Karolina Rasi

COLLAGEN XV AS A MATRIX ORGANIZER

ITS FUNCTION IN THE HEART AND ITS ROLE TOGETHER WITH LAMININ α4 IN PERIPHERAL NERVES
KAROLINA RASI

COLLAGEN XV AS A MATRIX ORGANIZER
Its function in the heart and its role together with laminin α4 in peripheral nerves

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Abstract

Collagen XV is a proteoglycan localized in the outermost layer of the basement membrane (BM) and in the fibrillar matrix. Although it is widely distributed in many tissues, its amount is generally low. It is characterized by a highly interrupted collagenous domain flanked by large globular domains and attached glycosaminoglycan chains, thus also being identified as a chondroitin sulphate proteoglycan. The C-terminal end of collagen XV, termed restin, has proved to have antiangiogenic effects. In view of its location in the outermost layer of the lamina densa and in association with interstitial collagen fibrils near BMs, it has been suggested that collagen XV may form a bridge between the fibrillar collagen matrix and the BMs. In vivo studies of mice lacking collagen XV have demonstrated that collagen XV is needed as a structural component to stabilize the skeletal muscle cells and capillaries.

The role of collagen XV in the extracellular matrix (ECM) was studied using a mouse model lacking the gene of interest (Col15a1−/−). The matrix of peripheral nerves, skeletal muscle, heart and the uterus during pregnancy was analysed at several time points, and peripheral nerve development was evaluated in a mouse model lacking collagen XV and the laminin α4 chain simultaneously as well as in Col15a1−/− mice. The function of collagen XV in the heart was analysed in young, adult and old mice separately.

The results indicate that collagen XV is needed for organizing collagen fibrils into proper bundles in the cardiac ECM. In the developing nerve it regulates collagen fibril size and the organization of the fibrils within the collagen bundle. In mature nerves and skeletal muscles it structures the BM–fibrillar matrix interphase, and in the uterus of pregnant mice it participates in fibrillar collagen remodelling, affecting the lateral fusion of collagens into thick fibrils. Even delicate changes in the matrix can lead to an alteration in the functioning of certain organs. Abnormal C-fibrils, polyaxonal myelination and decreased sensory conduction velocity were observed in the Col15a1−/− peripheral nerves, and a simultaneous lack of collagen XV and the laminin α4 chain resulted in more severe and permanent impairment of the segregation and myelination of the nerve axons. A complex cardiac phenotype was observed in the Col15a1−/− mice, predisposing them to cardiomyopathy.

Keywords: basement membrane, cardiomyopathy, collagen XV, extracellular matrix, laminin α4, myelination, peripheral nerve development
Rasi, Karolina, Kollageenin XV merkitys sidekudoksen järjestäymisessä. Merkitys sydämessä sekä yhdessä lamininii α4:n kanssa ääreishermoissa

Oulun yliopisto, Lääketieteellinen tiedekunta, Biolääketieteen laitos, Lääketieteellinen biokemia ja molekyylibiologia, PL 5000, 90014 Oulun yliopisto; Oulun yliopisto, Biocenter Oulu, Center for Cell Matrix Research, PL 5000, 90014 Oulun yliopisto

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**Tiivistelmä**


In vivo tutkimuksissa kollageenin XV poistotekijöissä hirsillä on osoitettu, että kollageenia XV tarvitaan rakenteellisena osana stabiloimaan luurankoli-hasta sekä hiussuonia.

Kollageenin XV merkitystä sidekudoksessa tutkittiin käyttäen hiirimallia, jolta puuttuu kyseinen geenin (Col15a1−/−). Ääreishermojen, luurankolihakseen, sydämen ja kohdun soluväliaineen analysoitiin eri-ikäisillä hirsillä. Lisäksi ääreishermojen kehitystä tutkittiin hiirimallillaa, jolta puuttuu sekä kollageeni XV että lamininii α4-ketju samanaikaisesti, sekä Col15a1−/− hiirimällä. Kollageenin XV tehtävää sydämessä analysoitiin nuorilla, aikuisilla ja ikääntyneillä Col15a1−/− hiirimällä.


**Asiasanat:** kardiomyopatia, kollageeni XV, lamininii α4, myelinisaatio, periferisen hermon kehitys, soluväliaine, tyvikalvo
The people who bind themselves to systems are those who are unable to encompass the whole truth and try to catch it by the tail; a system is like the tail of truth, but the truth is like a lizard; it leaves its tail in your fingers and runs away knowing full well that it will grow a new one in a twinkling

–Ivan Turgenev to Leo Tolstoy
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Oulu, September 2010

Karolina Rasi
Abbreviations

Ang II  angiotensin II
BM  basement membrane;
C-  carboxy-
Col XV  collagen XV
Col I  collagen I
Col III  collagen III
Col15a1  mouse collagen XV gene
COL15A1  human collagen XV gene
Col15a1-/-  knock-out mice lacking the collagen XV gene
DCM  dilated cardiomyopathy
DKO  double knock-out mouse
d.o.p.  day of pregnancy
E  embryonal day
ECM  extracellular matrix
HSAN  hereditary sensory and autonomic neuropathy
HR  heart rate (beats per minute)
IEM  immunoelectron microscopy
IVS  thickness of the intraventricular septum
kPa  kilo pascal
Lama4-/-  knock-out mice lacking the gene for laminin α4 chain
L-NAME  Nω-nitro-L-arginine methyl ester
LV  left ventricle
LV/BW  weight of the left ventricle normalized by body weight
LVEDD  left ventricular end diastolic diameter
LV EF  left ventricular ejection fraction
LVM  LV mass
LV PW  thickness of the left ventricle posterior wall
mRNA  messenger RNA
N-  amino
P  postnatal day
PCR  polymerase chain reaction
PNS  peripheral nervous system
S.D.  standard deviation
SEM  scanning electron microscopy
S.E.M.  standard error of means
TEM transmission electron microscopy
List of original articles

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


* Equal contributions
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1 Introduction

The human body consists of organs and tissues, which are composed of cells having various functions. The tissues do not solely consist of cells, however, as a substantial amount of the volume is taken up by extracellular matrix (ECM), the main components of which are collagens, laminins, proteoglycans, elastin and fibronectin. The ECM is needed to provide mechanical strength for the tissues, and it also affects the attachment, proliferation, migration, differentiation and metabolism of the surrounding cells. ECM-cell interactions have important implications for development and morphogenesis. The importance of the ECM has also been demonstrated in human diseases such as cardiac, liver or kidney fibrosis, cancer, diabetes and skin and skeletal diseases. In addition, there are a number of inherited connective tissue diseases caused by mutations in the ECM genes.

Collagen XV is a proteoglycan localized in the outermost layer of the BM and in the fibrillar matrix. It is widely distributed in tissues, albeit in low amounts compared with many other proteins. Its C-terminal end contains a restin domain, which may have anti-angiogenic effects. Previous studies have suggested in view of its location that collagen XV may serve as a bridge between the fibrillar collagens and the BM, and in vivo studies of mice lacking collagen XV have demonstrated that it is needed as a structural component to stabilize skeletal muscle cells and capillaries.

The goals of this research were to increase our knowledge of the biological function of collagen XV in the matrix of various tissues and in the heart, microvasculature and peripheral nerves and to form a more general view of the consequences of the lack of collagen XV in mutant mice, and hence to gain an idea of the human conditions that could potentially be caused by its absence or mutation. The results demonstrate that collagen XV acts as a matrix organizer in a number of tissues and that its roles in the matrix differ between development, maturity and ageing. Thus even delicate changes in the ECM architecture can lead to alterations in peripheral nerve development and function, while a lack of collagen XV in the heart leads to a complex cardiac phenotype, with cardiomyopathic changes visible in elderly mice. Moreover, a more detailed analysis confirmed the previous finding of abnormal capillary morphology and demonstrated that the functioning of the capillaries is severely affected.

These findings obtained with mutant mice suggest that collagen XV has important functions in a number of tissues and raise the possibility that a lack of
collagen XV or a mutation in the human gene could also lead to complex disorders in man, involving fragile capillaries, myopathy, impaired function of the sensory and autonomic nerves and a predisposition for cardiomyopathy.
2 Review of the literature

2.1 Human diseases and animal models

The mouse has become the model organism of choice for the study of human disease in recent years, as it shares 99% of the human genes (Rosenthal & Brown 2007). The mouse genome was characterized in terms of its nucleotide sequence in 2002 and the human genome in 2002, and the simultaneous development of techniques for manipulating the mouse genome has provided powerful tools for studying human genes and their functions. Thus the 2007 Nobel Prize in physiology or medicine was awarded to Drs Mario Capecchi, Martin Evans and Oliver Smithies for their work on genetic modifications in mice using embryonic stem cells. They had developed technologies that allow any gene in the nucleus to be either partly deleted (knock-out) or inserted (knock-in) whilst in the germline, making it possible to generate mouse lines that carry and express an altered gene (Hacking 2008).

Mice and humans share most physiological and pathological features, so that there are widely documented similarities in cardiovascular, musculoskeletal, nervous, immune, endocrine and other internal organ systems, but they can also be subjected to biomedical experimentation that is not possible in humans, making them valuable tools for studying human genetic diseases and initiating the testing of new treatment methods, including gene therapy. In addition, mice are easy to maintain, relatively cheap and have a rapid breeding time and large litter size (Rosenthal & Brown 2007). Among the various techniques for generating genetically engineered mouse lines, transgenic mice are ones in which the gene of interest is injected into a fertilized egg so that the offspring have extra copies and thereby over-express the transgene. This transgene can be tissue-specific or inducible with certain drugs. In knock-out mice, the gene of interest is “lost” by the homologous recombination of introduced DNA, i.e. the embryonic stem cells are transfected with a targeting construct which will homologously recombine with the resident gene and introduce a mutation or loss of the gene. In addition there are techniques for adding a new gene, silencing a gene, causing a mutation in an existing gene, replacing a coding region for an existing gene or down-regulating an existing gene (Eisen 2005). Despite the extensive toolkit available for generating genetically modified mice, the challenge is to determine the phenotype of each mutant mouse line and to translate the information into an
understanding of what could be the function of the gene of interest in humans and the symptoms caused by its modification. Dissimilarities between diseases in the genetically modified mice and those in humans could be caused by differences in mechanical and environmental stresses due to the small size, sedate lifestyle and short life-span of the laboratory mouse compared with humans and differences in basic physiology between mouse and human. In the case of many diseases that manifest themselves at advanced ages in humans, a mouse’s life is not long enough to develop such degenerative changes (Wynshaw-Boris 1996). But be that as it may, the lowly mouse, once detested as vermin and an agricultural pest, has now emerged as the leading mammalian model system for biomedical exploration.

2.2 The extracellular matrix

The ECM is a dynamic network of proteins. Hundreds of ECM proteins are encoded in vertebrate genomes, the primary components being fibrous structural proteins (e.g. collagens, laminins, fibronectin, vitronectin and elastin), specialized proteins (e.g. growth factors, small matricellular proteins and small integrin-binding glycoproteins) and proteoglycans. Their precise composition varies from tissue to tissue. The ECM is constantly undergoing remodelling, i.e. assembly and degradation – particularly during developmental processes, differentiation, wound repair, infections and many disease states. All cells make contact with the ECM, either continuously or at important phases in their lives, as stem cells or during migration or invasion. The ECM is well known for its function of providing structural support for organs and tissues, and for cell layers in the form of the BM, but it also regulates many aspects of cell behaviour, including cell proliferation and growth, migration, differentiation and survival. It has also been recognized more recently that the mechanical properties of the matrix, chiefly its stiffness, can affect cell behaviour (Engler et al. 2006, Winer et al. 2009, for reviews, see Daley et al. 2008, Hynes 2009).

2.3 Basement membranes

BMs are sheet-like specialized forms of the ECM covering the surfaces of individual cells, i.e. cardiomyocytes, myocytes, endothelial cells and Schwann cells in the peripheral nerves and underlining the epithelial cells. In addition to individual cells, BMs are found surrounding functional units in tissues such as muscle, kidney, adipose tissue and peripheral nerve tissue. Thus they are
widespread tissue components with a fine structure and composition that can vary from tissue to tissue or within the same tissue at different developmental periods and during repair. All BMs contain collagen IV, laminins, entactin-1/nidogen-1, and heparin sulphate proteoglycans. Collagen XV is localized in the outermost layer and collagen XVIII is an integral BM component. Other minor components include agrin, SPARC/BM-40/osteopontin and fibulins (Erickson & Couchman 2000 and references therein). A significant number of interactions contribute to the supramolecular assembly of BMs, so that the current BM model proposes two networks, one consisting of collagen IV and the second made up of multiple laminins (Fig. 1) (Yurchenco & O’Rear 1994, Timpl & Brown 1996). Cells bind to the BM via cell surface receptors such as integrins and dystroglycan, and the BM also binds to the adjacent fibrillar ECM outside the cells.

The BMs surrounding cells can be visualized by light microscopy when stained with periodic acid Schiff and silver staining or when immunostained with BM-specific antibodies, while at the ultrastructural level they have the appearance of thin sheets, usually with two distinct layers: the lamina densa, an electron-dense layer, and the lamina lucida, an electron-lucent layer between the dense layer and the cell. The thickness of a BM varies between 50 and 100 nm (Vracko 1974, Vracko & Benditt 1972), depending on the tissue and its developmental stage, since it is a dynamic structure that varies in composition and assembly during development, differentiation, wound repair and infections and in many disease states (Vracko et al. 1979, Kunze et al. 2009, for reviews, see Erickson & Couchman 2000, LeBleu et al. 2007, Yurchenco & Patton 2009).

2.3.1 Functions of the basement membrane

BM provide structural support and guide the polarity of cells during development. In mammalian kidneys they serve as semipermeable selective barriers that are necessary for glomerular blood filtration. As BM proteins have multiple binding sites for cell adhesion molecules, they serve as ligands for cell surface receptors. By binding to the latter a BM can influence cell behaviour by affecting the intracellular signalling pathways (Hynes 2002). BM components also guide cell differentiation and promote cell proliferation and migration (Aumailley & Krieg, 1996, Yang et al. 2005, Seghal et al. 2006). When the BM is damaged, from injuries such as wounds or pathogenic processes such as neoplasia, BM remodelling is initiated (LeBleu et al. 2007, Seppinen et al. 2008, Hagedorn et al. 2009). This consists of the synthesis of BM components, their self-assembly.
and BM network formation. Changes in BM composition affect cell behaviour in that tissue repair is activated by the recruitment of immune cells and the activation of fibroblasts. In addition, the BM is thought to bind growth factors that influence cell behaviour during BM remodelling. BM components such as collagen XVIII are found to have unique properties, in that the N-terminal domain (endostatin), an anti-angiogenic molecule that limits tumour growth, is released during neoplastic BM remodelling (O’Reilly et al. 1997). Other collagens also contain similar potentially anti-angiogenic domains that can be proteolytically released into the extracellular space (Kalluri 2002). Numerous studies involving the deletion of genes for BM components from genetically modified mice have provided information on the biological role of BMs and their constituent molecules in different organs. Mutations in its components can affect BM stability and continuity, and changes in its architecture have been shown to lead to developmental defects such as impaired myelination in the peripheral nerves of mice (Chen et al. 2003, Wallquist et al. 2002, for reviews, see LeBleu et al. 2007, Yurchenco & Patton, 2009).
2.4 The collagen superfamily

The name “collagen” is used as a generic term for proteins forming a characteristic triple helix of three polypeptide chains. Collagens and proteins with
collagen-like domains form a large superfamily comprising 29 types of collagen identified in vertebrates, some 20 additional proteins with collagen-like domains and some 20 isoenzymes of various collagen-modifying enzymes. To be classed as a collagen a protein must include a collagen-like structure and function as a structural protein in the extracellular matrix. Collagens are the main proteins of the ECM and the most abundant proteins in mammals, constituting ~30% of their protein mass. The collagens nevertheless differ greatly in size, function and tissue distribution (Table 1). They serve an important mechanical function, particularly in the connective tissue of skin, bone, tendon, cartilage and most other tissues, and they also play an important role in the cellular microenvironment and possess functions related to the regulation of cell signalling, proliferation, migration, survival and apoptosis (for reviews, see Gelse et al. 2003, Myllyharju & Kivirikko 2004, Heino 2007, Söderhäll et al. 2007, Gordon & Hahn 2010).

All collagens consist of three polypeptide chains, and at least one collagenous domain in each chain contains repeating Gly-X-Y sequences, where the X position is often occupied by a proline residue and the Y position by 4-hydroxy-proline residue. These Gly-X-Y repeats allow the formation of the characteristic triple helical structure. In some collagens all three chains are identical (homotrimers) while others contain two or three different chains (heterotrimers). The three chains are each coiled into a left-handed helix and are then wound around a common axis to form a triple helix with a shallow right-handed superhelical pitch, so that the final structure is a rope-like rod (for a review, see Myllyharju & Kivirikko, 2004).

The collagens are divided into eight subgroups based on their structural features (Fig. 2). The fibril-forming collagens consist of collagens I, II, III, V, XI, XXIV and XXVII, with the fibrils themselves able to consist of more than one type of collagen. Collagen I fibrils, for example, can contain small amounts of collagens III, V and XII. The FACITs, fibril-forming collagens with interrupted helices and related collagens, comprising collagens IX, XII, XIV, XVI, XIX, XX, XXII and XXVI are located on the surface of the fibril-forming proteins and mediate interaction with other matrix proteins but do not form fibrils themselves. The collagens VIII and X are grouped together as they form hexagonal networks, and the various isoforms of collagen IV are taken to make up a BM-forming group. One further group is formed by the collagens with transmembrane domains, namely collagens XIII, XVII, XXIII and XXV, another by collagen VII, which forms anchoring fibrils, and yet another by the isoforms of collagen VI, which form beaded filaments. Collagens XV and XVIII make up a subgroup of
their own, having a structure characterized by multiple triple helix domains and interruptions, and therefore referred to as “MULTIPLEXINs” (Oh et al. 1994a). New collagen types XXVIII and XXIX are not yet placed in any of the subgroups (for reviews of collagen types, see Myllyharju & Kivirikko 2004, Heino, 2007, Gordon & Hahn 2010).

Table 2. Collagen types, their genomic locations and tissue distributions.

