads to delayed hair follicle growth, preceded by impaired angiogenesis. It is known that angiogenesis is crucial for proper hair follicle growth, as inhibition of angiogenesis leads to retarded hair follicle growth, and accelerated growth has been observed in mice overexpressing VEGF (Yano *et al*. 2001). Moreover, overexpression of an angiogenesis inhibitor thrombospondin-1 leads to impaired growth of hair follicles and diminished perifollicular angiogenesis, which was detected already before the change in follicle growth (Yano *et al*. 2003), similarly as in our experiments with endostatin. It is known that excess of endostatin can lead to morphological changes in capillaries during cutaneous wound healing, but no other morphological alterations except disorganized capillary BMs were observed in dermal capillaries of 17-day-old ES-tg mice. In healing wounds we observed

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delayed maturation of hemidesmosomes in the ES-tg mice (see study I), and also in hair follicles we could see an increased proportion of immature hemidesmosomes in these mice, suggesting that the excess of endostatin affects the maturation of the epidermal-dermal junction during hair follicle cycling.

Lack of collagen XVIII was observed to lead to a faster catagen of the first hair cycle and faster start of the first real anagen. A similar result was obtained with the *Col18a1*$^{\Delta 3/\Delta 3}$ line lacking the two longer variants, suggesting that the changes seen in the *Col18a1*$^{-/-}$ mice are due to the lack of the long variants of collagen XVIII. We also generated mice specifically lacking the shortest form of collagen XVIII. In these mice the catagen also occurred slightly faster, and the first real anagen started earlier than in the controls. FGF-5 and EGF are important regulators of catagen induction (Murillas *et al*. 1995, Hansen *et al*. 1997, Suzuki *et al*. 2000), and also TGF-β1 and 17β-estradiol are known to induce catagen (Foitzik *et al*. 2000, Ohnemus *et al*. 2005). There are only few mouse lines in which the hair follicle cycling is affected, but premature initiation of hair follicle cycling has been observed in mice overexpressing noggin, which is an inhibitor of bone morphogenetic protein. These mice entered the first catagen earlier than the controls, and they showed also an accelerated induction of the first anagen. (Guha *et al*. 2004.) An acceleration in hair follicle cycling is also seen in mice which are homozygous for the Iasi congenital atrichia allele of the fuzzy gene (fz^{ica}/ fz^{ica}). However, there is no knowledge of the mechanism through which the fuzzy gene product exerts its effects. (Mecklenburg *et al*. 2005.)

The localization of the short variant of collagen XVIII is found in BMs of the skin vasculature, and it is possible that lack of this variant affects angiogenesis in the skin. This will be studied more in detail in our future experiments. Microarray studies have revealed upregulation of collagen XVIII in the bulge region of hair follicles (Tumbar *et al*. 2004, Blanpain *et al*. 2004), and we could see with immunofluorescence staining that the long variants of collagen XVIII are localized in the hair follicle bulge, and also in the BM surrounding the dermal papilla. It is possible that collagen XVIII affects hair follicle cycling by many different mechanisms. The function of the long variants in the BM surrounding the dermal papilla is not known, but it is possible that lack of these variants can cause premature apoptosis of the hair follicle cells. The lack of collagen XVIII from the bulge may allow cells to start proliferation earlier, and thus lead to premature start of anagen. Reporter mouse lines bred to *Col18a1*$^{-/-}$ mice could bring more insight into the effects of collagen XVIII deficiency on the activation status of the Wnt/β-catenin signalling pathway in the bulge region. Optimization

of staining conditions for antibodies against the frizzled variant of collagen XVIII is ongoing. These antibodies will let us distinguish between the middle and the long variant, and help to evaluate the possible biological role of these variants in different parts of the hair follicle.

In conclusion, our results show that overexpression of endostatin leads to delayed hair follicle growth and impaired hair follicle associated angiogenesis. Lack of collagen XVIII leads to an accelerated first hair cycle, and the same observation was made in mice lacking the long variants of collagen XVIII. A milder change is seen in mice lacking the short variant of collagen XVIII, but also in these mice the anagen, and to some extent also the first catagen, are accelerated. The presence of collagen XVIII in the bulge suggests a possible role for the longest frizzled variant of collagen XVIII in regulating the behavior of bulge stem cells by modulating the Wnt-signalling in hair follicles.

7　Future perspectives

This work has provided more insight into the role of collagen XVIII and endostatin in regulating physiological angiogenesis and the processes dependent on it, but also shed light on the role of the different N-terminal variants of collagen XVIII. Our results show that endostatin can delay the healing of cutaneous wounds. This is an important issue considering the fact that endostatin is currently being tested as a chemotherapeutic agent against cancer. As several cancer patients also need surgery, it will be important to know how endostatin affects wound healing in man, and clinical studies may be needed in the future to test this. Accelerated wound healing in mice lacking collagen XVIII suggests that endogenous collagen XVIII may be needed physiologically as a negative regulator of angiogenesis. This gives a reason to study also other, so far unexamined, angiogenic processes in collagen XVIII-deficient mice, such as rheumatoid arthritis and psoriasis. Collagen XV has a C-terminal domain which is highly homologous with endostatin. In the future we are planning to perform wound healing experiments with mice lacking collagen XV, as it is also possible that collagen XV may regulate angiogenesis through its C-terminal domain.

Our results did not reveal a significant role for collagen XVIII or endostatin in bone development. However, we observed that endostatin can affect osteoblast behavior, and it is known from previous experiments that endostatin can also affect osteoclast behavior. Our results strengthen the previous assumption that lack of collagen XVIII or excess of endostatin do not have major impacts on mice in an unchallenged situation. However, if the mice are challenged by an environmental factor or another genetic predisposition, the collagen XVIII/endostatin mutation can become biologically relevant. Examples of this are skin wounds as an environmental challenge in which we did see changes in mutant mouse lines, or the formation of hydrocephalus in mice lacking collagen XVIII in a certain inbred background. Therefore, it is possible that collagen XVIII and endostatin can affect for example bone fracture healing, even though they are not required for the development of the skeleton. Further studies are needed to elucidate this.

The generation of promoter-specific knockout mouse lines expressing exclusively either the short or the two longer variants of collagen XVIII have provided new tools to discover the biological significance of these variant forms. In this study we have used these mouse lines to study the role of collagen XVIII on hair follicles. Our results indicate a special role for the long variants of

collagen XVIII in regulating hair follicle cycling. This study will be continued by phenotyping transgenic mice overexpressing the differing N-terminal noncollagenous domains of collagen XVIII, as well as double mutants generated by crossing the transgenic mice with the knockout mice. The results obtained in this study have also raised the question of a possible role of collagen XVIII as a modulator of Wnt-signalling in hair cycling, and in future experiments we will study this in more detail in hair follicles and also in other organs of the body. The promoter-specific knockout lines are also being used for other studies in our laboratory, and it seems that the three variants of collagen XVIII have very distinct roles in the body. Therefore, one major aim of our group now is to better understand the biological roles of the different variants, and determine the molecular mechanisms through which these variants mainly fulfill their roles in the body.

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