<table>
<thead>
<tr>
<th>Type</th>
<th>Genes (genomic localization)</th>
<th>Tissue distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibril forming collagens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>COL1A1 (17q21.31–q22), COL1A2 (7q22.1)</td>
<td>heart, bone, dermis, tendon, ligaments, cornea</td>
</tr>
<tr>
<td>II</td>
<td>COL2A1 (12q13.11–q13.2)</td>
<td>cartilage, vitreous body, nucleus pulposus</td>
</tr>
<tr>
<td>III</td>
<td>COL3A1 (2q31)</td>
<td>heart, skin, vessel wall, reticular fibres of most tissues (lungs, liver, spleen, etc.)</td>
</tr>
<tr>
<td>V</td>
<td>COL5A1 (9q34.2–q34.3)</td>
<td>heart, lung, cornea, bone, fetal membranes; together with type I collagen</td>
</tr>
<tr>
<td>XI</td>
<td>COL11A1 (1p21), COL11A2 and COL11A3 (6p21.3)</td>
<td>cartilage, vitreous body</td>
</tr>
<tr>
<td>XXIV</td>
<td>COL24A1 (1p22.3)</td>
<td>embryonic expression in eye and skeleton (mouse)</td>
</tr>
<tr>
<td>XXVII</td>
<td>COL27A1 (8q32-33)</td>
<td>cartilage, ear, eye, colon and lung (in mouse)</td>
</tr>
<tr>
<td>Basement membrane collagens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>COL4A1 (13q34)</td>
<td>basement membranes</td>
</tr>
<tr>
<td>Microfibrillar collagens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>COL6A1 (21q22.3)</td>
<td>widespread: heart, dermis, cartilage, placenta, lungs, vessel wall,</td>
</tr>
<tr>
<td></td>
<td>COL6A2 (21q22.3)</td>
<td>intervertebral disc</td>
</tr>
<tr>
<td></td>
<td>COL6A3 (2q37)</td>
<td>widespread</td>
</tr>
<tr>
<td></td>
<td>COL6a4 (only in mouse)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>COL6A5 (3q22.1)</td>
<td>lung, testis, colon</td>
</tr>
<tr>
<td></td>
<td>COL6A6 (3q22.1)</td>
<td>heart, muscle, lung, kidney, liver, spleen, thymys</td>
</tr>
<tr>
<td>Anchoring fibrils</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VII</td>
<td>COL7A1 (3p21.3)</td>
<td>skin, dermal–epidermal junctions; oral mucosa, cervix</td>
</tr>
<tr>
<td>Type</td>
<td>Genes (genomic localization)</td>
<td>Tissue distribution</td>
</tr>
<tr>
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<tr>
<td><strong>forming collagens</strong></td>
<td></td>
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<tr>
<td>VIII</td>
<td>COL8A1 (3q12–q13.1)</td>
<td>endothelial cells, Descemet’s membrane</td>
</tr>
<tr>
<td></td>
<td>COL8A2 (1p34.3–p32.3)</td>
<td></td>
</tr>
<tr>
<td>X</td>
<td>COL10A1 (6q21–q22.3)</td>
<td>hypertrophic cartilage</td>
</tr>
<tr>
<td><strong>FACIT and related collagens</strong></td>
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<tr>
<td>IX</td>
<td>COL9A1 (6q13), COL9A2 (1p33–p32.2)</td>
<td>cartilage, vitreous humor, cornea</td>
</tr>
<tr>
<td>XII</td>
<td>COL12A1 (6q12–q13)</td>
<td>perichondrium, ligaments, tendon</td>
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<tr>
<td>XIV</td>
<td>COL14A1 (6q23)</td>
<td>dermis, tendon, vessel wall, placenta, lungs, liver</td>
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<tr>
<td>XVI</td>
<td>COL16A1 (1p34)</td>
<td>fibroblasts, amnion, keratinocytes</td>
</tr>
<tr>
<td>XIX</td>
<td>COL19A1 (6q12–q14)</td>
<td>muscle, spleen, prostate, kidney, liver, placenta, colon, skin</td>
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<tr>
<td>XX</td>
<td>not in human</td>
<td>corneal epithelium, embryonic skin, sternal cartilage, tendon</td>
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<tr>
<td>XXI</td>
<td>COL21A1 (6p12.3–11.2)</td>
<td>blood vessel wall</td>
</tr>
<tr>
<td>XXII</td>
<td>COL22A1 (6q24.23–q24.3)</td>
<td>tissue junction in skeletal and heart, cartilage and hair follicle.</td>
</tr>
<tr>
<td>XXVI</td>
<td>COL26A1/EMID2 (7q22.1)</td>
<td>testis and ovary</td>
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<td><strong>Transmembrane collagens</strong></td>
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<tr>
<td>XIII</td>
<td>COL13A1 (10q22)</td>
<td>epidermis, hair follicle, endomysium, intestine, chondrocytes, lungs, liver</td>
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<tr>
<td>XVII</td>
<td>COL17A1 (10q24.3)</td>
<td>dermal–epidermal junctions</td>
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<tr>
<td>XXIII</td>
<td>COL23A1 (5q35)</td>
<td>amnion, lung, cornea, kidney and lung</td>
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<tr>
<td>XXV</td>
<td>COL25A1 (4q25)</td>
<td>neurons, Alzheimer disease plaques</td>
</tr>
<tr>
<td><strong>Multiplexins</strong></td>
<td></td>
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<tr>
<td>XV</td>
<td>COL15A1 (9q21–q22)</td>
<td>endothelial cells, heart, muscle, nerve, fibroblasts, kidney, pancreas</td>
</tr>
<tr>
<td>XVIII</td>
<td>COL18A1 (21q22.3)</td>
<td>vascular and epithelial BMs, lungs, liver, kidney, muscle, retina and fibroblasts.</td>
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<tr>
<td><strong>New</strong></td>
<td></td>
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<tr>
<td>XXVIII</td>
<td>COL28A1 (7p21.3)</td>
<td>dorsal root ganglia, peripheral nerves</td>
</tr>
<tr>
<td>XXIX</td>
<td>COL29A1 (3q21)</td>
<td>skin, lung, small intestine, colon, testis</td>
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2.4.1 Collagen synthesis

The biosynthesis of fibrillar collagens involves several steps. The polypeptide chains are synthesized on membrane-bound ribosomes and secreted into the lumen of the endoplasmic reticulum. This is followed by a number of post-translational modification steps involving several enzymes. First the signal peptides are cleaved. Collagen prolyl-4-hydroxylase, located in the endoplasmic reticulum (ER), plays a central role in this, as the 4-hydroxypoline residues are essential for the formation of triple helical molecules \textit{in vivo}. Three pro-collagen chains are assembled into a triple helix in the ER and the procollagen is secreted in transport vesicles through the Golgi. After the newly formed procollagen molecules have been secreted into the extracellular space, the N and C-terminal...
propeptides are cleaved and the procollagens start to assemble into collagen fibrils. Finally, covalent cross-links are formed (Fig. 3; for a review of collagen synthesis, see Myllyharju & Kivirikko, 2004). For the non-fibril forming collagens the synthesis is somewhat different. For example collagen IV does not undergo cleavage after the secretion (Khoshnoodi et al. 2010).

Fig. 3. The main steps in the synthesis of fibril-forming collagen. The polypeptide chains are synthesized on membrane-bound ribosomes and secreted into the lumen of the endoplasmic reticulum, where the main steps in biosynthesis are: (i) cleavage of the signal peptides (not shown); (ii) hydroxylation of certain proline and lysine residues to 4-hydroxyproline, 3-hydroxyproline and hydroxylysine; (iii) glycosylation of some of the hydroxylysine residues to galactosylhydroxylysine and glucosylgalactosylhydroxylysine; (iv) glycosylation of certain asparagine residues in the C propeptides, or both the N and C propeptides, by reactions similar to those in many other proteins; (v) association of three C propeptides directed by specific recognition sequences; and (vi) formation of intramolecular and intermolecular disulphide bonds. A nucleus for the assembly of the triple helix is formed in the C-terminal region after the C propeptides have become associated and ~100 proline residues have been hydroxylated to 4-hydroxyproline in each of the chains, and the triple helix is then propagated towards the N-terminus in a zipper-like fashion. The procollagen molecules are transported from the endoplasmic reticulum through the Golgi stacks, during which they begin to aggregate laterally to form secretory vesicles. The subsequent steps are cleavage of the N and C propeptides, spontaneous self-assembly of the resulting collagen molecules into fibrils, and formation of covalent crosslinks initiated by oxidation of the 1 amino group in certain lysine and hydroxylysine residues into reactive aldehyde derivatives From Myllyharju and Kivirikko, in TRENDS in Genetics, 2004. Reprinted by permission of Elsevier.
2.4.2 Collagen assembly

The formation of higher-order structures is best known for the fibril-forming collagens. Purified collagen spontaneously assembles into fibrils in vitro because collagen molecules are free to bind other collagens and there are no other partners available. In vivo, the fibrillar collagens have up to 50 known binding partners (Di Lullo et al. 2002, Kadler et al. 2008) and the situation is quite different. Faced with so many potential binding partners, the collagen molecules might easily be sequestered into dead-end molecular interactions, and no free collagen molecules would be available to form fibrils (Kadler et al. 2008). Thus several matrix organizers are needed for collagen fibrillogenesis and for cell-BM-fibrillar matrix interplay. Cells need collagen V and XI to nucleate collagen fibrils, and fibronectin and integrins to specify the site of assembly. By localizing fibril formation near the cell plasma membrane, the cells can maintain control over the process (Kadler et al. 2008).

Numerous proteins, glycoconjugates and small proteins have been shown to influence the rate of assembly, size and structure of collagen fibrils formed in vitro. In addition, some mouse models have been generated for in vivo analysis. The molecules that have received the most attention are the N-propeptide of collagen I, collagen V, crosslinking enzymes such as lysyl oxidase, tenascin-X, thrombospondin 2, cartilage oligomeric proteins (COMP), matrilins, perlecan, decorin, biglycan, fibromodulin and lumican (Bornstein et al. 2000, Svensson et al. 2002, Wiberg et al. 2003, Wenstrup et al. 2004, Kvist et al. 2006).

As an example of such regulation, tenascin-X deficiency does not interfere with the synthesis or processing of collagen, but it can regulate fibril assembly, as the packing density of collagen fibrils was reduced in null mice and there was a 30% reduction in the collagen content of the skin (Burch et al. 1997, Mao et al. 2002). Interestingly, the tenascin-X gene was the first disease-causing gene in the Ehlers-Danlos syndrome that did not encode a fibrillar collagen or an enzyme processing the procollagens (Burch et al. 1997).

2.4.3 Collagens in human diseases

The importance of collagens is illustrated by the wide spectrum of diseases caused by changes in collagen biosynthesis or mutations in collagen genes. So far more than 1000 mutations, involving 26 genes in 13 collagen types, have been identified (Myllyharju & Kivirikko 2001, Söderhäll et al. 2007, Lanfranconi &
Mutations in collagen genes may lead to genetic diseases such as Ehlers-Danlos syndrome, Alport syndrome, Bethlem myopathy, osteogenesis imperfecta, certain types of epidermolysis bullosa and Knobloch syndrome. In addition, collagens play important roles in more common diseases such as osteoarthritis, osteoporosis and aortic aneurysm, in pathological conditions leading to fibrosis, in wound healing and in the healing of fractures. The severity of the phenotype ranges from lethal at the embryonic stage to phenotypes that are difficult to distinguish from more common disorders. About 75% of the mutations characterized are single base substitutions that either change the codon of a critical amino acid or lead to abnormal RNA splicing. Most of the single base substitutions are found in the Gly-X-Y triplet, leading to impaired folding of the triple helix or an interruption in it. Other mutations include gene deletions, insertions, duplications and complex re-arrangements. Mutations that cause abnormal polypeptide chains cause a more severe phenotype than null alleles (Myllyharju & Kivirikko 2001).

Ehlers-Danlos syndrome (EDS) is a heterogeneous group of inheritable disorders resulting from mutations in genes for collagen V, collagen I, collagen II, tenascin-X and lysyl hydroxylase. These disorders are characterized by skin abnormalities, joint hypermobility, skeletal deformations and ruptures in hollow organs. The vascular type of EDS is characterized by thin, fragile skin, easy bruising and haematomas, and patients suffer from arterial complications such as ruptures and aneurysm in vessels of all calibres. Most of the mutations leading to this severe connective tissue disease are found in the collagen III gene (Germain 2007).

Alport syndrome is caused by various mutations in the BM collagen IV. The onset, prognosis and severity of the kidney disease will depend on the inheritance pattern, type and location of the mutation. The kidney is the most commonly affected organ, with haematuria, interstitial nephritis and progressive renal failure caused by glomerular BM thickening and splicing into lamellae, although sensorineural deafness and retinopathy have also been observed in some patients (Van Agtmael & Bruckner-Tuderman 2010).

Bethlem myopathy and Ullrich congenital myopathy are muscle diseases caused by mutations in collagen VI chains. The disease is characterized by progressive muscle weakness and wasting, respiratory compromise and contractures in joints (Collins & Bonnemann 2010). Osteogenesis imperfecta, a disease characterized by brittle bones, abnormal teeth, thin skin, blue sclera, weak tendons and hearing loss, is caused by mutations in collagen I, but mutations in
the enzymes needed for the modification of collagens can also lead to this disease. The most severe forms are embryonically lethal, while the mildest cases can be hard to distinguish from common osteoporosis (Shapiro & Sponsellor 2009). The skin blistering diseases known as epidermolysis bullosa are caused by mutations in collagens VII and XVII, while Knobloch syndrome, characterized by encephalocele and ocular abnormalities, is attributable to mutations in collagen XVIII (for a review, see Myllyharju & Kivirikko 2001). Corneal endothelial dystrophies, characterized by painful loss of vision, have been shown to be caused by mutations in the COL8A2 gene encoding collagen VIII (Biswas et al. 2001). More recently, mutations in the COL4A1 gene encoding collagen IV have been identified as a cause of familial vasculopathy, cerebral small vessel disease, porencephaly and infantile hemiparesis (Lanfranconi & Markus 2010). Mutations in the COL4A1 gene have also been shown to lead to HANAC syndrome, a hereditary angiopathy with neuropathy, aneurysm and cramps (Plaisier et al. 2007). COL29A1 gene, encoding for epidermal collagen has been shown to be the gene responsible for atopic dermatitis, a common chronic skin disease. It is thought that collagen XXIX plays an important role in keratinocyte cohesion and lack of it may allow antigen penetration through the skin (Söderhäll et al. 2007).

2.5 Collagen XV

Primary structure of collagen XV

Collagen XV belongs to the group of non-fibril-forming collagens, making up, together with collagen XVIII, the subgroup of multiplexins (multiple triple helix domains and interruptions) within the collagen superfamily (Myers et al. 1992, Abe et al. 1993, Kivirikko et al. 1994, Muragaki et al. 1994, Oh et al. 1994a, Oh et al. 1994b, Rehn & Pihlajaniemi 1994, Rehn et al. 1994). Collagen XV is thought to be a homotrimer consisting of three \(\alpha_1\) (XV) chains (Rehn & Pihlajaniemi 1994, Sasaki et al. 2000). The human \(\alpha_1\) (XV) chain contains nine collagenous domains, whereas the mouse equivalent contains seven. The collagenous domains are the most highly interrupted regions in the collagens and include the Gly-X-Y triplets (where ‘X’ and ‘Y’ can be any amino acid, but are often proline and hydroxyproline, respectively). In collagen XV only two-thirds of the sequences represent collagenous domains, the longest collagenous segments being only 114 and 71 amino acids in length. The collagenous regions
have several non-collagenous interruptions of varying sizes (Kivirikko et al. 1994, Myers et al. 1992) (Fig. 4). The human and mouse amino acid sequences exhibit an overall identity of 72%, but this homology is not distributed equally, being highest close to the C-terminal end (Hägg et al. 1997b). The C-terminal restin domain of collagen XV is structurally related to the endostatin domain of collagen XVIII. Even though the restin and endostatin domains are homologous in terms of sequence, they are not comparable in their anti-angiogenic effects (Ramchandran et al. 1999). Rotary shadowing EM of recombinant collagen XV revealed rod-like molecules with a mean length of 241.8 nm and a globular domain at one end (Hurskainen et al. 2009). Human tissue-derived purified XV monomers had a curled/eight/pretzel configuration and an ability to self-assemble into higher-order structures (Myers et al. 2007). In contrast to the other collagens, collagen XV was able to curl and twist, suggesting that the many interruptions in the collagenous region are responsible for making this possible. The authors also found that collagen XV monomers could arrange themselves into clusters, while some were arranged in simpler oligomers. Collagen XV molecules could also create a cruciform pattern, probably possessing interaction through internal binding sites within the collagenous region (Myers et al. 2007).

Collagen XV is the only known proteoglycan/collagen bearing CS (chondroitin sulphate) GAG (glycosaminoglycan) chains, and it also exists as a novel hybrid of CS and HS (heparan sulphate) chains with a differential ratio depending upon the tissue (Fig. 4) (Li et al. 2000). It has been suggested that the HS chains may be involved in the binding of growth factors and other cytokines, while the CS chains could be involved in maintaining a hydrated environment (Deepa et al. 2004). The CS-HS forms have been shown to bind collagens I and II and to organize monomers to form fibrils (Kvist et al. 2006).
Fig. 4. Schematic figure of collagen XV and a model of its location in the ECM. A) The amino-terminal (N) non-collagenous region, containing the thrombospondin-like domain (TSP) is 530 residues. The carboxy-terminal (C) domain, 245 residues, contains the restin domain. The collagenous domains, totally 577 residues, are displayed as yellow, while the interruptions are black. The locations of the 11 glycos-amino-glycan (GAG) chains are shown. The sequence information was reported by Myers et al. (1992) and Kivirikko et al. (1994). B) Collagen XV is located in the outermost layer of the BM as well as to the fibrillar collagens in the ECM. It is suggested that the collagenous parts with interruptions are the interacting domains. Collagen XV is also able to bend and make 8-like figures.
The collagen XV gene

The human COL15A1 gene is about 145 kb in size and contains 42 exons (Hägg et al. 1998). The human gene has been mapped to chromosome 9q21-q22 (Huebner et al. 1992), while its mouse counterpart has been localized to chromosome 4, B1-3 (Hägg et al. 1997b).

Tissue distribution and localization of collagen XV

Collagen XV has a widespread tissue distribution in both humans and the mouse. An in situ hybridization study of collagen XV in 20-gestational-week human foetuses revealed that its main producers are mesenchymally derived cells, especially fibroblasts, muscle cells and endothelial cells. Also, some epithelial cells can produce collagen XV (Kivirikko et al. 1995). The collagen XV protein has been localized by light microscopy to most epithelial and all nerve, muscle, fat and endothelial BM zones, except for the glomerular capillaries and hepatic and splenic sinusoids (Myers et al. 1996). The localization of collagen XV in the mouse differs depending on the developmental stage, the capillaries in the heart and skeletal muscle being positive from the foetal to the mature stage, whereas most of those in the brain lose their collagen XV expression and only some larger vessels there remain positive. Similarly, collagen XV is expressed in the capillaries of the foetal lung but is lost with maturity. The skeletal muscle contained collagen XV from E13.5 onwards, thus coinciding with primary myotube formation, while the first signs of it in the heart appeared at E14.5, around the BM zones of the cardiac muscle. The expression then gradually expanded from the periphery inwards. In the peripheral nerves collagen XV has been detected in the perineurium and endoneurium of both foetal and adult mice, but it is absent from the synaptic BM of the neuromuscular junction (Muona et al. 2002). Immuno-electron microscopy (IEM) of human tissues (placenta, colon and kidney) has shown that collagen XV is not an integral BM component, as it is localized both in the outermost layer of the BM and on the surface of large collagen fibrils (Fig. 4). It has been proposed that it may serve to link the collagen fibrils to each other and to the BM (Amenta et al. 2005a).

During expression, collagen XV is folded in the endoplasmic reticulum. To initiate trimerization, the multiplexins, including collagen XV, must have a sufficient association constant, higher than that of the fibrillar collagens. The trimerization domain is present in the N-terminal end of the NC1 domain, and
being very small, about 30 residues, these domains can form trimers at very low (picomolar) concentrations (Boudko et al. 2009).

Myers et al. (2007), who extracted collagen XV from the umbilical cord, one of its most abundant sources, estimated that it accounts for approximately $1 \times 10^{-4}$% of total protein in this tissue. It is regarded as the second rarest collagen to be isolated from tissues to date, exceeded only by collagen XIX (Myers et al. 2007).

**Binding partners of collagen XV**

Even though based on the location of collagen XV in association with fibrillar collagens, collagen XV has not been shown to bind collagen I or III *in vitro*, but interaction has been demonstrated between the restin part of collagen XV and nidogen-2 and 1, fibulin-2 and 1 and perlecan (Sasaki et al. 2000), and between recombinant collagen XV and fibronectin, laminin and vitronectin (Hurskainen et al. 2009). Nidogens are BM glycoproteins, interacting with wide range of other BM proteins. They are thought to link proteins within the BMs. Nidogen 1 is found in all BMs, while Nidogen 2 is restricted to BMs of skeletal and heart muscle. Nidogens 1 and 2 are thought to compensate each other, while simultaneous lack of both results in BM defects leading to failure in cardiac function in mice (Bader et al. 2005). Fibulin 1 and 2 are small glycoproteins associated with BMs, elastic fibers and other matrices. Fibulin 1 is exists widely in BMs, in association of microfibrillar and elastic fibers. Fibulin 2 expression is more restricted to BMs of heart, placenta, ovary and cartilage. They are thought to function as intermolecular bridges. Fibulin 1 knock-out mice show bleedings and the mice die around birth (de Vega et al. 2009). Perlecan is a heparin sulphate proteoglycan found in BMs of developing cartilage, blood vessels and heart. Its function is to stabilize BMs against mechanical forces and knock-out mice die around E10.5 due to massive hemopericarditis (Sasse et al. 2008). Fibronectin is a dimeric glycoprotein and has an ability to bind collagens, heparin, fibrin and proteoglycan molecules (Singh et al. 2010). It can simultaneous bind cells to the surrounding matrix molecules. Mice studies have shown that fibronectin is required for proper embryonic cardiovascular development and total lack of fibronectin leads to embryonic lethality with several developmental deformities, including the heart (George et al. 1993). The function of laminins is described in section 2.7. Vitronectin is a glycoprotein present in both blood and ECM. It has been shown to bind to collagen, heparin, integrins, plasminogen and thrombin-
antithrombin III complex. The major functions of vitronectin are multiple; it is participating in haemostasis, immune defence, fibrinolysis, cell adhesion, migration and proliferation as well as extracellular anchoring. However, mice lacking this protein are found to be normal with respect to development, fertility and survival (Schwartz et al. 1999).

**Biological function of collagen XV**

No human disease has been found to date to be caused by a lack of or mutation in the COL15A1 gene, but some information on the function of collagen XV has been gathered from analysing null mice (Col15a1−/− mice) and from zebrafish models and *in vitro* models. It has also been analysed in human samples representing several disease models. Eklund et al. (2001) have shown in Col15a1−/− mice that collagen XV functions as a structural component that is needed to stabilize skeletal muscle cells and capillaries, and also that it is needed for the proper functioning of the heart. The Col15a1−/− mice studied were from the 129SV background and were fertile and had a normal lifespan, but from 3 months onwards their skeletal muscles showed focal areas of degeneration, macrophage infiltration, regeneration and variation in fibril size characteristic of myopathic disorders, the back and paraspinal muscles being more affected than the other muscle groups. No signs of fibrosis were detected. Both the skeletal and cardiac muscle showed defects in the form of collapsed, irregularly shaped, swollen or degenerating capillaries. When the mice were exercised in a treadmill higher β-glucuronidase activity was observed in the mutants than in controls, indicating greater susceptibility to muscle injury, and there were more degenerative fibrils and a stronger inflammatory response. The exercise also led to increased apoptosis, β-glucuronidase activity and pro-MMP levels in the Col15a1−/− hearts, whereas pro-MMP levels were lower in the mutant mouse heart under normal conditions. Isolated and perfused hearts showed normal basal contractility, but the maximal pressure developed when stimulated with isoproterenol was significantly lower in the 6 and 12-month-old Col15a1−/− hearts. These *in vitro* results pointed to a potential effect of collagen XV on the physiological functioning of the heart (Eklund et al. 2001).

A zebrafish model has also been used to study the function of collagen XV in muscle development. In the zebrafish collagen XV is primarily located in the peri-notochordal BM where it is needed for notochord differentiation. Morpholino-mediated Col15a1 knock-down consequently showed defects in
notochord differentiation and in fast and slow muscle formation, disorganization of the peri-notochodal BM and myofibrils, and a U-shaped myotome (Pagnon-Minot et al. 2008). Collectively, these data suggest that in addition to having a structural role in providing stability to the skeletal muscle, collagen XV also plays a role in muscle development.

Collagen XV and human fibrotic diseases

Some data are available on the involvement of collagen XV in human diseases. Its role in fibrotic diseases has been studied in patients suffering from glomerular diseases with interstitial fibrosis, where an interstitial accumulation of collagen XV is seen in the glomerular segments with small, thick-walled capillaries, while the reaction in the fibrillar collagen matrix of normal healthy interstitial tissue was either very weak or non-existent (Hägg et al. 1997a). In Dupuytren’s contracture, the most common inherited connective tissue disease in humans, fibroproliferation and contractures occur in the palmar fascia due to aberrant wound healing. When total RNA was isolated from the diseased palmar fascia and compared with samples from healthy patients, the COL15A1 gene was nevertheless found to be down-regulated in the fibrotic samples, indicating that over-expression of collagen XV is not universal to all fibrotic diseases and may vary with tissue type and localization (Satish et al. 2008).

Collagen XV and tumours

It has been proposed on the grounds of gene location that collagen XV may be a tumour suppressor (Harris 2003), and complete suppression of tumour formation has been shown in vitro by the presence of high levels of collagen XV. Highly tumorigenic human cervical carcinoma cells that do not normally express collagen XV were transfected by means of an expression vector carrying the full-length cDNA of the human COL15A1 gene and subsequently transfected into nude mice. The tumours expressing collagen XV were significantly smaller in volume than in the controls and the mice survived longer (Harris et al. 2007). A hypothesis that collagen XV may suppress the growth of malignant tumours has also been put forward in conjunction with the observation that it disappears from the BM before invasion by ductal carcinoma cells (Amenta et al. 2003), and a similar disappearance is seen in skin cancers and melanomas (Fukushige et al. 2005). Furthermore, the expression of collagen XV is down-regulated in the BMs
of colonic adenocarcinomas, but increased in the surrounding interstitium, possibly making the stroma more suitable for tumour cell attachment, migration and infiltration (Amenta et al. 2000). In addition, it has been shown in a high-density gene expression microarray that the COL15A1 gene is frequently methylated in renal cell carcinoma, indicating that this tumour suppressor candidate gene is inactivated in that particular type of cancer (Morris et al. 2010).

2.6 Collagen XVIII

Collagens XV and XVIII are closely related in structure, as both have a thrombospondin sequence homology, seven homologous collagenous domains and similar C-terminal ends, termed endostatin domains (Kivirikko et al. 1994, Rehn & Pihlajaniemi 1994). Collagen XVIII differs from collagen XV by having three isoforms, by being localized only to BMs and by being a heparin sulphate proteoglycan (Halfter et al. 1998) whereas collagen XV is a chondroitin sulphate proteoglycan (Li et al. 2000). The trimerization domain of collagen XVIII has been crystallized and its structure has been compared with a model for collagen XV. Despite the primary sequence similarity between the two collagens, the differences in the hydrophobic residues in their respective trimerization domains suggest that their proteins can be co-expressed in the same cells without being mixed (Boudko et al. 2009).

Collagen XVIII has several biological functions. A mutation in the human COL18A1 gene leads to Knobloch syndrome, characterized by high myopia, vitreoretinal degeneration with retinal detachment, macular abnormalities and occipital encephalocele (Passos-Bueno et al. 2006). Mice lacking collagen XVIII also show other ocular abnormalities, namely delayed regression of the hyaloid vessels, poor retinal angiogenesis and reduced susceptibility to high oxygen-induced retinal neovascularisation, and in a specific genetic background, susceptibility to the development of hydrocephalus (Fukai et al. 2002, Ylikarppa et al. 2003 Utriainen et al. 2004, Hurskainen et al. 2005). In addition, it has been shown that a lack of collagen XVIII enhances atherosclerosis (Moulton et al. 2004). Endostatin, the C-terminal domain of collagen XVIII, has been shown to inhibit angiogenesis and tumour growth (O'Reilly et al. 1997). Thus mice overexpressing endostatin show a decrease in the number of dermal lymphatic vessels in the skin and in papillomas and skin carcinomas. These mice also have a reduced number of lymph node metastases (Brideau et al. 2007). Endostatin has been extensively studied for potential cancer therapy and it is used as a treatment
for lung and colon cancer in some countries. Clinical trials among patients with metastatic cancer have shown low efficacy, however, compared with that seen in experimental studies (Sund & Kalluri 2009). It has been shown recently that a lack of collagen XVIII accelerates wound vascularization, whereas excessive endostatin delays wound healing in mice (Seppinen et al. 2008).

2.7 Laminins

Laminins are the major components of BMs and have been shown to have crucial functions in development and disease. Laminins bind to cell surface receptors and connect the BM tightly to the cell. This promotes the specific functions of the BM, such as stabilizing cellular structures, serving as a barrier and regulating the intracellular signalling cascades. It has been suggested that laminins may be principally responsible for organizing BM assembly on cell surfaces. In the absence of other components, purified laminins can assemble to form sheet-like matrices on cell surfaces. All in all, laminins function as the primary scaffold for the BMs, while collagen IV, nidogen and perlecan enhance BM stability, at least during development (Yurchenco & Patton 2009).

Laminins are heterotrimers consisting of one α, one β and one γ chain (Fig. 5), the trimers being named according to their chain compositions, so that laminin-423 is a trimer consisting of α4, β2 and γ3 chains. Their synthesis and assembly occurs inside the cell, but further extracellular proteolytic processes may occur in some laminins before they reach their final form. Each laminin has one long arm and two short arms, with the latter, consisting of N-terminal globular LN domains, having the ability to form independent networks. The C-terminal part, consisting of a globular domain, binds several cell surface receptors and some ECM ligands (for a review, see Scheele et al. 2007). Interestingly, laminins containing the laminin α4 chain lack the α-subunit short arm (see Fig. 5) and are therefore thought not to polymerize (Cheng et al. 1997, Frieser et al. 1997).

Laminins have a tissue-specific distribution. Laminin-111 and laminin-511 are necessary during embryonic development, so that laminin α1 chain deficiency is lethal at an early embryonic stage in mice, most probably because of defects in the extra-embryonic BM called Reichert’s membrane. In addition to the embryonic BMs, the α1 chain is also expressed in developing epithelial cells and in a few epithelial BMs in adult mice. The laminin α2 chain is mainly expressed in muscles and Schwann cells, while the α3 chain is found in the epidermis (Durbeej 2010) and the α4 chain is expressed around endothelial cells, Schwann
cells, the neuromuscular junction, developing muscle fibres, smooth muscle, fat cells and bone marrow (Thyboll et al. 2002). The laminin α5 chain is widely expressed in a number of tissues. Mice lacking the α3, β3 or γ2 chains die around birth due to severe skin blistering, while mice without laminin α2, α4 or β2 chains survive but defects become visible after birth.

Several human diseases caused by mutations in laminin genes have been described. Mutations in laminin α2 cause type 1A congenital muscular dystrophy, characterized by severe muscle weakness, hypotonia, dysmyelinating peripheral neuropathy and brain defects. Pierson’s syndrome is caused by mutations in the laminin β2 chain, leading to glomerular defects, renal failure and early mortality. Epidermolysis bullosa, a skin blistering disorder, can arise on account of mutations in laminin-332 or auto-antibodies to the protein, while laminin α4 mutations have been identified recently in cases of cardiomyopathy. In addition, laminins have a role in cancers and in infections, as several invasive microbes and viruses use laminin binding to enter the cell (for a review, see Durbeej, 2010).
The laminin α4 chain is part of laminin-411 (Fig. 5) and laminin-421, which are widely distributed in the BMs of peripheral nerves, endothelial cells, the heart, the developing kidney and skeletal muscle, and in non-BM locations such as the developing brain and bone marrow (Frieser et al. 1997, Iivanainen et al. 1997, Gu...
et al. 1999, Lathia et al. 2007). The laminin α4 chain has been located by ultrastructural analysis in the ECM, near the capillaries, rather than in the BM (Talts et al. 2000). It can already be detected in the capillaries of skeletal muscle at embryonic day E11 and is expressed there during development and into adulthood (Thyboll et al. 2002).

The laminin α4 chain has several biological functions. Mice lacking in it (Lama4−/− mice) have haemorrhages in subcutaneous tissues and muscle during the embryonic and neonatal periods, demonstrating that it has a significant role in the formation of capillaries and their subsequent integrity. Thus the capillary BMs of the null mice were discontinuous and had delayed synthesis of collagen IV and nidogen (Thyboll et al. 2002).

In the adult murine heart the laminin α4 chain is mainly expressed in the blood vessels and the peripheral sarcolemma of cardiomyocytes. Mice lacking this chain gradually develop cardiac hypertrophy, dilated ventricles and cardiac dysfunction, most likely due to poor microvascularization and cardiac ischaemia (Wang et al. 2006b).

The laminins in the peripheral nerves differ in composition during development and in adulthood. The laminin α4 chain is expressed in the dorsal root ganglia at E15.5 and in the peripheral nerves at all embryonic stages, but its levels have decreased significantly by adulthood (Lentz et al. 1997). Adult peripheral nerves contain the laminin α4 chain only in the perineurium, the BM sheets surrounding the fascicles of the nerve fibres, whereas it is almost totally lacking in the endoneurium, the BMs surrounding the Schwann cells (Patton et al. 1997).

The neuromuscular junctions, active zones and junctional folds in Lama4−/− mice are not precisely apposed to each other, suggesting a mild defect that does not explain the motor defects (Patton et al. 2001). In a behavioural analysis Lama4−/− mice had a tendency to extend their legs backward in a spastic-like manner, and they also showed greater variation in step size. They were able to swim, but had frequently pauses in which they were only floating in the water. Morphological analysis of the peripheral nerves revealed thinner myelin sheaths, polymyelinating axons and impaired segregation of the axons, and therefore less myelinating fibres. The spinal roots were less affected than the distal nerves, mostly because the sorting and myelination in the roots is more dependent on laminin-211 than on laminin-411. The spinal cord and central nervous system had no abnormalities in myelination, nor were any defects seen in the endoneural BM. Schwann cells cultured on laminin-411 showed faster proliferation than on
laminin-111 or laminin-211, suggesting that laminin-411 promotes Schwann cell proliferation. Decreased Schwann cell proliferation is seen at 1.8 and 3.5 days from birth in mice lacking both \( \alpha_4 \) and \( \alpha_2 \). These results are consistent with a hypothesis that increasing cell density in a nerve activates the Schwann cells to start the segregation of axons from the embryonic axon bundles (Wallquist et al. 2005, Yang et al. 2005).

So far no human neurological disease caused by mutations in the laminin \( \alpha_4 \) chain has been reported, but such mutations can lead to severe dilated cardiomyopathy (DCM). When the LAMA4 gene was sequenced from 180 patients with DCM and compared with a control group three mutations were found in the patients. Histological analysis of myocardial biopsy samples from these affected patients revealed a loss of endothelial cells as the primary cause of cardiomyopathy. Laminin \( \alpha_4 \) is connected with ILK (integrin-linked kinase) via integrins, and this pathway is thought to be crucial for directing extracellular signals into the cell. Mutations in this pathway affect the survival of endothelial cells and cardiomyocytes. It has been proposed that disturbances in the interaction of endothelial cells and cardiomyocytes with the ECM could predispose individuals to heart failure and cardiomyopathy (Knoll et al. 2007).

2.8 Matrix organization and remodelling in the uterus and heart

The ECM is critical for the viability of many tissues. For vessels and capillaries it provides a scaffold for maintaining the organization of the vascular endothelial cells into blood vessels. Endothelial cell adhesion to the ECM is needed for endothelial cell proliferation, migration, tube formation and survival and for vessel stabilization (Davis & Senger 2005). The ECM plays an important role in the development of cardiac tissue, in maintaining its structure and function, and in cardiac remodelling during various cardiac diseases (Spinale 2007). In addition, extensive matrix remodelling occurs in physiological situations such as early pregnancy, when the uterine microenvironment is adapting to embryo implantation (Stumm & Zorn 2007).

*Extracellular matrix remodelling in the pregnant uterus*

When embryo implantation occurs, the blastocyst is attached to the inner wall of the uterus, the endometrium. The endometrial stroma of the implantation site undergoes extensive changes, called decidualization, which leads to cell
proliferation and growth, ECM degradation and a decrease in the extracellular spaces are decreased. The pre-existing thin collagen I and III fibrils in the decidualized area are thickened, in a process called lateral fusion, and various proteoglycans and glycoproteins change their expression pattern and localization in the uterine tissue (Spiess & Zorn 2007, Stumm & Zorn 2007). This extensive remodelling of the endometrium ECM is important for successful implantation, although the significance and function of the lateral fusion of collagen fibrils is still unclear. Thickening of collagen fibrils has been described in ageing, however, and in pathological conditions such as damaged blood vessels, liver fibrosis and tumours (Staubesand & Fischer 1980, Itoh et al., 1981).

**Extracellular matrix of the heart**

The major fibrillar collagens in the heart are collagens I and III, which are synthesized by cardiac fibroblasts. Approximately 85% of the total myocardial collagen is collagen I and 11% is collagen III, the other components of the cardiac ECM being smaller amounts of various collagens, including collagens XV, V and VI, elastin, fibronectin, laminins and various proteoglycans. Collagens I and III maintain the alignment of the myofibrils within the cardiomyocytes via collagen-integrin-cytoskeleton-myofibril connections, and convert the cyclic translation of myocyte shortening into a coordinated contraction of the heart. Other components of the cardiac ECM are also important in mediating this function (Jugdutt 2005). Collagen I aggregates into thick fibrils, whereas collagen III forms thin fibrils. Collagen I and III also form aggregate struts of varying thickness that are widely distributed among the cardiomyocytes, connecting them together (Ju & Dixon 1996). Scanning electron microscopy has revealed the three dimensional architecture of the cardiac ECM, showing how bundles of collagen fibrils surround and support individual cardiomyocytes and fascicles formed by them (Spinale 2007). It has been shown that there are numerous collagenous bundles that extend between adjacent cardiomyocytes. The insertion point of the bundle, also called a strut, is constantly just off the Z-band of the cardiomyocyte. Before insertion the collagen struts divide into two or three smaller bundles, and when these adhere to the BM they loosen and spread out laterally. In the diastole, when the cardiomyocytes lengthen and the diameter increases, the struts are straight, allowing the cells to separate slightly, while in the systole the struts produce wavy bundles and there is less tension in them as the cardiomyocytes come closer (Caulfield & Borg 1979). It is thought that the collagen bundles have the function
of supporting the cardiomyocytes and vessels and maintaining cardiac stiffness and the geometry of the ventricular chambers in the heart.

In pathological conditions the ECM undergoes remodelling, which includes the accumulation of ECM proteins, and changes in their content proteins. In hypertrophic hearts an increase in fibrillar collagens (fibrosis) is seen, leading to increased diastolic stiffness and reduced systolic stiffness. Changes in the ratio of collagen I to collagen III mark a transition that takes place in many pathological conditions. By end-stage heart failure in humans a decrease in collagen cross-linking, increases in collagens I, III, IV and VI, fibronectin, laminin and vimentin levels, myocyte loss, eccentric hypertrophy and a decrease in the LV mass to volume ratio are to be seen (Jugdutt 2005). It has become increasingly clear that the ECM has an important dynamic, independent role in various cardiac pathological processes.

2.9 Human cardiomyopathies

Cardiomyopathies are defined as myocardial disorders in which the heart muscle is structurally and functionally abnormal in the absence of any coronary artery disease, hypertension, valvular disease or congenital heart disease attributable to a myocardial abnormality. They may be divided into five groups according to the ventricular morphology and function, namely hypertrophic (HCM), dilated (DCM), restrictive (RCM), arrhythmogenic right ventricular cardiomyopathy (ARVC) and unclassified cardiomyopathies, as laid down by the European Society of Cardiology working group on myocardial and pericardial diseases (Fig. 6). Each group is then divided into familial and non-familial forms, where familial forms are hereditary diseases or de novo mutations in a gene and are mostly caused by a single gene mutation, while non-familial forms include idiopathic and acquired cardiomyopathies (for a review, see Elliott et al. 2008). The various types of cardiomyopathy are briefly outlined below, and the dilated form, which is most relevant to the results of the enclosed original papers, is presented in more detail.
Fig. 6. Classification of cardiomyopathies. HCM indicates hypertrophic cardiomyopathy, DCM dilated cardiomyopathy, RCM restrictive cardiomyopathy and ARVC arrhythmogenic right ventricular cardiomyopathy. Each subgroup is divided into familial and non-familial forms.

**Hypertrophic cardiomyopathy**

HCM is the most common cardiomyopathy, affecting 1 in 500 of the general population. It is also the most common cause of sudden cardiac death in young persons and an important cause of heart failure disability at any age. It is defined by the presence of an increased thickness of the ventricular wall as detected in an echocardiogram or by magnetic resonance imaging (MRI). The ventricular cavity is normally diminished in size and the patients show increased fractional shortening. Loading conditions such as hypertension and valvular diseases must be excluded. In a minority of patients the hypertrophy progresses to ventricular dilatation and systolic dysfunction. The familial causes include mutations in several sarcomeric proteins such as cardiac troponin, titin, actin and α-tropomyosin. Few non-sarcomeric protein mutations have been found. Congenital syndromes, inherited metabolic disorders or neuromuscular diseases are to be found in young patients (Maron et al. 2006, Elliott et al. 2008).
Restrictive cardiomyopathy

RCM is characterized by increased myocardial stiffness, a normal LV wall thickness, impaired ventricular filling and normal systolic function. This is the least common type of cardiomyopathy. Familial forms of RCM have been reported, caused by the troponin or the desmin gene, and non-familial forms may result from systemic disorders such as amyloidosis, sarcoidosis or scleroderma or the use of anthracycline drugs (Maron et al. 2006, Elliott et al. 2008).

Arrhythmogenic right ventricular cardiomyopathy

ARVC an uncommon familial cardiomyopathy, affecting only 1 in 5000 in the general population. The right ventricle is affected, with progressive loss of cardiomyocytes and their replacement by fibrosis or fat and resulting in focal or global abnormalities. Patients have arrhythmias, syncope, cardiac arrest and global or segmental chamber dilatation or wall motion abnormalities. Diagnosis requires a 12-lead ECG, an echocardiogram, right ventricular angiography, cardiac MRI and computer tomography, in addition to a personal and family history. A myocardial biopsy is a sensitive means of diagnosing the fibrosis and fatty infiltrations. ARCV is a frequent cause of sudden cardiac death in young people in Italy. The Naxos and Carvajal syndromes, caused by mutations in genes encoding plakoglobin and desmoplakin, have been recognized, and other defects have been reported in genes coding for cardiomyocyte desmosomes (Maron et al. 2006, Elliott et al. 2008).

Unclassified cardiomyopathies

This group includes left ventricular non-compaction (LVNC), in which the myocardial wall is thickened and in some cases there is ventricular dilatation and systolic dysfunction, which can be transient in newborn infants. LVNC is sometimes associated with neuromuscular diseases. Takotsubo cardiomyopathy is characterized by transient regional systolic dysfunction, leading to angina-like chest pain in the absence of obstructive coronary disease. Symptoms often occur after emotional or physical stress and in most cases affect post-menopausal women (Maron et al. 2006, Elliott et al. 2008).
Dilated cardiomyopathy

DCM is defined by the presence of left ventricular dilatation with normal left ventricular wall thickness and left ventricular systolic dysfunction. Hypertension, valve disease, coronary artery disease or other diseases that lead to systolic dysfunction must be excluded. The prevalence is estimated to be 1 in 2500, making it the third most common cause of heart failure and the most common cause of heart transplantation (Maron et al. 2006, Elliott et al. 2008). 30–50% of the cases are thought to be familial (Fatkin et al. 2010). Mutations are found in genes coding for sarcomeric proteins, Z-band proteins, cytoskeletal proteins and the nuclear membrane (lamin A/C). A familial disease should be suspected in patients with a family history of sudden cardiac deaths, conduction system disease or skeletal myopathy (Elliott et al., 2008). Genetic linkage and candidate gene screening have resulted in the identification of nearly 40 chromosomal loci and disease genes in adult-onset DCM patients and their families, including the chromosomal locus 9q13-q22 (Krajinovic et al. 1995, Fatkin et al. 2010). Even though the inherited gene mutation is present from birth, the DCM phenotype appears later in adult life. This age-dependent penetrance suggests that there must be an accumulation of changes in the myocardium before the clinical threshold is reached.

The symptoms of DCM may include signs of heart failure, dyspnoea, fatigue, palpitations or peripheral oedema. Prodrome signs such as conduction abnormalities, atrial fibrillation and supraventricular arrhythmias can occur before clinical DCM occurs. Some families have a phenotype with mitral valve prolapse, or extracardiac findings such as skeletal myopathy or sensorineural hearing loss. The disease history varies depending on the response to medical treatment. Heart transplantation is needed in cases of progressive disease. Patients with conduction abnormalities can require a pacemaker. Sudden cardiac death can sometimes be the first sign of DCM (Fatkin et al., 2010). Clinical screening of unaffected family members (ECG and echocardiogram) have resulted in increased knowledge of the pre-stage of DCM. 29% of asymptomatic relatives have echocardiographic abnormalities and 27% of these develop DCM (Baig et al. 1998).
2.9.1 Pathological findings in dilated cardiomyopathy

The pathophysiology of DCM is characterized by an increase in the ratio of the left ventricular (LV) chamber radius to wall thickness, resulting in myocardial wall stress. This is thought to promote further dilatation. There are significant changes in the myocardial cells, cardiomyocytes and ECM, with the ECM alterations including changes in collagen fibril architecture, in that there are less collagen struts connecting adjacent cardiomyocytes and collagen cross-linking is reduced, rendering the collagen more easily degradable. These early changes in the ECM are likely to contribute to the progression of dilatation and dysfunction (Spinale 2007). In addition, several studies have shown that changes in collagen content, typically measured in terms of the collagen I/collagen III ratio, occur in DCM patients (Bishop et al. 1990, Marijjanowski et al. 1995). Collagen III forms an elastic network that stores kinetic energy, whereas collagen I is considered to be a stiffer fibrillar protein that is needed for tensile strength. An increase in the collagen I content leads to myocardial stiffness, compromising the diastolic and systolic ventricular functions in DCM patients (Pauschinger et al. 1999). Cardiac remodelling, whether detected as changes in gene or protein expression or in cellular and interstitial features, leads to changes in cardiac size, shape and function and occurs both in cardiomyopathy and in other pathological conditions (Cohn et al. 2000). Cardiomyocytes from patients with DCM show an increase in length without any increase in diameter. Under normal conditions the cardiomyocytes are connected laterally by collagen struts that are inserted into the BM and the cardiomyocyte sarcolemma to form an ECM-sarcolemmal-cytoskeletal network capable of rapidly transferring mechanical signals to the cardiomyocyte nucleus, resulting in myocyte growth in a longitudinal or transversal direction. In DCM, however, the cardiomyocytes do not respond with an appropriate increase in transverse area, probably due to changes in the ECM. A similar increase in the longitudinal direction is seen in early neonatal development. It has been shown that these long, slender cardiomyocytes have a reduced ability to shorten and develop force (Gerdes & Capasso 1995). Slippage of cardiomyocytes has been seen in end-stage DCM, and changes in cardiomyocyte orientation have been thought to be responsible for the enlargement of the LV chamber cavity (Beltrami et al. 1995). In addition to these structural alterations in cardiomyocyte orientation and shape, the contact sites of the cardiomyocytes are also affected, leading to changes in the intercalated discs. Force transmission between the cardiomyocytes occurs via the adherence
junctions, which are made up of transmembrane proteins belonging to the cadherin family. The electrical coupling is coordinated through gap junctions and mechanical stability is provided by desmosomes. There are several mouse models in which alterations in intercalated disc proteins have been shown to lead to dilated cardiomyopathy (Perriard et al. 2003), but very little data is available on intercalated discs in human DCM samples. The expression of metavinculin, an adherence junction protein, is down-regulated in some DCM patients, and the intercalated discs are reported to have been irregular and fragmented in a patient with a mutation in the metavinculin gene (Olson et al. 2002).

2.9.2 Mouse models for dilated cardiomyopathy

The number of mouse models for DCM is extensive. Animal models are valuable for studying early and pathological changes, and it is hoped that they will help to find DCM patients in early stages of the disease, before the heart has dilated and the patient has developed severe symptoms. Based on studies with mutant mice, human mutations have been identified in proteins such as the muscle LIM protein and the Cypher protein expressed in the Z-line of striated muscles (Sheikh et al. 2007). In addition, there are several mouse models for which no corresponding human mutations have yet been found. Mice over-expressing tropomodulin serve as a good model for the calcium overload that occurs in DCM, but these mice die 2–3 weeks after birth (Sussman et al. 1999). Mice lacking in thrombospondin-2 (TSP-2), a non-structural matricellular protein, regulating cell-matrix interactions, serve as a good model for studying age-related DCM, as they are healthy when young and the pathological changes accumulate during ageing. These null mice show increased inflammation and MMP-2 activity and decreased cross-linking, contributing to impaired cardiomyocyte survival and increased cardiac dilation with age. In addition, Sirius red polarization microscopy has revealed a looser mode of assembly of the collagen bundles (Swinnen et al. 2009). A mouse model that produces mutant collagen that is resistant to collagenase digestion has been generated to study the role of collagens in cardiomyopathy and heart failure. The mutant hearts of older mice showed increased fibrosis and collagenolysis and decreased cardiac contractility and the passive diastolic function. In addition, the mutant mice had hypertension and bradycardia and other conduction system failures leading to dilatation and heart failure (Miller & Tyagi 2002). So far very few human mutations in ECM proteins have been reported as leading to DCM, but those that have been mentioned include mutations in the BM component
laminin α4 chain in human DCM patients. Mice lacking the laminin α4 chain develop ischaemic cardiomyopathy with elevated levels of vascular endothelial growth factor and hypoxia-inducible factor 1α and malformed blood vessels with wide pericapillary ECM spaces, suggesting that microcirculation defects were the cause of the cardiomyopathy. In contrast to human patients, the mutant mice developed cardiac hypertrophy but no LV dilatation (Wang et al. 2006a).

2.10 Microvessel integrity

The capillaries in the heart are connected to the adjacent cardiomyocytes via collagen bundles or struts arising from the entire circumference of the capillary BM. These struts are inserted into the cardiomyocyte BM at the Z-lines, whereas they are inserted more tangentially in the capillary BM. The struts extending between the capillaries and the cardiomyocytes are longer than those connecting two cardiomyocytes, and it is thought that they are needed to keep the capillaries open at high pressure, as most of the myocardial capillary flow occurs during the systole (Caulfield & Borg 1979).

The impaired vessel integrity, fragile capillaries and leakage reported in human inheritable connective tissue disorders are attributable to mutations in genes encoding collagens I, III or V or enzymes responsible for the post-translational modification of these collagens. In Ehlers-Danlos syndrome (also described in section 2.4.3) the capillaries may be abnormal, with a lack of the normal perivascular collagen, resulting in poor support. Bruising, leakage and vessel ruptures are common in these patients. Patients with mutations in collagen IV (the COL4A1 gene) show porencephalic cavities in the brain, caused by haemorrhages, cerebral small vessel disease, an important cause of stroke, and vascular cognitive impairment, microbleeds, and also HANAC syndrome, with abnormal vasculature and haematuria (Lanfranconi & Markus 2010, Van Agtmael & Bruckner-Tuderman 2010). Kidney biopsies from these patients have revealed interruptions and thickening of the capillary BMs (Lanfranconi & Markus 2010). Easy bruising is occasionally seen in patients with osteogenesis imperfecta, due to mutations in collagen I, and in Marfan patients, with mutations in the fibrillin-1 gene, but as no fragility is seen in the capillaries, there could be other reasons for the bruises, such as thin skin or less subcutaneous fat (Malfait & De Paepe 2009). Moreover, several studies with mutant mice have shown that the lack of an ECM protein can lead to abnormal or fragile capillaries. Elimination of collagen (α1) IV in mice leads to an early lethal phenotype due to ruptures in Reichert’s membrane.
The blood vessels in these mice show abnormal branching and are fragile and leaky (Poschl et al. 2004). A missense mutation in the mouse Col4a1 gene leads to defects in both capillaries and large vessels. The BM composition is altered, causing detachment of endothelial cells in the aorta and affecting the vascular tone and blood pressure control. These mice also show microbleeds, probably reducing the erythrocyte count and blood volume (Van Agtmael et al. 2010). Mice lacking collagen XV have previously been reported to have structurally abnormal capillaries with degenerating endothelial cells. In addition, erythrocytes and thrombocytes were frequently seen in the extracellular space, indicating leakage from the microvessels (Eklund et al. 2001). Fibulin knock-out mice and mice lacking of perlecan in their endothelial BMs are reported to have an embryonically lethal condition involving dilatation and bleeding in the capillaries while the larger vessels remain normal (Hallmann et al. 2005). BM components such as the laminin α5 and α4 chains have been shown to be essential for vessel stability. As described previously (section 2.7.1), mice lacking laminin α4 experience haemorrhages postnatally and have a discontinuous endothelial cell BM. The subcutaneous bleedings are less prominent in the adult stage, but the mice still have dysfunctional microcirculation in the myocardium, leading to ischaemic cardiomyopathy (Thyboll et al. 2002, Wang et al. 2006a). Laminin α5 knock-out mice die before birth due to abnormalities in the placental and foetal vasculature. These animals, like those lacking in laminin α4, have increased lumen diameters (Miner et al. 1998).

### 2.11 Peripheral nervous system

The peripheral nervous system (PNS) consists of the nerves and ganglia that lie outside the central nervous system (the brain and spinal cord). The PNS transfers motor and autonomic signals from the central nervous system to the organs and limbs and sensory signals from back to the central nervous system. The neurons of the PNS and their specialized projections, or axons, carry electrical information rapidly over considerable distances. The axons can be up to 1.8 m in length (as in a giraffe) and are supported and protected by layers of connective tissue. The outermost layer on the peripheral nerves is a loose form of ECM called the epineurium, which has the function of connecting the nerve trunk to the adjacent tissue. Inside the epineurium the nerve is divided into fascicles, bundles of axons, each surrounded by an ECM layer called the perineurium, which is thought to act
as a selective diffusion barrier and protect the nerve against stretch. The endoneurium is the BM surrounding every single Schwann cell in the nerves (Bunge, 1993). The cell body of each neuron in the PNS is located in the ganglia, and when an axon reaches the end organ, the heart, muscle or skin, it branches into axon terminals, which form the synapses (Fig. 7). The synapses transform the signal to the individual target cell. Each axon is covered by Schwann cells, and those in myelinated axons form fatty myelin sheaths with short gaps, the nodes of Ranvier, along their whole length. Electric signals are transferred very fast in these myelinated axons, as impulses can cross the axon membrane only at the nodes of Ranvier. Motor axons and some of the sensory nerves are myelinated. Non-myelinated axons are also covered by Schwann cells but no myelin is formed, and several axons are grouped together by a single Schwann cell. In the non-myelinated axons the electric signals progress smoothly in continuous waves, resulting in a more slow signal transmission compared to myelinated axons. The non-myelinating axons, also called C-fibres or Remak-bundles, transfer temperature or pain sensations and autonomic functions (Hoitsma et al. 2004, Pocock & Richards 2001).

Fig. 7. Schematic presentation of a peripheral nerve. The cell body is located in the ganglia. The axon is covered by Schwann cells and the gaps between the Schwann cells are called nodes of Ranvier. When reaching its destination the axon is divided into several axon terminals.

2.11.1 Peripheral nerve development

The neural plate converts to a hollow neural tube that differentiates into the brain and spinal cords. Neural crest cells arise from the embryonic neural tube, detach
and migrate to various locations in the body, and the PNS ganglia are mostly derived from these neural crest cells (for a review, see Henderson & Copp 1997). During the development of peripheral nerves the neural crest cells generate Schwann cell precursors, which accompany and support the axons from the moment these project out from the periphery. Before myelination the immature Schwann cells form circles around the embryonic axon bundles. The Schwann cells that are in contact with large-calibre axons form myelin sheaths, while the small axons are grouped into C-fibers by one Schwann cell and no myelin is formed. After the Schwann cells have formed the BM, they start a radial sorting process. The BM is important both for this radial sorting and for the initiation of myelination, as the BM establishes an axis of polarity and define the orientation of the Schwann cells with respect to the extracellular environment and to the axonal membrane to be myelinated (for a review, see Simons & Trotter 2007).

In radial sorting (Fig. 8), each Schwann cell sends cytoplasmic processes into the axon bundle and segregates an axon into a 1:1 relationship (Webster et al. 1973). When the large axons have been segregated, a second group of Schwann cells associate with the rest of the axons in the bundle and form non-myelinating Schwann cells (Webster 1971). An excessive proliferation of Schwann cells takes place during radial sorting in order to achieve the correct number of cells to match the number of available axons (Martin & Webster 1973). After a Schwann cell has embraced an axon, it starts to form a specific lipid-rich myelin compound consisting mainly of cholesterol, phospholipids and glycosphingolipids together with the myelin basic protein (MBP) needed for the assembly of myelin. The myelin sheath is wrapped around the axon, resulting in a spiral membrane (for a review, see Simons & Trotter 2007).

Radial sorting followed by myelination continues until all the axons in the embryonic bundles have been segregated. In mice this lasts until around 3 weeks after birth (Webster et al. 1973), while in humans the radial sorting and myelinations of the peripheral nerve begin during foetal life and maturation continues during infancy and varies with age. The motor and sensory nerve conduction velocity at birth is a half of that in adults, but it increases rapidly during the first year of life and reaches adult values between 3 and 5 years of age. The increase in the conduction velocity during maturation reflects largely the increase in large myelinated fibers (García-García & Calleja-Fernández 2004).
Fig. 8. Development of the peripheral nerve. I) Before myelination the immature Schwann cells encircle the embryonic axon bundle. II) The Schwann cells start to send cytoplasmic processes into the axon bundle and segregate the larger axons one by one. When a Schwann cell has wrapped itself around an axon it starts to form myelin. III) Most axons are myelinated, but the Schwann cells form C-fibers from some small axons in the embryonic axon bundle and in these cases each Schwann cell process wraps itself around a group of axons.

2.11.2 Roles of BM components in peripheral nerve development

Segregation and myelination have been intensively studied in animal models and it has been suggested the BM or its components may have a crucial role in the initiation of these processes. The most extensively studied components are the laminins, which are thought to have a signalling capacity. Laminin-mediated signals regulate Schwann cell proliferation and survival and the actin cytoskeleton dynamics that are essential for radial sorting and myelination. Collagens and their receptors have been shown to promote neurite outgrowth and Schwann cell adhesion, spreading and myelination (for a review, see Chernousov et al. 2008).

Laminin

Laminins seem to have a crucial role in the Schwann cell proliferation that promotes the radial sorting process. The laminin α4 chain may also be critical for the segregation of single axons by Schwann cells or for controlling the initiation
of myelin formation, as these two functions fail in the absence of the α4 chain, resulting in polyaxonal myelination (Yang et al. 2005). Meanwhile, deletion of the laminin γ1 chain, which is present in most laminin heterotrimers, blocks the axonal sorting process almost completely, causing the mice to retain embryonic axon bundles in their peripheral nerves, so that they display tremor, muscle weakness and hind limb paralysis and most of them do not reach adulthood (Chen & Strickland 2003). Several mouse strains are available with mutations in the laminin α2 chain, which is a major component of the Schwann cell BM. The peripheral nerves of these mice contain embryonic non-segregated axon bundles, the BM is discontinuous, and the spinal roots and cranial nerves are more affected than the peripheral nerves (Yang et al. 2005). Mutations in the human LAMA2 gene cause about 1/3 of all cases of congenital muscular dystrophy, as described in more detail below.

**Laminin receptors**

In addition to laminins, the integrins that act as their receptors have been shown to affect peripheral nerve development. Conditional knock-out of Schwann cell β1 integrin results in inability of the Schwann cells to segregate axons in the peripheral nerve. These mice have a similar phenotype to those lacking the laminin γ1 chain (Feltri et al. 2002). Conditional knock-out mice lacking dystroglycan, another laminin receptor, in their Schwann cells are characterized by abnormalities in myelin sheath folding (Saito et al. 2003).

**Collagen**

An important role for collagens has been suggested in a recent *in vitro* study with co-cultures of Schwann cells and dorsal root ganglia, where collagen V (or more specifically its α4 chain) was lacking or its receptor, glypican, was down-regulated. Suppressed expression of either of these molecules leads to significantly inhibited myelination, *i.e.* less myelin segments could be detected in the co-cultures and a reduction in the steady state level of MBP was observed in an immunoblot analysis of lysates from the co-culture. The α4(V) collagen chain and glypican were localized in the tube-like matrix structures where myelination assembly occurred, suggesting that Schwann cell-ECM interaction is important for myelination (Chernousov et al. 2006). Unfortunately, no mutant mouse model with a peripheral nerve phenotype is available for collagen V. Collagen α1(V)
homozygous null mice succumb at the embryonic stage, while mice lacking the \( \alpha_3(V) \) chain have normal amounts of collagen V in their nerve tissue (Chernousov et al. 2008). In vitro studies suggest that collagen IV, a major BM component, can participate in Schwann cell differentiation and the axonal growth of peripheral neurons (Lein et al. 1991), but again total knock-outs are embryonically lethal and no conditional knock-out mice have reported to exist so far.

**Is an intact BM needed for myelination?**

Although it was previously thought that an intact BM is needed by the Schwann cells in order to form myelin, it has now been reported that double mutants lacking the laminin \( \alpha_4 \) and \( \alpha_2 \) chains show some myelination in the spinal roots despite the absence of a BM (Yang et al. 2005). In addition, mice lacking the laminin \( \alpha_4 \) chain have impaired segregation and myelination despite the presence of a continuous BM (Wallquist et al. 2005, Yang et al. 2005), and the peripheral nerves of mice lacking dystroglycan have intact BMs but abnormal myelination (Saito et al. 2003). These results suggest that myelination can occur in some instances under conditions of an apparent lack of any BM, at least as seen in EM analysis. It is likely that the BM components are still present but organized to a lesser degree than normal. This suggest that it is the interaction and signalling between the different BM components and the Schwann cells that is essential for myelination rather than proper assembly of the BM itself (Court et al. 2006).

### 2.12 Human peripheral neuropathies

Peripheral neuropathy may involve damage to a single nerve or nerve group (mononeuropathy) or affect multiple neurons (polyneuropathy). The symptoms depend on which nerve type is affected: sensory, motor or autonomic nerves. Damage to the sensory nerves results in changes in sensations, burning sensations, pain, tingling or numbness. When the motor nerves are affected it can cause muscle weakness or muscle control problems such as difficulties in breathing or swallowing, paralysis, falling, lack of muscle control, muscle atrophy and muscle cramp. If the autonomic nerves are involved, signalling to organs and glands will be affected, leading to symptoms such as heat intolerance, diminished ability to sweat, diarrhoea or constipation, urination difficulties, male impotence and dizziness due to abnormal blood pressure control. There are numerous causes of peripheral neuropathies, including hereditary ones, which will be described in
more detail later in this section. Other causes can be systemic diseases such as diabetes, cancer, dietary deficiencies, kidney diseases leading to uraemia and alcoholism. Infections and auto-immune diseases such as sarcoidosis, Sjögren syndrome, amyloidosis, syphilis and AIDS can cause nerve damage and neuropathies. Several drugs can cause neuropathy and injuries, and prolonged exposure to cold temperatures or prolonged pressure on the nerve can lead to neuropathy (Shy et al. 2007).

The hereditary neuropathies can be divided into those where the neuropathy is the major finding and more widespread neurological disorders or syndromes where other organs are also affected. The hereditary neuropathies can be classified into three groups; 1. hereditary motor and sensory neuropathy (HMSN), also termed as Charcot-Marie-Tooth disease, 2. distal hereditary motor neuropathy (dHMN), and 3. hereditary sensory and autonomic neuropathy (HSAN; for a review of hereditary peripheral neuropathies, see Reilly 2007).

**Hereditary motor and sensory neuropathy**

The most common group of hereditary peripheral neuropathies, HMSN, is characterized by distal muscle wasting and weakness, reduced tendon reflexes, impaired distal sensation and foot deformities, and by both sensory and motor nerve disturbances in neurophysiological tests. Mutations in 24 genes have been identified so far, including those coding for peripheral myelin protein, myelin protein zero, early growth response 2, neurofilament light protein, lamin A/C and many others (for a review, see Reilly 2007).

**Distal hereditary motor neuropathy**

The dHMNs resemble the HMSN but lack any sensory involvement (for a review, see Reilly 2007).

**Hereditary sensory and autonomic neuropathy**

HSANs are much rarer than HMSN and are characterized by distal sensory loss that predominantly affects the lower limbs and leads to recurrent injuries, ulcerations, osteomyelitis and the need for amputation. Autonomic disturbances such as sweating disturbances, postural hypotension and gastro-oesophageal reflux are common on account of autonomic nerve damage. Patients occasionally
complain of spontaneous shooting or lancinating pain. The peripheral sensory and autonomic nerves are mostly affected, but there is also motor involvement. HSAN I is characterized by autosomal dominant inheritance, while the other HSAN types are present at birth and are transmitted as autosomal recessive disorders. They affect both sexes, and the onset of the disorder is during the second or third decade of life, although it may occasionally start later. It often starts with a sensory loss, first affecting the toes but then spreading to the distal parts of the lower limbs. The severity of the disease varies from family to family. The clinical findings consist of changes in sensation, muscle strength and reflexes. Sometimes loss of pain and thermal sense is striking, suggesting that the small nerve fibres are mostly affected. Neurophysiological tests show nerve conduction in the lower limbs to be more affected than that in the upper limbs. The conduction velocity in the sensory nerves is more affected, and conduction can be totally absent in the sural nerve. Conduction velocities in the motor nerves are slightly reduced, and the compound motor action potential amplitudes are reduced. Common to all HSAN patients is the lack of a normal axon flare in a histamine test. When 0.1 ml of histamine is injected intradermally into a normal subject, the skin capillaries become vasodilated and a bright red flare is observed within 5 minutes. The dysfunctional C-fibers alter the axon reflex in the dermal nerves of HSAN patients, however, so that no flare is seen. The small fibers in sural nerves may be more involved than the large fibers (for reviews, see Axelrod & Gold-von Simson 2007, Auer-Grumbach 2008).

**HSAN I**

HSAN disorders are divided into subgroups, where each disorder is likely to be caused by different genetic errors that primarily affect small fibre nerve development. Rotthier et al. sequenced seven known mutation-causing genes in 100 HSAN patients and found mutations in 4 of these, namely SPTLC, RAB7, WNK1/HSAN2 and NTRK1, in a total of 19 patients. Thus the currently known disease-causing genes explain at most 19% of the genetic causes of HSAN (Rotthier et al. 2009). Three genetic loci 9q22.1-22.3, 3p22-24, and 3q21 and mutations in 2 genes have been identified so far in the HSAN I subgroup (Nicholson et al. 1996, Dawkins et al. 2002, Klein et al. 2005). The genetic loci 9q22.1-22 were identified by means of linkage studies in four Australian kinships with the HSAN 1 disorder (Nicholson et al. 1996). The SPTLC gene, coding for serine palmitoyltransferase, explains many, but not all of the mutations in this
region, as 11 out of 24 families examined were found to carry mutations in this gene (Dawkins et al. 2001).

### 2.12.1 Congenital muscular dystrophy with peripheral neuropathy

Given that the hereditary neuropathies are divided into primary neuropathies and more widespread neurological disorders or syndromes in which other organs are also affected, it should be noted that a mutation in the LAMA2 gene, coding for the laminin α2 chain, leads to congenital muscular dystrophy, characterized by muscle necrosis, muscle atrophy and regeneration, reduced peripheral motor nerve conduction velocity and a thinning of the white matter in the brain (Helbling-Leclerc et al. 1995, Shorer et al. 1995, Geranmayeh et al. 2010). As the laminin α2 chain is expressed in muscle BMs, the neuromuscular synapses, Schwann cells and the brain, the disorder not only affects the peripheral nerve but rather is a more widespread syndrome with several organs affected.
3  Outlines of the present study

There is no human disease to date that is known to be caused by a mutation in the COL15A1 gene. The primary structure, distribution in developing and adult tissues and localization of collagen XV are well known, and by the time the present work was commenced previous successful attempts at generating and analysing a mouse model lacking in collagen XV (Col15a1−/−) had revealed an important function for this collagen in capillaries and skeletal muscle. A detailed study of its function at the BM-fibrillar matrix interphase was lacking, however, even though previous research had suggested that it might serve as bridge between the BM and the fibrillar matrix and it had been suggested on the grounds of the Col15a1−/− mouse phenotype that collagen XV could be needed to provide structural strength for the capillaries and myocytes. The high level of collagen XV expression in the heart prompted us to study the functioning of the heart in more detail in these null mice and to look for similarities to human cardiac pathologies. In particular, the finding that collagen XV has a role in organizing the fibrillar collagen matrix prompted us to study the effects of induced cardiac load on Col15a1−/− mice and to investigate the possible development of hypertrophy and fibrosis. When the present research started, we considered the Col15a1−/− mouse phenotype to be so mild that generating a double knock-out mouse with a simultaneous lack of the laminin α4 chain and collagen XV could provide important new information on the roles of these ECM molecules. The rationale for generating this double mutant line lay in the similar tissue distribution of the two molecules. Both single knock-out mice were viable, and the double knock-out mice also proved to be viable, with a normal lifespan, but they were afflicted with severe motor defects. As both molecules were expressed in the peripheral nerves, we set out to analyse the development of these. At that stage there were no reports of impaired peripheral nerve development in single laminin α4 knock-out mice. Altogether, the general aim of this work was to gain a better and more detailed understanding of the biological function of collagen XV in the matrix of certain tissues, the heart, microvasculature and peripheral nerves, and thereby to evaluate its potential roles in human pathological conditions. The specific aims were:

1. to investigate the function of collagen XV in the BM-fibrillar matrix interplay occurring in developing and mature tissues and under circumstances of collagen remodelling,
2. to analyse the consequences of a lack of collagen XV in the murine heart and vasculature, and
3. to generate a mouse line simultaneously lacking in collagen XV and the laminin α4-chain and to evaluate the function of each molecule and their combined effects on peripheral nerve development and function.
4 Materials and methods

The material and methods used in this thesis are summarized in Table 3. Detailed descriptions with references can be found in the original papers I-III.

Table 3. Material and methods.

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5 Results

5.1 Ultrastructural analysis of the roles of collagen XV in the BM – fibrillar matrix interphase

In view of the reported localization of collagen XV both in association with BMs and adjacent to pericellular collagen fibrils, we decided to analyse closely the cell-matrix interphase in Col15a1<sup>−/−</sup> mice. This mutant mouse line had been generated earlier (Eklund et al. 2001). In the peripheral nerves ultrastructural analysis of 15-day-old sciatic nerves from Col15a1<sup>−/−</sup> and age-matched WT controls revealed smaller collagen fibrils with rough surfaces in the mutants (I, Fig. 1a-b). The mean collagen fibril diameter was 28.34±3.2 nm in the WT nerves and 25.58±3.8 nm in the Col15a1<sup>−/−</sup> nerves (means±S.D. p =0.001), and when the fibrils were grouped according to size we could see a negatively skewed distribution in the mutants (I, Fig. 1g). Also, the bundles of collagen fibrils had a more disorganized appearance, and the inter-fibrillar spaces were increased (32.8±10.0 nm and 22.3±5.8 nm for Col15a1<sup>−/−</sup> and WT, respectively, means±S.D. p =0.001, I, Fig. 1h). Interestingly, these findings were not permanent, as after the segregation of axons and myelination had concluded at the age of 1 month no difference in collagen fibril diameter or organization could be seen between the controls and mutants. Instead, the collagen fibrils in the Col15a1<sup>−/−</sup> nerves seemed to be directly adhering to the BM, while in the WT nerves a distance of 100–200 nm could always be distinguished between the BM and fibrillar collagens when the area analysed was a perfect transverse cut (I, Fig. 1c-d). There was an increase in collagen fibrils in the ECM of the peripheral nerves in the 1 and 2-year-old WT mice, whereas no comparable age-dependent fibrosis could be seen in the Col15a1<sup>−/−</sup> nerves (I, Fig. 1e-f). Instead the collagen fibrils were seen to be aggregated close to the BM in the same manner as at 1 month of age. These findings suggest that collagen XV has specialized roles in the organization of the fibrillar collagens during the nerve development while in the mature nerve it is needed to keep the collagen fibrils at a distance from the BM.

Skeletal muscle was analysed to see whether similar packing of collagen fibrils at the BM surface would occur as in the sciatic nerve. Myocytes of 3-month-old WT mice were covered by a thin BM, and very few collagen fibrils were seen in the adjacent ECM (I, Fig. 2a). In contrast, the myocyte BM in the Col15a1<sup>−/−</sup> skeletal muscle was covered with densely packed collagen fibrils, rendering the BM
sometimes impossible to recognize (I, Fig. 2b). The amount of collagen fibrils increased with age, as they were abundant in the matrix of the 1-year-old WT skeletal muscle, although occurring at a distance from the BM (I, Fig. 2c). There was no apparent increase in the number of collagen fibrils in the old Col15a1−/− muscle, and those seen were still adhering to the myocyte BM, as in the younger null mice (I, Fig. 2d). These findings regarding collagen fibril organization in skeletal muscle are in accordance with the findings in the peripheral nerves, and taken together they suggest that collagen XV is needed in the BM-fibrillar matrix space, possibly to link the fibrillar collagens to the BM, but at the same time to build the 3D matrix architecture in such a way that the collagen fibrils are not packed together, glued and bound to the BM.

Analysis of matrix network in the absence of collagen XV by scanning electron microscopy

We used SEM to obtain a better understanding of the effects of the lack of collagen XV on collagen fibril organization and matrix architecture. Both sciatic nerves and skeletal muscle from 2-month-old mice were analysed. In the control nerve the axons were covered by thick fibrillar collagen bundles, and often wavy bundles were seen to connect the axons to each other, whereas in the Col15a1−/− nerve the axons were covered with fibrillar collagen bundles that were looser in appearance, and there were larger numbers of separate, single collagen fibrils between the axons (I, Fig. 3a-b). Even though there seems to be a similar amount of fibrillar collagen in the Col15a1−/− nerves, the organization of the collagen bundles is disturbed and as a result there is a very loose bundle structure compared with the wavy, densely packed bundles seen in WT (I, Fig. 3c-d).

The collagenous ECM is less extensive in the skeletal muscle of WT mice than in the peripheral nerves; there is a delicate network of thin collagen fibrils associated with the skeletal myotubes and the bundles connecting the individual myotubes are thinner (I, Fig. 3e). This evenly distributed fibrillar collagen network is largely lacking in the Col15a1−/− muscle and is replaced by a coarser network of thick, densely packed, aggregated collagen bundles (I, Fig. 3f). Only a few large collagen bundles were detected in WT as compared with Col15a1−/− (I, Fig. 3g-h). The SEM analysis coincides with TEM findings in which we could see that the Col15a1−/− myotubes are covered with densely packed collagen bundles that are in close contact with the BM. Altogether the SEM analysis highlights the
distinct nature of the fibrillar collagen network in the ECM of the two tissues, albeit in both cases the lack of collagen XV results in an altered fibrillar matrix.

**Role of Collagen XV in collagen remodelling in the uterus**

Implantation of the embryo requires extensive adaptations of the uterine microenvironment. It has been shown that lateral fusion of collagen I and III fibrils to form “thick fibrils” occurs in the decidualized area of the endometrial stroma after the 6th day of pregnancy (d.o.p.) (Spiess & Zorn 2007). Previous studies have shown that collagen XV is known to occur in large amounts in the placenta (Muragaki et al. 1994, Myers et al. 1996). Considering the robust collagen remodelling process that takes place in the uterus during pregnancy, it was attractive to study the endometrium at 7 d.o.p in Col15a1+/− and WT mice. Many of the fibrillar collagen bundles in the decidualized area of the Col15a1−/− endometrium were cauliflower-like in appearance in transverse sections (I, Fig. 4b), and when the fibrils were oriented in a longitudinal fashion it appeared that this abnormal shape resulted from incomplete lateral fusion of the small collagen fibrils to form thick fibrils. Moreover, many of the thick collagen fibrils in the Col15a1−/− mice had frayed ends (I, Fig. 4d). In contrast, most of the thick fibrils in the WT mice were fused completely, and only few were seen to divide into smaller fibrils at the ends (I, Fig. 4a and c). Nevertheless, the mean diameter of the thick fibrils (collagen fibrils measured in the decidualized area) was the same in both genotypes, namely 143.8±33.6 nm in WT and 141.7±16.1 nm in the Col15a1−/− decidualized endometrium, means±S.D., p = NS.

Closer examination of the thick WT collagen fibrils showed delicate strands binding them to each other and to the cells (I, Fig. 4e). Each strand was located at an exact distance from the previous one, coinciding with the electron-dense bands (d-bands) of the thick collagen fibril. In contrast, these delicate strands were essentially lacking from the thick collagen fibrils in the Col15a1−/− mice chains (I, Fig. 4f). These interesting findings prompted us to examine the ultrastructural localization of collagen XV in the endometrium. Immunoelectron microscopic (IEM) signals for collagen XV were found in association with the thick collagen fibrils as well as with the BMs and non-collagenous microfibrils (I, Fig. 5). Interestingly, the large fibrils located close to the BM showed more IEM labelling than the fibrils in more distant parts.
5.2 Changes in the Col15a1−/− heart extracellular matrix

Ultrastructural analysis revealed that, in addition to the fibrillar collagen matrix, a small amount of non-fibrillar proteins was observed in the cardiac interstitium of the young WT mice (1 month of age), which was then reduced in amount during maturation and increased again in the older mice (Fig. 9a and c). These non-fibrillar protein deposits were more readily detectable in the interstitium of the young Col15a1−/− mice (1 month of age), and in contrast to WT, they accumulated extensively during ageing (Fig. 9b and d). The non-fibrillar protein deposits contained ECM and serum proteins, including fibronectin, collagen VI and fibrin (II, Online Fig. 1). In the case of fibrin, increased perivascular staining was detected in the Col15a1−/− by comparison with the WT LVs (II, Online Fig. 1e-f). The structure and distribution of the fibrillar collagen matrix was further studied using SEM, in which the fibrillar collagen bundles in the WT mice were seen to have formed characteristic waves and struts covering the cardiomyocytes (Fig. 9e). In contrast to the well-organized pattern in WT, the fibrillar collagen bundles in the Col15a1−/− endomysium were loose and disorganized (Fig. 9f). A lower proportion of thick collagen bundles in the Col15a1−/− LVs were also evident in picrosirius red stained samples analysed by polarized light microscopy (II, Online Fig. 2a-b). The mean green / red ratios (representing thin and thick collagen fibrils, respectively) were 1.10±0.06 for WT and 1.41±0.11 for Col15a1−/− (means±S.E.M., p < 0.01) at age 5 months and 1.18±0.04 and 1.25±0.07 respectively at 1 month (means±S.E.M., p =NS, II, Online Fig. 2c). These data indicate that a lack of collagen XV in the heart is not pro-fibrotic under unstressed conditions, but rather the fibrillar matrix is poorly organized in the absence of collagen XV and contains marked interstitial deposition of non-fibrillar protein aggregates. Longitudinal analysis suggested an ageing-dependent accumulation of non-fibrillar protein deposits and some alteration in fibrillar collagen phenotypes.

To test whether collagen XV deficiency and the observed changes in the matrix affect cardiac elasticity, stiffness measurements were performed on the WT and Col15a1−/− LVs using a microprobe indenter device. 2 mm slices were indented in steps using a needle probe indenter and the stiffness was calculated from plots of force in kPa vs. indentation depth. The stiffness of the Col15a1−/− LV was 27.1±1.4 kPa, while the value in the WT mice was 21.4±2.3 kPa (means±S.D. p <0.05), indicating an increase in myocardial stiffness in the Col15a1−/− mice (Fig. 9g). This observation was verified by using liver as a softer, collagen XV-negative control tissue. The WT and Col15a1−/− mice had virtually equal stiffness in the
liver, the mean force being 11.5±0.4 kPa and 11.2±0.5 kPa (means±S.E.M.), respectively.

Fig. 9. Heart Non-fibrillar protein deposits, poorly organized collagen bundles, and increased stiffness in the Col15a1−/− LV. The cardiac interstitium of young (1 month of age) WT (A) and Col15a1−/− (B) mice contains non-fibrillar proteins, marked with asterisks. That of 1 year old WT mice (C) contains a higher amount of fibrillar collagen
(arrows), while non-fibrillar protein deposits (asterisks) fill the perivascular space in the Col15a1−/− heart (D). CM indicates cardiomyocytes and the arrowheads in B and D point to oedematous cardiomyocytes. The inserts in C and D show the boxed areas at a higher magnification. SEM of a 5 month old WT heart (E) shows fibrillar collagen organized into bundles of various sizes (arrowheads) covering the cardiomyocytes. The collagen bundles are poorly organized and thinner in the Col15a1−/− heart (F, arrowheads). Cardiac elasticity measurements using a needle probe indenter revealed a 22.0% increase in stiffness in the Col15a1−/− LVs relative to WT (G). * P<0.05, means±S.D). Reprinted by permission of Wolter Kluwer Health.

5.3 Microvessels and circulation in the Col15a1−/− mice

In the initial characterization of the Col15a1−/− mice structural and morphological changes in the microvessels and ischaemic damage in the cardiomyocytes had been reported, including endothelial cell degeneration and swelling of the heart capillaries (Eklund et al. 2002). To study the heart microvessels in more detail, a SEM analysis was carried out. The capillaries in the WT mice showed intact surfaces, whereas in the mutant mice there were frequent ruptures in the capillary walls, observed in 10 to 20% of the capillaries analysed (Fig. 10a-d). In addition, the mutant capillaries were tortuous and showed variations in thickness (Fig. 10d). In addition to the structural abnormalities detected by SEM, we could see extravasated erythrocytes and ischaemic damage in some of the cardiomyocytes, as revealed by TEM. To investigate whether the vascular patterning and ruptures were associated with poor vascular maturity, including defective perivascular cell recruitment, LV whole mounts were immunostained with antibodies to CD31 and NG2 to detect endothelial cells and pericytes, respectively. Similar to the observations in SEM, the vessel pattern in the Col15a1−/− samples was disorganized, with irregular alignment of the capillaries as compared with that in the WT mice, although the mutant microvessels showed a normal pericyte covering, suggesting normal maturation (Fig. 10e-f). Quantification of the capillary density using histological sections stained with CD31 showed the Col15a1−/− mice to have a 32% increase in capillary density compared with the WT (p <0.01) at 5 months of age (Fig. 10g). The increased capillary number in Col15a1−/− was found to be age-dependent, since the capillary densities in the 1–month-old Col15a1−/− and WT mice were similar. (Fig. 10g).

To investigate the potential involvement of the larger vessels, namely the aorta and coronary arteries, in the Col15a1−/− cardiac phenotype, WT tissue samples were first stained with collagen XV antibodies. In contrast to the
endothelial BM location in the capillaries, collagen XV was found to be located in the outermost connective tissue layer (the tunica adventitia) in the aorta and the coronary arteries (II, Online Fig. 3a-d). The lack of collagen XV immunoreactivity in the endothelial BM in WT was in line with the observation that the endothelial cell layer in the Col15a1−/− arteries and aorta was intact (II, Online Fig. 3e-h). Moreover, analysis of the levels of Bnip-3 and Glut-1 mRNAs, known markers of an ischaemic myocardium due to coronary artery occlusion (Brosius et al. 1997, Regula et al. 2002), showed no differences between the genotypes (II, Online Fig. 3i-j). These data suggested an important role for collagen XV, especially in the capillary BM underlying the endothelial cell lining. The defect in capillary structure was not sufficient to induce marked ischaemic damage at the level of the whole LV, however.

These morphological alterations suggested dysfunctional microvessels in the Col15a1−/− mice. Our previous immunostaining studies had indicated that collagen XV is deposited around the capillaries of adults in specific vascular beds, including the heart and the skin (Muona et al. 2002). To investigate microvascular function in vivo, intravital microscopy, which readily allows analysis of the superficial vasculature, as in the skin and brain, was used with living mice. A decrease in the capillary perfusion index, reduced red blood cell velocity and a lower microvascular blood flow rate were observed in the Col15a1−/− skin, indicating decreased microcirculatory function (II, Fig. 3). Furthermore, increased microvascular permeability was observed in the Col15a1−/− mice (II, Fig. 3d). The quantified results regarding capillary density in the skin show no differences between the WT and Col15a1−/− mice (II, Fig. 3e). Since previous studies have indicated that collagen XV occurs in only a few larger capillaries in the adult brain (Muona et al. 2002), the brain was used as a control for the haemodynamic abnormalities observed in the Col15a1−/− skin. As expected, the lack of collagen XV did not alter the functional parameters of the cerebral microcirculation as visualized through the cranial window (II, Online Fig. 4 and 5).
Fig. 10. Ruptured and deranged capillaries in the Col15a1−/− LVs. SEM of the cardiac vasculature shows intact capillaries located between the cardiomyocytes in WT (A and C), but frequent ruptures in the capillary wall in the Col15a1−/− samples (B and D). In WT the capillaries are aligned with the myocytes (C), while in Col15a1−/− they are
tortuous and vary in thickness (D). Intact capillaries are marked with arrows in A-D and ruptures in the capillaries with arrowheads. Vascular patterning and pericyte recruitment were studied using CD31 and NG2-stained LVs from WT (E) and Col15a1−/− (F) mice. The double labeling shows that the pericytes (NG2, red) are covering the capillaries (CD31, green) in both the WT and Col15a1−/− hearts. The vascular pattern is disorganized, however, and shows increased variation in the diameter and alignment of the microvessels in the Col15a1−/− heart. The capillary density / microscopy field 0.06 mm² (40x) is higher in 5 months old Col15a1−/− (G, black bar) than in the WT (G, white bar) heart, at 1 month no difference was seen between the genotypes (**P<0.01, means±S.E.M.). Reprinted by permission of Wolter Kluwer Health.

Echocardiographic analysis of Col15a1−/− heart function

We used echocardiographic and telemetric analysis to investigate cardiac structure and function, heart rate and blood pressure in living mice. Echocardiography revealed a smaller cardiac size in the aged Col15a1−/− mice (14 months). This was associated with a thinner interventricular septum and posterior wall of the Col15a1−/− LV than in the WT mice (II, Table 2, Online Table 1 and Online Fig. 9). No differences in LV volume or in systolic or diastolic function were observed between the aged Col15a1−/− and WT mice (14 months), however. For a longitudinal view, cardiac function was analysed at 1 and 7 months of age (II, Online Table 1 and Online Figure 9). Systolic dysfunction was observed in the juvenile Col15a1−/− mice (1 month old), i.e. a reduced ejection fraction (EF: 53.6±0.1 vs. 65.0±3.1 in WT, means±S.E.M., p <0.05) and fractional shortening (FS: 27.0±0.6 vs. 34.9±2.3, p <0.05). Likewise, the echocardiographic measurements revealed altered LV geometry in the Col15a1−/− mice at this age, including an increased LV end diastolic dimension (LVEDD: 2.6±0.1 mm vs. 2.1±0.1 mm, P<0.05) and volume (LV Vol: 25.3±1.8 μl vs. 16.7±2.8 μl, p<0.05), and a decrease in the thickness of the LV posterior wall in the systole (LVPW: 0.7±0.04 mm vs. 0.8±0.03 mm, WT and Col15a1−/−, p <0.05). These abnormalities in heart function and dimensions in the Col15a1−/− mice were substantially improved at the age of 7 months. Collectively, the longitudinal echocardiographic analysis revealed an early-onset cardiac phenotype suggestive of DCM, recovery of LV function and geometry with time, and age-dependent thinning of the LV wall in Col15a1−/− mice.
5.4 Alterations in heart weight, cellular architecture and intercalated discs in the Col15a1−/− left ventricle

Cardiac mass was followed during ageing from 19 to 78 weeks (II, Table 1). The LV and total heart mass and their ratio to body weight were unchanged in the young (average age 7 months) and middle-aged (11 months) Col15a1−/− mice relative to the WT mice, but in the older mice (16 months) the cardiac weights were found to be lower in the Col15a1−/− individuals. Echocardiography confirmed the smaller cardiac size in the aged Col15a1−/− mice as described earlier (previous section). Since several mechanisms for ventricular thinning have been proposed, such as myocyte loss and atrophy, and alterations in cellular architecture, including an increase in length, variability in size and lateral slippage of cardiomyocytes leading to cellular misalignment (Beltrami et al. 1995, Schaper et al. 1995), we investigated the cellular mechanisms that may underlie the lower weight and thinner LV wall in the Col15a1−/− mice further by measuring the numbers of cardiomyocytes and their dimensions (diameter, length and shape).

No statistically significant difference in cell density (61.0±4.7 cells per field for Col15a1−/− vs. 59.8±6.3 for WT) or myocyte diameter (14.79±1.4 μm vs. 14.82±1.3 μm) was observed, indicating that the thinner Col15a1−/− LV in the old mice (12–14 months) does not result from smaller cell size (II, Fig. 4b). Instead, the Col15a1−/− LV in the 1-month-old mice showed decreased cardiomyocyte diameters (11.8±0.2 μm) relative to WT (12.5±0.1 μm, means±S.E.M., p < 0.01), but no change in cell density (93.0±4.5 and 93.0±4.0 cells per field, respectively, II, Fig. 4a). To study the cellular organization of the cardiomyocytes, 50 μm LV sections were stained with laminin β2 (BMs) and N-cadherin (intercalated discs) to visualize the lateral sides of single myocytes and their cell-cell junctions, followed by optical sectioning with confocal microscopy (II, Fig. 4e-f). No change in the number of intercalated discs was observed between the genotypes in the old mice (12–14 months), although the Col15a1−/− intercalated discs were shorter, (42.7±2.9 μm as compared with WT 52.8±2.2 μm, means±S.E.M., p <0.05), fragmented and unevenly distributed along the cardiomyocytes (II, Fig. 4c-d). Moreover, the cardiomyocytes in the Col15a1−/− samples were found to be irregularly shaped and elongated (II, Online Fig. 6). For comparison, cellular organization was studied in juvenile mice at the age of 1 month. Here the spatial organization of cardiomyocytes was normal in the Col15a1−/− individuals, although the cardiomyocytes were shorter than in WT (187.0±4.9 μm vs. 210.4±4.0 μm for WT, means±S.E.M., p<0.001). In addition, the Col15a1−/− mice
had shorter intercalated discs (39.04±0.37 μm vs. 42.83±0.38 μm in WT, means±S.E.M., p <0.001, II, Fig. 4d). Ultrastructural analysis revealed fragmented and irregular intercalated discs, and specifically disarrangement of the filaments at the adherence junctions, in the Col15a1−/− LVs as opposed to the continuous, well-defined intercalated discs and adherence junctions in the WT (II, Fig. 4g-h). Collectively, these data indicate that the early-onset defects (at age 1 month) due to a lack of collagen XV included smaller cardiac myocytes and shorter intercalated discs. Interestingly, a reversal in cardiomyocyte size was concurrent with an improvement in cardiac function and dimensions and an increase in capillary density, while an altered cellular organization of the cardiomyocytes emerged with increasing age.

5.5 Col15a1−/− heart under hypertrophic conditions

To study the Col15a1−/− and WT cardiovascular systems under acute loading conditions, we next used two well-characterized models for experimental hypertension, namely angiotensin II (Ang II)-induced pressure overload and nitric oxide (NO) inhibition with Nω-nitro-L-arginine methyl ester (L-NAME). In addition to raising the peripheral resistance through vasoconstriction, Ang II also has direct hypertrophic effects on cardiomyocytes (Sadoshima & Izumo 1993) and marked profibrotic effects in the heart (Gonzalez et al. 2002), while L-NAME primarily induces sustained systemic arterial hypertension by inhibiting NO synthesis in vascular endothelial cells (Gardiner et al. 1992). To gain insights into the role of collagen XV in experimental hypertension, we first measured its expression in sham-operated and loaded WT mice. The left ventricular collagen XV/18S RNA ratio was elevated 3.2±0.4-fold after a 3-day Ang II treatment (p <0.001 vs. sham), while L-NAME increased the left ventricular collagen XV/18S RNA ratio 1.34±0.09- and 1.87±0.10–fold after 3 days and 2 weeks of treatment, respectively, as compared with sham-operated control mice (p <0.05 and p <0.001 vs. sham. II, Fig. 5a-b). Ultrastructurally, collagen XV accumulated along the fibrillar collagens in the ECM and in the non-fibrillar proteins between the cardiomyocytes and endothelial cells (II, Fig. 5c-d). These data indicated that expression of collagen XV is up-regulated under hypertensive conditions and that it is deposited in the interstitium and associated with the fibrillar collagen matrix.

As the fact that Col15a1−/− cardiac weight did not follow the WT pattern in an unstressed state could be due to either a lack of sufficient growth stimuli or low responsiveness to the stimuli, we tested cardiac growth responses under
hypertensive conditions following Ang II and L-NAME loading. Three days of Ang II treatment increased the LV / bodyweight (LV/BW) ratio in the WT mice by 55±4% (means±SD), and a comparable increase, 54±6%, was observed in the Col15a1⁻/⁻ mice (II, Fig. 6a). Furthermore, an increase in LV/BW was observed after 3 days of L-NAME treatment in both the Col15a1⁻/⁻ and WT mice (3±2% and 3±3%), and the values remained elevated at 2 weeks (10±4% and 10±3%, WT and Col15a1⁻/⁻ mice, respectively. II, Fig. 6a). Cardiac dimensions and function under hypertensive conditions were investigated by means of echocardiographic measurements (II, Table 2). Consistent with the gravimetric data, echocardiographic measurements revealed an increase in LV weight that was evident in the Col15a1⁻/⁻ mice as compared with the WT mice, the change from the baseline being even higher in the Col15a1⁻/⁻ mice than in the WT ones. This indicated that although cardiac weight in the Col15a1⁻/⁻ mice did not follow the normal pattern of development during ageing, these animals reacted normally to a hypertrophic stimulus, i.e. cardiac expression of collagen XV is not necessary for hypertrophic responses. Interestingly the heart rate that was higher in the Col15a1⁻/⁻ mice in basal conditions (333±26 vs. 285±60 in WT, means±SD, not statistically significant) did not elevate similarly under the hypertrophic stimulus in the Col15a1⁻/⁻ mice, the change from baseline being only 21±5% in Col15a1⁻/⁻ mice vs. 49±9% in WT (means±SD, p <0.05). To investigate cardiac loading and hypertrophic responses in more detail, we measured mRNA levels of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), widely used markers of stretch-induced cardiac loading (Chien et al. 1993). The ratios of ANP mRNA (0.127±0.016 and 0.156±0.023 in WT and Col15a1⁻/⁻, respectively, means±S.E.M.) and BNP mRNA to 18S mRNA (0.248±0.052 and 0.275±0.035 in WT and Col15a1⁻/⁻, respectively, means±S.E.M.) did not differ significantly between the unstressed WT and Col15a1⁻/⁻ mice (II, Fig. 6b-c). Three-day treatment with Ang II led to 21.0±2.0 and 11.7±1.8 -fold induction of the ANP and BNP mRNAs relative to 18S mRNAs in the LVs of the WT mice, while the induction of ANP and BNP gene expression in the Col15a1⁻/⁻ mice was attenuated by 30±7% and 47±6%, respectively, relative to WT (II, Fig. 6b-c). These findings suggest that Ang II induces different loads in Col15a1⁻/⁻ than in WT mice, or that Col15a1⁻/⁻ hearts are unable to respond to Ang II-induced loading in the normal way. To test for equal loading, α-SKA mRNA levels were measured (Stilli et al. 2006). These were found to be similar at the basal level (αSKA mRNA to 18S mRNA 0.096±0.012 and 0.110±0.017 in WT and Col15a1⁻/⁻.
respectively, means±S.E.M) and to be elevated to the same degree in both in response to Ang II.

*Experimental hypertension leads to an altered ECM response in Col15a1−/− mice*

The observed induction of collagen XV expression and its accumulation in the outermost layer of the BM and in the interstitial collagen fibrils near the BM after a hypertrophic stimulus, combined with the morphological alterations observed in the fibrillar collagen matrix, suggested a role for collagen XV in fibrillar matrix remodelling. To ascertain whether the ultrastructural changes observed in unstressed mice are associated with altered expression of fibrillar collagens, and whether collagen XV deficiency predisposes the individual to fibrosis under loading conditions, we measured collagen I and collagen III mRNAs, which represent the most abundant ECM proteins in the heart and are hence widely used as markers of cardiac fibrosis (Weber 1989). We also examined the ratio between them, as an indicator of myocardial stiffness and a marker of cardiac dysfunction (Pauschinger et al. 1999). The mRNA levels of collagen I (ratio of Col1a1 mRNA to 18S mRNA; 0.291±0.051 and 0.304±0.049 in WT and Col15a1−/−, respectively) and collagen III (ratio of Col3a1 mRNA to 18S mRNA; 0.190±0.024 and 0.215±0.026 in WT and Col15a1−/−, respectively, means±S.E.M.) were similar in the two groups at the basal level (II, Fig. 6e). Although both collagen I and collagen III expression was induced in the Ang II-treated WT and Col15a1−/− mice, the induction of collagen III expression was blunted in Col15a1−/−, leading to an abnormally increased collagen I/III ratio (II, Fig. 6e). Consistent with the mRNA data, collagen staining with picrosirius red analysed by bright field microscopy did not reveal any quantitative differences in total fibrillar collagen content between the Col15a1−/− and WT hearts at the basal level or after two weeks of Ang II treatment. Nevertheless, picrosirius red staining followed by polarized light microscopy demonstrated a striking qualitative difference in the collagen phenotype between the genotypes after Ang II treatment, with a slight increase in the green / red ratio (small / large fibrils) in the WT mice relative to sham (+10.5±7.5%), while in the Col15a1−/− mice this ratio decreased (-30.3±2.6%, means±S.E.M., p <0.001. II, Online Fig. 8a-d). In contrast to the total amount of fibrillar collagens, accumulation of the non-fibrillar protein was strongly induced in the Col15a1−/− hearts after Ang II treatment (II, Online Fig. 8e-h). Taken together, these findings suggest that collagen XV plays a role in matrix
remodelling in the heart and participates in the organization of the collagen fibrils. Furthermore, in terms of the quality of the fibrillar collagen matrix, the Col15a1−/− mice responded abnormally to increased haemodynamic loading, which may predispose a subject to pathological responses under cardiac stress.

5.6 Peripheral nerve development in Col15a1−/− and Lama4−/− mice

Another tissue with high collagen XV expression is that of the peripheral nerves, and thus we studied the effects of a lack of this collagen in sciatic nerves. Collagen XV and another BM-associated molecule, the α4 chain of laminin, have similarities in their tissue expression patterns (Iivanainen et al. 1997, Muona et al. 2002). We therefore compared the findings in Col15a1−/− mice with those in Lama4−/− mice lacking the α4 chain of laminin. Like the Col15a1−/− mice, Lama4−/− mice are also viable (Eklund et al., 2001, Thyboll et al., 2002). Light microscopy of the sciatic nerves revealed similar, incompletely myelinated areas in the WT and mutant mice at 3–15 days of age (III, Fig. 1a-d). These areas, representing embryonic axon bundles, disappeared by the age of 1 month in the WT and Col15a1−/− mice, by which time the axons in the bundles were segregated and myelination was complete. Non-segregated areas were conspicuous at the age of 15 days in the Lama4−/− mice, however, and non-segregated axon bundles were still clearly visible at 2 months (III, Fig. 1g). At 14 months the non-segregated axon bundles were smaller and surrounded by numerous small, recently myelinated axons (III, Fig. 1k and Figure 11). Ultrastructural analysis confirmed the presence of large bundles of non-segregated axons in the Lama4−/− mice and closer examination of these structures indicated that Schwann cell processes segregate the axons in most, but not all, of the bundles in these mice when they are over 14 months old (III, Fig. 2a-b). In the Col15a1−/− mice the segregation and myelination of the axons proceeded normally for the majority of the axons, but polyaxonal myelination (i.e. numerous small axons enclosed within a single, abnormally thin myelin sheath) was found in the young and adult Col15a1−/− nerves (III, Fig. 2c and Figure 11). Similarly, polyaxonal myelination was found in adult Lama4−/− nerves (III, Fig. 2d), and also in 3–15-day-old WT mouse nerves in some cases. This is typical of an ongoing myelination process, and we found it to be corrected by remodelling during normal development in the adult WT nerves. This is suggestive of a defective remodelling process and a diminished ability to establish the correct 1:1 axon-Schwann cell relationship in the nerves of all the mutant mouse lines.
Most axons in sensorimotor nerves such as the sciatic nerve are normally ensheathed by myelin-forming Schwann cells in a 1:1 axon-Schwann cell relationship, but a specific set of axons, the C-fibres (representing the autonomic nerves and some of the sensory nerves), are surrounded by non-myelinating Schwann cells, which form amyelin sheaths around them. The defect in axon segregation involved both myelinating and non-myelinating Schwann cells. C-fibres could be distinguished in WT and \textit{Col15a1}^{-/-} sciatic nerves at 21 days after birth, whereas only a few C-fibres were found in the \textit{Lama4}^{-/-} mice, although the latter also had a normal complement of C-type fibres at 14 months. Although C-fibres could be found in normal amounts in the adult \textit{Col15a1}^{-/-} nerves, the axons were more loosely packed than in the WT mice, and the Schwann cell cytoplasm was less electron-dense (III, Fig. 2h and Figure 11).

5.7 Effect of a simultaneous lack of collagen XV and laminin $\alpha$4 on peripheral nerve development

The single knock-out \textit{Col15a1}^{-/-} and \textit{Lama4}^{-/-} mouse lines were crossed to generate a knock-out (DKO) mouse line in order to assess whether the two molecules were functionally involved with each other. The simultaneous lack of collagen XV and laminin $\alpha$4 chains did not impair the viability of the mice, but it did lead to non-segregated areas in the peripheral nerves that were clearly visible at 2 and 14 months of age (III, Fig. 1h and l). No significant differences in the number or size of the non-segregated axon bundles were seen between the \textit{Lama4}^{-/-} mice and the DKO mice at the ages of 15 days, 1 month or 2 months, but they were still abundant in the DKO mice at 14 months (III, Fig. 1l). Ultrastructural analysis revealed that all the bundles of axons in the DKO mice remain non-segregated at the age of 14 months and are significantly larger than those in the \textit{Lama4}^{-/-} mice ($p < 0.0002$)(III, Fig. 2b). In addition, the 2-month-old DKO mice lacked C-fibers, and only a few were found at 14 months, all of which appeared to be abnormal (III, Fig. 2f). Like the single knock-out mice, the DKO mice showed polyaxonal myelination (III, Fig. 2e). The simultaneous lack of collagen XV and the laminin $\alpha$4 chain apparently does not allow for the repair of defects in myelination, nor for the slow progress of myelination that occurs in any case when the laminin $\alpha$4 chain alone is lacking (Figure 11).

To gain a better understanding of this mechanism, we used Ki67 staining to examine Schwann cell proliferation rate in developing and adult nerves. We found that there were significantly less proliferating cells in the DKO and \textit{Lama4}^{-/-}
nerves at P7 than in the WT and Col15a1−/− nerves (p = 0.02 for WT vs. DKO and p = 0.007 for WT vs. Lama4−/−), while very few proliferating cells could be seen in the 1-month-old nerves of the WT and Col15a1−/− mice, i.e. after the active myelination period had been completed (perineural cells were not included in the calculations) (III, Fig. 3). The Lama4−/− nerves still contained many proliferating cells at this time point, indicating that proliferation had started and continued later in these nerves (III, Fig. 3b). As in the WT nerves, Schwann cell proliferation was low in the 1-month-old DKO nerves (p = 0.25 for WT vs. DKO. III, Fig. 3b).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>WT</th>
<th>Col15a1−/−</th>
<th>Lama4−/−</th>
<th>DKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time point</td>
<td>P5</td>
<td>1 year</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Summary of findings

<table>
<thead>
<tr>
<th>BM:</th>
<th>Normal</th>
<th>Occasional protrusions</th>
<th>Irregular folds</th>
<th>Partly broadened</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myelinated axons:</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Polymylinated axons:</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Non-segregated axon bundles:</td>
<td>No</td>
<td>Slightly disorganized</td>
<td>Few, small</td>
<td>Yes</td>
</tr>
<tr>
<td>C-fibers:</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Few, irregular</td>
</tr>
</tbody>
</table>

Fig. 11. Summary of findings in Col15a1−/−, Lama4−/− and DKO sciatic nerves. At P5 the myelination process is ongoing in the WT and mutant mice, and the nerves of all genotypes contain non-segregated axon bundles. The characteristic defects in the BM (drawn in red) are already seen in all the mutants at P5. At the age of 1 year the WT mice show myelinated axons and C-fibers, the Col15a1−/− nerves show normally myelinated axons, polymylinated axons and disorganized C-fibers and the Lama4−/− nerves contain normally myelinated axons, C-fibers, polymylinated axons and small non-segregated axon bundles, but abnormal BM folds have accumulated in the extracellular space. The DKO nerves contain normally myelinated axons, but only a few C-fibers of a very abnormal appearance, polymylinated axons and large, non-segregated axon bundles. An irregular, partly broadened BM covers the Schwann cells. From Rasi et al. 2010, reprinted by permission of the Society of Neuroscience.
5.8 BM formation

In view of the fact that both collagen XV and laminin are BM-associated molecules, the formation of the BM around the non-segregated axon bundles, the myelinated axons, the non-myelinating Schwann cells and the polymyelinated axons was examined by electron microscopy in all of the mutant mice. As in the WT mice, the BM in the Col15a1−/− mouse nerves was continuous and even in appearance, with the exception of occasional protrusions, suggesting a mild impairment of BM assembly (III, Fig. 4b). Irregular, BM-like folds adjacent to a normal-looking BM were already frequently observed around both the non-segregated axon bundles and the myelinated axons in the Lama4−/− mouse nerves at the age of 3 postnatal days (III, Fig. 4c). These irregular folds, which appeared to branch off from the BM and accumulate in the extracellular space, were identified as BM material by collagen IV staining in IEM (III, Fig. 4e). This defect was still apparent at the ages of 2 months and over 1 year. The BM around the Schwann cells in the DKO mice did not form irregular folds, but it varied in thickness and contained poorly organized BM-like material dispersed in the extracellular space (III, Fig. 4d).

Collagen XV has previously been detected in the endoneurium, perineurium and epineurium in both developing and mature nerves (Muona et al., 2002) by immunofluorescence labelling. IEM revealed that collagen XV is localized to the outer zone of the Schwann cell BM in the sciatic nerve and in the adjacent fibrillar collagen matrix (III, Fig. 4f). Previous studies have shown that laminin α4 expression is up-regulated in developing nerves (Patton et al. 1997, Patton et al. 1999, Nakagawa et al. 2001) and is expressed at least in the perineurium of adult mice (Nakagawa et al. 2001).

In order to test whether the loss of collagen XV and laminin α4 affects the composition of the endoneural BMs, we stained cryosections of WT, Col15a1−/−, Lama4−/− and DKO sciatic nerves with antibodies for dystroglycan, laminin β1, laminin γ1, integrin β1 and collagen IV. No differences were observed between the WT and mutant samples. An unaltered pattern of laminin β1, α1, α2 and α5, entactin, perlecan and agrin immunolabelling in the BM of the Lama4−/− sciatic nerves has been reported previously (Yang et al. 2005).
5.9 Distribution of axon diameters, g-ratio and myelin structure in the nerves

A count of axons in the 2-month-old nerves showed that only the DKO mice had a statistically significant decrease in their number compared with WT (p = 0.0017. III, Fig. 6a). The Lama4^{-} mice tended to have fewer axons than WT, but the difference was not statistically significant (p = 0.16. III, Fig. 6a). The axon diameter distribution was slightly altered in the Col15a1^{-} nerves, which had more small axons while the Lama4^{-} mice tended to have a larger average axon diameter than the WT individuals (III, Fig. 6b). The DKO nerves had more very small axons (less than 2 μm in diameter) than WT, but there were fewer axons than normal in all the other size groups (III, Fig. 6b).

Myelin thickness, measured as the g-ratio (axon diameter/total fiber diameter), was lower in the small, medium and large-diameter axons in all the mutant mouse lines, the mean g-ratios being 0.51, 0.56, and 0.62 for Col15a1^{-}, 0.50, 0.59, 0.63 for Lama4^{-} and 0.47, 0.59 and 0.63 for the DKO mice as compared with 0.47, 0.53 and 0.57 for the WT nerves (values corresponding to increasing axon diameter. III, Fig. 6c). The differences relative to the WT mice were highly significant except for the small-diameter axons in the DKO mice.

The areas occupied by non-segregated axon bundles were measured in sciatic nerves from 2-month and 12–14-month-old mice and found to be larger in the 2-month-old Lama4^{-} mice than in the DKO mice (p = 0.046). At the latter age the non-segregated bundles remained large in the DKO mice but had diminished in size in the Lama4^{-} nerves (p < 0.0002. III, Fig. 6d). We conclude that the lack of collagen XV alone slightly altered the axon diameter distribution, and in particular the g-ratio, while the simultaneous lack of both collagen XV and laminin α4 resulted in impaired axonal segregation and fewer myelinated axons.

X-ray diffraction analysis confirmed that there was significantly less myelin in the DKO and Lama4^{-} mice, findings that correlated well with the presence of large non-segregated axon bundles observed in the microscopic analyses. Diffraction from the fixed sciatic nerves showed strong X-ray scatter. The mean myelin period (± standard deviation; SD) in the WT mice was 197.8 ± 2.1 Å, and the proportion of the total scatter that was accounted for by the multilamellar myelin (calculated as M/(M+B)) was 0.21 ± 0.05. Optic nerves were used as controls, since these represent the central nervous system, which lacks the molecules studied here. Here too, the X-ray scatter was strong, with orders of 2–4 conspicuous. The mean period was 160.8 ± 1.1 Å, and the proportion of the total
scatter that was attributable to myelin was 0.15 ± 0.04. The differences in period and in the proportion of myelin between the sciatic and optic nerves taken as two groups were statistically significant (p < 0.0001). When the genotypes were correlated with the data, there was no statistically significant difference among the four groups concerning the optic nerves, but the genotypes of the sciatic nerves could be clearly distinguished, with a rank order (from the strongest to the weakest pattern) of Col15a1<sup>−/−</sup> > WT > Lama4<sup>−/−</sup> > DKO.

Assessment of the myelin periodicity data with Student’s T-test indicated significant differences in the sample means between the WT (196.5 Å) and DKO mice (199.5 Å) and between the WT and Lama4<sup>−/−</sup> mice (198.5 Å) (p < 0.0002 and p < 0.015, respectively), whereas there was no significant difference between the WT and Col15a1<sup>−/−</sup> mice (196.7 Å, III, Fig. 5). Comparing the proportions of myelin among the genotypes, we found that the only significant differences were between the WT (0.227) and DKO groups (0.157) (p < 0.0002) and the WT and Lama4<sup>−/−</sup> groups (0.194) (p < 0.04, III, Fig 5).

5.10 Electrophysiological measurements of the peripheral nerves

The mice were subjected to electrophysiological analysis to gain insight into the significance of the morphometric and behavioural findings (described in the next section). Both the motor and sensory nerve conduction studies showed substantial differences between the genotypes. A marked reduction occurred in the compound muscle action potentials (CMAP) of the tibial nerve in both mutants lacking laminin α4, indicating that the number of normally functioning motor axons is reduced when laminin α4 is absent. The tibial CMAP amplitudes in the WT mice were 14.7 mV and 17.7 mV when stimulated at the sciatic notch and at the ankle, respectively, whereas those in the Lama4<sup>−/−</sup> mice were 5.62 mV and 7.22 mV, respectively, and those in the DKO mice 4.39 mV and 5.30 mV (III, Table 1). Although the DKO mice showed a trend for less functioning motor axons than in the Lama4<sup>−/−</sup> mice, the difference was not statistically significant (p = 0.38 and 0.27 for stimulations at the sciatic notch and ankle, respectively), probably because the Lama4<sup>−/−</sup> and DKO mice were still morphologically similar at 3 months of age. The Col15a1<sup>−/−</sup> mice showed no changes in tibial CMAP amplitudes (11.4 mV and 15.2 mV) relative to the WT mice (III, Table 1). Motor nerve conduction velocity profiles were normal in all the mice, and studies using repetitive stimulation also showed normal responses.
The sensory nerve action potential was increased in both the \textit{Lama4}^{-/-} and DKO mice relative to the WT mice, the values being 328 µV, 345 µV and 261 µV, respectively, but the lack of laminin α4 had no significant effect on the sensory nerve conduction velocity (SCV) as measured from the tail (III, Table 2). The SCV value was significantly lower in the \textit{Col15a1}^{-/-} mice than in the WT, \textit{Lama4}^{-/-} or DKO mice (28.7 m/s; 36.0 m/s; 33.8 m/s and 33.4 m/s, respectively), indicating that a lack of collagen XV alone results in poorer conduction in the sensory nerves (III, Table 2). The ultrastructure of the nodes of Ranvier was studied by transmission electron microscopy, but no differences that would explain the poorer conduction in the \textit{Col15a1}^{-/-} sensory nerves were observed.

5.11 Behavioural analysis

The mice were subjected to tests of motor coordination and balance, which revealed impaired performance in the mutant mice in a graded fashion. In a round beam test, in which the mice were placed on a beam and their movements were observed, all three mutants fell off the beam and were less active than their WT littermates (III, Table 3). Performance was most severely compromised in the DKO mice, followed by the \textit{Lama4}^{-/-} and \textit{Col15a1}^{-/-} mice. The \textit{Lama4}^{-/-} and DKO mice took longer to cross the beam and made more hind limb slips. Similar graded impairment was found when the mice were placed on a rotating rod, the ‘rotarod test’, as the DKO and \textit{Lama4}^{-/-} mice differed significantly from their WT littermates (III, Fig. 7a).

The hind limb reflexes were clearly impaired in both the \textit{Lama4}^{-/-} and DKO mice (III, Online movies), but intriguingly, the \textit{Lama4}^{-/-} and \textit{Col15a1}^{-/-} genotypes appeared to have additive effects in extension reflexes that depend on postural sense (III, Fig. 7c), nor did simultaneous lack of both molecules worsen the results in all cases, as \textit{Col15a1}^{-/-} tended to alleviate the \textit{Lama4}^{-/-} genotype in a grasping reflex that draws on cutaneous sensation (III, Fig. 7d). On the other hand, both the \textit{Lama4}^{-/-} and DKO mice were impaired in their tactile sensory threshold as assessed in terms of von Frey hairs (III, Fig. 7e). Only the DKO mice exhibited a significantly enhanced sensitivity to heat pain in the hot plate test (III, Fig 7f).

The motor impairment observed in the \textit{Lama4}^{-/-} and DKO mice did not prevent them from moving around. No genotype differences were observed in exploratory movements between the arms in the elevated plus-maze test (III, Table 3). Furthermore, a possible confounding effect of anxiety on sensorimotor
test performance could be ruled out as similar scores were recorded for all the genotypes on parameters measuring anxiety (III, Table 3).
6 Discussion

The focus in this thesis has been on the gaining of a better and more detailed understanding of the biological functions of collagen XV and a more general view of the roles of collagen XV in vivo. The new information could help us to evaluate what human pathological conditions could be brought about by a lack of the COL15A1 gene or mutations in it.

Collagen XV is widely distributed in tissues, occurring in the BM zones of skeletal muscle, the heart, capillaries, the uterus and peripheral nerves and most epithelial BMs (Kivirikko et al. 1995, Hägg et al. 1997a, Myers et al. 1996, Muona et al. 2002). Previous immunolocalizations of collagen XV in placental, kidney and colon tissues have traced it to the outermost zone of the BM, and it is often found in association with large fibrillar collagens in the extracellular matrix (Amenta et al. 2005a). The localization data suggest a role for collagen XV in fibril formation and collagen bundle organization, but no direct data exist to support this hypothesis. In vitro studies using recombinant human collagen XV have failed to identify binding to collagen I or III, rendering it possible that some other component could link it to the fibrillar collagens. In order to elucidate the function of collagen XV in the matrix further, we decided to analyse selected tissues with known high levels of collagen XV expression in a mouse model lacking this collagen.

As collagen XV is expressed in the matrix of several tissues in both mice and humans, it is possible that mutations in the Col15a1 gene could lead to a complex phenotype with several organs affected. The present work adds to our knowledge of the roles of collagen XV in the formation of the fibrillar collagen matrix and its physiological functions in the heart, microcirculation and peripheral nerves, and it also points to roles for collagen XV in certain rather common diseases such as cardiac hypertrophy.

6.1 Collagen XV as a matrix organizer

Our immunoelectron microscopic studies of sciatic nerves and uteri confirm the previous notion of a collagen fibril-associated location for collagen XV (Amenta et al. 2005b). Based on our analysis of several tissues in Col15a1−/− mice, we now make the claim that collagen XV acts as a regulator of fibrillar collagen assembly in development and remodelling processes by affecting the size and organization of collagen fibrils during development and their lateral fusion during tissue
remodelling. In addition to its regulatory function, collagen XV also appears to acts as a link between the cell and the fibrillar matrix. In particular, it appears to be required for the formation of a proper interphase between the cellular BM and the adjacent fibrillar matrix. Since recombinantly produced collagen XV does not bind to collagen I or III (Hurskainen et al. 2009), the effect of collagen XV could be indirect and require other molecules capable of binding to the fibrillar collagens, such as fibronectin, vitronectin, fibulin or others. Alternatively, the chondroitin sulphate side chains of collagen XV (which were lacking in the recombinant protein) may be required for its activities.

Previous studies have shown that Col15a1−/− mice are viable and fertile, but exhibit progressive histological changes that are characteristic of mild muscular diseases, so that the muscles of mutants are more vulnerable to exercise-induced muscle injury (Eklund et al. 2001). The structurally abnormal capillaries (Eklund et al. 2001) and the abnormal microcirculation reported here (II) may contribute to the myopathic phenotype in Col15a1−/− mice. We now extend this phenotypic data and demonstrate that the mice also suffer from impaired peripheral nerve development. Based on the first characterization of Col15a1−/− mice, it was suggested that collagen XV may function as a structural component needed to stabilize the skeletal muscle cells (Eklund et al. 2001). It has also been suggested that collagen XV may serve as a bridge between the BM and fibrillar collagens (Amenta et al. 2005a). To find out how a lack of collagen XV can lead to such phenotypic changes, we focused on the matrix changes that occur in skeletal muscle and peripheral nerves, and showed that collagen XV serves as a bridge, not only by connecting the fibrillar collagens to the BM, but also by creating an interphase between the collagen bundles and the BM (Fig. 12a). Mature collagen fibrils are normally covered with fibronectin or other matrix glycoproteins. This prevents direct contact between the collagens and the cells. On the other hand, newly formed collagen molecules can interact with the cells to induce intracellular signalling and alter gene expression (Di Lullo et al. 2002, Kovanen 2002). We suggest that collagen XV, like fibronectin, covers the collagen fibrils, either directly or via another molecule, and that its absence may result in these collagen fibrils being glued to the BM, which in turn could alter certain cellular functions. In addition to this abnormal BM-fibrillar matrix interaction, collagen XV seems to organize the fibrillar collagen bundles. In the mutant skeletal muscle, however, the collagen fibrils were packed so tightly together within the fibrillar bundles so that it was not always possible to distinguish individual fibrils (Fig. 12a). This was seen in both TEM and SEM analysis, but it remains unclear how
this bundle organization affects the structural or tensile properties that are needed in the muscle.

In the peripheral nerve a lack of collagen XV leads to abnormal C-fibers (the non-myelinated axons that signal for temperature, pain and autonomic functions) and polymyelinated axons (i.e. several axons within a single myelin sheet). There are several reports of a lack of ECM molecules, mainly BM components, or mutations in them leading to impaired development of the peripheral nerve (Nakagawa et al. 2001, Feltri et al. 2002, Wallquist et al. 2005, Yang et al. 2005, Yurchenco & Patton 2009). In the Col15a1−/− nerves, however, the BM was essentially intact, with only minor irregularities in structure. Instead, we found that collagen XV affected the fibrillar matrix differently at different time points in development. At very early time points (P15) the size of the collagen fibrils and their organization within the bundle was altered, while in the mature nerve the collagen fibrils did not differ in diameter between the WT and mutant mice, suggesting that the expression of some other ECM protein compensated for the loss of collagen XV by the age of 1 month. As in skeletal muscle, the collagen fibrils in the mature nerve also adhered to the BM in the mutant, in addition to which a lack of collagen XV resulted in irregularly organized collagen bundles connecting adjacent axons (Fig. 12b). It is tempting to consider this abnormal packing of collagen fibrils into the BM to be one of the most important pathobiological consequences of a lack of collagen XV. The altered fibrillar collagen architecture may account for the “swelling” observed in the Col15a1−/− C-fibers. Moreover, the altered pericellular milieu could change the cellular functions, leading to the observed lower synthesis of fibrillar collagen in the old Col15a1−/− mice (1 and 2 years old) compared with controls.

In contrast to the peripheral nerves and skeletal muscle, the role of collagen XV in the cardiac ECM was quite different. At all time points studied the Col15a1−/− heart showed a prominent accumulation of non-fibrillar protein, attempts to identify the nature of which proved unsuccessful. It thus remained open as to whether it was due to leaking capillaries or represented attempts by the cells to compensate for the loss of collagen XV. The fact that both young (aged 1 month) and old (aged 14 months) WT hearts showed a similar accumulation of non-fibrillar proteins in the ECM leads one to think that other proteins should be considered in addition to those from leaking capillaries. It should be noted however, that the accumulation is not a sign of fibrosis, as the picrosirius red staining for fibrillar collagens did not reveal any changes in their quantity. Likewise, mRNA expression data suggested that the expression of collagens I and
III was similar in the Col15a1−/− and WT mice. Previous studies have demonstrated that collagen XV is accumulated in human fibrotic kidneys (Hägg et al. 1997a), suggesting that it may have a role in fibrotic processes. We demonstrate here that although collagen XV expression is up-regulated in a hypertrophic, fibrotic heart, a lack of collagen XV in the heart does not inhibit or lead to excessive fibrosis. Even though collagen XV does not participate in the regulation of fibrosis, i.e. the accumulation of fibrillar collagen, it is necessary for organizing the fibrillar collagens into bundles in the heart as elsewhere. The present SEM analysis shows that a lack of collagen XV results in poorly packed and organized collagen bundles in the heart, indicating that collagen XV is needed for proper fibrillar collagen bundle organization in the ECM. This was confirmed by picrosirius red staining and analysis with a polarized microscope, showing more green color, indicative of thin collagen bundles, in the Col15a1+/+ LVs than in their WT counterparts. The importance of properly organized collagen bundles has been emphasized earlier (Caulifield & Borg 1979, Ju & Dixon 1996, Spinale 2007), and it is attractive to speculate that the thin, poorly organized collagen bundles in the Col15a1−/− LVs lead to an abnormal development of force in the cardiomyocytes. The bundles participate in myocyte shortening in the contracting heart, as they straighten out in the diastole, allowing the cells to separate and the ventricles to fill up with blood, while in the systole they have a wavy appearance and allow the cardiomyocytes to come closer together (Caulifield & Borg 1979). Isolated and perfused hearts from Col15a1−/− mice did not develop any maximal pressure when perfused with isoprenol comparable to that in the WT hearts (Eklund et al. 2001). The poorly organized collagen bundles are also very likely the cause of the lengthening and irregular shape of the cardiomyocytes in old Col15a1−/− LVs.

The importance of collagen XV for the capillaries has been shown previously at the ultrastructural level (Eklund et al. 2001) and it is demonstrated here that the morphological alterations are accompanied by changes in the microcirculation, with altered permeability and perfusion in these capillaries. It remains unclear whether the microvessel impairment is due to abnormal development and angiogenesis, or whether the primary healthy capillaries are suffering from incomplete structural support from the poorly organized collagen bundles. It is thought that collagen bundles are needed to keep the capillaries open during the systole (Caulifield & Borg 1979). When the lumen is partly closed, or the capillary has curves, as in the Col15a1−/− hearts, it is possible that the blood flow
will damage the capillaries, resulting in ruptures and extravasations of erythrocytes.

As collagen XV was involved in organizing the fibrillar collagens in the matrix, it was attractive to study collagen remodelling in a tissue where collagen XV is strongly expressed. The endometrial stroma of the implantation site undergoes extensive changes called decidualization, leading to cell proliferation and growth, ECM degradation and a decrease in the extracellular spaces. The collagen fibrils in the decidualized area are thickened relative to the pre-existing thin collagen I and III fibrils in a process called lateral fusion (Spiess & Zorn 2007, Stumm & Zorn 2007). We show that collagen XV is localized to the thick-fibrils in the decidualized area of the endometrium, and it also affects the lateral fusion process, as when it is lacking the thick fibrils are incompletely fused, resulting in cauliflower-like collagens and frayed ends in the collagen fibrils when viewed in longitudinal section. These changes could impair the structural properties of the microenvironment for the implantation, although in practice the Col15a1−/− mice are fertile and the litters of normal size, with only occasional breeding difficulties. Hence it remains unclear whether these problems are due to the inbred nature of the strain or changes in implantation.

Collectively, the data indicated that even delicate changes in the matrix architecture and BM-matrix communication can lead to accumulating defects in several tissues in the mutant mice.
Fig. 12. Collagen XV as a matrix organizer. Schematic presentation of a cell, the surrounding BM and adjacent fibrillar collagens in the skeletal muscle (A). Lack of collagen XV results in adherence of collagen fibrils to the BM, and the BM – fibrillar matrix interphase is altered, including the fibrillar organization within the collagen bundles. The axons in the peripheral nerve (B) are covered by collagen bundles, and by some of the bundles that connect adjacent axons with each other. A lack of collagen XV results in thinner, irregularly organized collagen bundles. Col indicates collagen fibrils.

6.2 Lack of collagen XV leads to cardiomyopathic changes in Col15a1−/− mice

Animal models, particularly transgenic mouse models, are valuable tools for studying the in vivo function of the gene of interest, the goal being to translate the information into human pathologies. So far no human disease has been reported to be caused by a mutation in the COL15A1 gene. Our results demonstrate the importance of collagen XV in the heart, and this detailed study of the Col15a1−/− heart could help to identify cardiac pathologies that are attributable to changes in collagen XV in humans.
The main findings were ventricular wall thinning and lower cardiac weight, even though the left ventricle diameter remains the same in old Col15a1−/− mice (aged 14 months). Cardiac function, as analysed by echocardiography, is normal in these old mice, and although the young mice (aged 1 month) showed decreased EF and FS, but this mild systolic dysfunction was reversible. The cardiomyocytes were smaller in the young Col15a1−/− heart, but their diameter normalized with time, and longer, irregularly shaped cardiomyocytes were observed in the old mutant mice, as compared with age-matched WT controls (Fig. 13). These changes in the cellular architecture may be the reason for the shorter, more fragmented intercalated discs seen in the Col15a1−/− LVs. Electrical conduction in the heart was not assessed here, but such a measurement could verify whether the ultrastructural findings of abnormal adherence junctions at the intercalated discs are of functional significance. However, as the gap junctions were normal, it is possible that the electric transmission may be normal, while the adherence junctions are needed for force transmission from one cardiomyocyte to another.

Previous in vitro studies of Col15a1−/− mice have suggested that there could be a defect in generating the maximal force in perfused isolated hearts during isoproterenol infusion (Eklund et al. 2001). These changes together indicate that a lack of collagen XV will lead to cardiomyopathic changes and that not only is the ECM affected but also the cardiac dimensions and the cellular architecture.

The role of the abnormal microvasculature and possible poor perfusion and increased permeability in the cardiac tissues remains partly open. We cannot rule out the possibility that the cardiomyopathic changes could be due to poor circulation. The ischaemic changes we see are local, however, and no large areas of ischaemic, necrotic or fibrotic tissues are seen in the Col15a1−/− LVs. Bnip-3 and Glut-1 mRNAs, markers of an ischaemic myocardium caused by coronary artery occlusion (Brosius et al. 1997, Regula et al. 2002) were not altered, indicating that no global ischaemia was apparent in the Col15a1−/− LVs. The increase in capillary density in the mature mice (5 months old) may partly compensate for the dysfunctional microvasculature and could even be a possible mechanism for the recovery from the systolic dysfunction seen in young mice.

Comparing the phenotype of mice lacking the possible bindings partners of collagen XV, namely, perlecan, nidogen 1 and 2, fibulin 1 and 2 and vitronectin with the Col15a1−/− mice, there are some similarities (Khoshnoodi et al. 2008, Bader et al. 2005, Vega et al. 2009, Sasse et al. 2008, Singh et al. 2010, George et al. 1993, Schwartz et al. 1999). It would be interesting to speculate if some of the cardiac findings like abnormal capillaries and bleedings are due to lack of
interaction between the BM components, laminin, perlecan or nidogens. However, the BMs of Col15a1−/− heart are intact indicating that lack of collagen XV is not sufficient to disturb the BM architecture and the function of these components. Even though the vascular phenotype of fibronectin knock-out mice is severe, the mice being early embryonic lethal, it is interesting to speculate if the vascular phenotype in Col15a1−/− mice is depending on defects in collagen XV - fibronectin interaction, as fibronectin has been shown to be critical for the vascular morphogenesis, especially the formation of the lumens and modulating the interaction between the endothelial and perivascular cells (Astrof et al. 2009). The in vivo interaction of fibulin and vitronectin with collagen XV, would be interesting to study in more detail as they are also interacting with collagens and microfibrils in the ECM (de Vega et al. 2009, Schwartz et al. 1999). Both fibulin 1 and collagen XV knock-out mice show capillary phenotype, however the latter not being lethal. The reason for abnormal capillaries in fibulin 1 knock-out mice is thought to result due to abnormal cell-matrix interactions (Kostka et al. 2001).

Considering the typical presentations of human cardiomyopathies, the Col15a1−/− phenotypic changes resemble those found in mildly symptomatic or asymptomatic relatives of DCM patients (Baig et al. 1998, Matsumura et al. 2006). The Col15a1−/− cardiac phenotype is too mild to unequivocally fulfil the diagnostic criteria for DCM (Elliott et al. 2008), but the cardiomyopathic changes seen in the old Col15a1−/− LVs bear similarities to those seen in DCM patients, namely poorly organized collagen bundles (Spinale 2007), abnormalities in the intercalated discs (Olson et al. 2002, Perriard et al. 2003), irregularly organized cardiomyocytes (Beltrami et al. 1995, Schaper et al. 1995), increased myocardial stiffness (Cabrera 1999, Wu et al. 2002) and dysfunctional microcirculation (Roura & Bayes-Genis 2009, Wang et al. 2006a). Echocardiography revealed certain characteristics of DCM in juvenile Col15a1−/− mice, namely increased LV volume and depressed EF and FS, albeit this condition was not progressive, in contrast to the classic presentation of DCM. As in the collagen XV-deficient mice, a bimodal pattern of clinical presentation is observed in certain inherited forms of DCM. This is especially evident in a recent extensive study demonstrating that a mutation in the alpha-tropomyosin gene can cause either severe symptoms in early life or mild asymptomatic LV dilation in adulthood (Lakdawala et al. 2010). In these families a subset of affected infants showed an improvement in LV contractility which was unlikely to be related solely to receiving basic medical therapy (Lakdawala et al. 2010). Additionally, improvements in cardiac function with time have been documented in follow-up studies of mildly affected DCM
family members and in rare cases of patients with acute/recent onset idiopathic DCM (Steimle et al. 1994, Mann-Rouillard et al. 1999). The mechanisms underlying the improved cardiac function in some patients and the distinct age-dependent clinical manifestations are currently unknown.

The classification of cardiomyopathies is undergoing changes and it is highly likely that new types will be presented in the near future. Our aim is to characterize the cardiomyopathic changes in the Col15a1^-^- LV in further detail in order to be able to assess their correlations with cardiac phenotypes found in humans.

It should be kept in mind that the human and mouse phenotypes do not always go hand in hand, as seen in the mice lacking the laminin α4 chain and the human patients with a mutation in that chain. The former developed ischaemic cardiomyopathy and in contrast to the human patients, the mutant mice developed cardiac hypertrophy and no LV dilatation (Wang et al. 2006a). It is for this reason that a mouse model must only be regarded as a tool, showing a direction. The limitation with that particular study was that only female mice were used for the functional assessments. Other recent cardiac phenotype studies have revealed that female C57BL6 mice survive better and have a milder phenotype than male mice from the same background (Swinnen et al. 2009). The reasons are not known, but could be the same as those affecting the human genus-dependent differences in cardiac diseases.

When conducting a search for cardiomyopathies in the same genetic region where collagen XV is encoded we found that the chromosomal region 9q13-q22 is reported to contain an unidentified gene causing familiar DCM (Krajinovic et al. 1995). Even though several years have elapsed since that publication, no human gene has been reported to be responsible for this mutation. Intriguingly, the gene encoding human collagen XV has been mapped to that same genomic region, 9q21-22 (Huebner et al. 1992). Altogether, our findings suggest that the COL15a1 gene should be regarded as a candidate gene for human cardiomyopathies.
Fig. 13. Lack of collagen XV affects the cardiac cell architecture and intercalated discs. Regular cardiomyocyte shape and continuous intercalated discs in a WT heart (A). Elongated and irregularly shaped cardiomyocytes in a Col15a1−/− heart, where the intercalated discs are shorter and more fragmented due to the altered cell shape (A). A schematic illustration of WT and Col15a1−/− intercalated discs (B). The mutant intercalated discs show similar gap junctions and desmosomes to the WT controls. The distribution of adherence junctions is altered and in many cases totally lacking in the Col15a1−/− intercalated discs. CM indicates cardiomyocyte; GJ, gap junction, des, desmosome and AJ, adherence junction.

6.3 Collagen XV in peripheral nerves and its possible role in neuropathies.

As collagen XV is not expressed only in the heart, it should be kept in mind that a possible human phenotype lacking collagen XV or having a mutation in it, may lead to a complex phenotype including at least the capillaries, skeletal muscles and peripheral nerves as well. We evaluated the role of collagen XV in the peripheral nerves and found minor defects in their development, principally their segregation and myelination, resulting in polyaxonal myelination in Col15a1−/− nerves. Even though most of the axons were normally myelinated, the myelin
sheath was thinner when measured in terms of the g-ratio, an increase in which was observed in small, medium and large-diameter axons, indicating that both sensory (small) and motor (large) axons had a thinner myelin sheath. In addition, we found that the C-fibers, i.e. several axons within a Schwann cell without a myelin sheath were abnormally formed. The axons were irregularly shaped and the Schwann cell cytoplasm normally segregating the axons and tightly wrapping around them was oedemic and loosely packed in the Col15a1−/− C-fibers. Since the function of C-fibers is to transfer signals for temperature, pain sensation and autonomic functions (Pocock & Richards 2001, Hoitsma et al. 2004), we tested whether these functions were changed in the Col15a1−/− nerves by performing a behavioural analysis and the hot plate test. To our surprise, the time at which the Col15a1−/− mice started licking their paws in the hot plate test was unchanged relative to the WT mice, but the Col15a1−/− mice were less willing to walk on a round beam and had significantly more falls than the WT mice. Electrophysiological tests revealed normal motor conduction velocities in the Col15a1−/− mice and only a statistically non-significant tendency for decreased motor nerve compound action potentials in the tibial nerve. The sensory nerve action potential measured in the tail was unaffected, but the conduction velocity in the sensory nerves was significantly decreased compared with the WT individuals. Altogether, these results demonstrate that a lack of collagen XV affects peripheral nerve development, and that despite the very mild morphological changes, the function of the nerves in the Col15a1−/− mice is changed.

In addition to collagen XV, we also focused on the role of the laminin α4 chain in the peripheral nerves, as we considered these two BM-associated proteins expressed largely in the same tissues to be possibly functionally linked. This led us to deepen our understanding of the roles of the laminin α4 chain in peripheral nerves. Our work with the Col15a1−/−; Lama4−/− double knock-out line generated for this purpose indicated that the simultaneous lack of collagen XV and the laminin α4 chain results in a more severe phenotype than seen in the respective single knock-outs. Even though the need for a properly formed BM to ensure peripheral nerve development has been widely discussed in the recent literature, and there are several studies indicating that segregation and myelination can occur even without a BM (Wallquist et al. 2005, Yang et al. 2005, Court et al. 2006), our results suggest that the more seriously affected BM in the DKO mice compared with the Lama4−/− mice leads to permanent defects in the segregation and myelination of the axons. While proliferation occurred in the peripheral
nerves of the Lama4\(^{-}\) mice at the age of 1 month, when myelination had come to an end in the WT and Col15a1\(^{-}\) mice, no proliferation was seen in the WT, Col15a1\(^{-}\) or DKO mice. This could indicate that the recovery seen in the Lama4\(^{-}\) nerves and the increase in proliferation are brought about by a compensation mechanism and that the laminin \(\alpha_4\) chain is not necessary in mature nerves. When there is a simultaneous lack of these two molecules, however, this compensatory mechanism does not function.

Although no human neurological disease caused by a mutation in the gene encoding the laminin \(\alpha_4\) chain has been reported, the peripheral nerve phenotype resembles the laminin \(\alpha_2\) mutations in mice to the extent that it is very likely that a disease with similar features in the nerves to congenital muscular dystrophy could be found in humans (Yang et al. 2005, Geranmayeh et al. 2010).

The peripheral nerve phenotype in Col15a1\(^{-}\) mice has features that suggest that collagen XV may be involved in human hereditary sensory and autonomic neuropathies (HSAN), where it is the sensory and autonomic nerves that are mostly affected. Conduction velocities in the sensory nerves decrease in a similar manner to those in Col15a1\(^{-}\) mice, while the motor nerves are less affected. Unique to HSAN patients is the lack of a normal axon flare in a histamine test due to the non-functioning state of the C-fibers (Auer-Grumbach et al. 2003, Axelrod & Gold-von-Simson 2007). We were not able to perform this test on our transgenic mice, but in view of the morphological findings at the ultrastructural level we can say that the C-fibers are clearly affected. While the Col15a1\(^{-}\) mice showed a normal pattern in the hotplate test, the DKO mice, with even more severely affected C-fibers, were experiencing allodynia and were extremely sensitive. In the DKO mice the axons that should have been C-fibres were still unsegregated in the axon bundles, so that signalling in them was probably not finely controlled.

The findings in the Col15a1\(^{-}\) mice lead us to suggest that mutations in the human COL15A1 gene could be responsible for sensory and autonomic neuropathies, myotonia and capillary defects in several tissues, including the skin, skeletal muscle and heart, and predispose patients to cardiomyopathy.
7 Future perspectives

Our findings in the Col15a1−/− mice lead us to suggest that mutations in the human COL15A1 gene could give rise to sensory and autonomic neuropathies, myotonia and capillary defects in several tissues, including the skin, skeletal muscle and heart, and predispose patients to cardiomyopathy. In addition to searching for families with heritable mutations in the COL15A1 gene, it would be of great interest to analyse the possible involvement of collagen XV in more common diseases, categories that could include coronary artery disease, various cardiomyopathies and diabetic neuropathies. Col15a1−/− mice could be used as a tool for analysing the heart phenotype further, e.g. in mice older than one year, in ischaemic models or where the cardiac phenotype is worsened by the use of cardiotoxic drugs such as anthracycline or by myocarditis. In addition to the tissues studied in this thesis, the kidney contains relatively high levels of collagen XV, and thus an inquiry into kidney function in Col15a1−/− mice would be warranted. Furthermore, it would be of interest to target possible abnormalities in blood coagulation, in the circulation in the uterus and in bone formation in these mice.

The TEM and SEM analyses of the matrix in Col15a1−/− mice provided an interesting picture of the roles of collagen XV there. We now think that collagen XV has the function of organizing the collagen bundles and maintaining an interphase between the BM and fibrillar matrix. Many questions remain still open, however: which are the active partners, are there others in addition to the fibronectin, vitronectin, laminin, perlecain, nidogen 1 and 2, and fibulin 1 and 2, identified in in vitro studies as binding to collagen XV, and are any of these physiologically significant? What is the mechanism by which collagen XV organizes the fibrillar collagen bundles? The findings in the in vivo model raise many questions that would require in vitro studies to find an answer. In addition, based on knowledge from human cancer studies one important source of information could be the matrix ultrastructure in Col15a1−/− mice in the presence of in situ and invasive malignancies.

In conclusion, this work has provided a further insight into the roles of collagen XV in the heart and the peripheral nerves and has focused on its function in the matrix of several tissues during development, adulthood and ageing. Together with previous studies on Col15a1−/− mice, it widens our view of the pathological changes that result from a lack of collagen XV. The findings also underscore the importance of a properly organized ECM and show that even
delicate changes in the matrix can lead to alterations in the functioning of an organ.
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Original papers


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The original publications are not included in the electronic version of this thesis.
1067. Mäkinen, Johanna (2010) Systematic search and evaluation of published scientific research: implications for schizophrenia research
1070. Tanskanen, Päivikki (2010) Brain MRI in subjects with schizophrenia and in adults born prematurely: the Northern Finland 1966 Birth Cohort Study
1075. Kinnunen, Urpo (2010) Blood culture findings during neutropenia in adult patients with acute myeloid leukaemia: the influence of the phase of the disease, chemotherapy and the blood culture systems
1076. Saarelä, Ville (2010) Stereometric parameters of the Heidelberg Retina Tomograph in the follow-up of glaucoma
1077. Reponen, Jarmo (2010) Teleradiology—changing radiological service processes from local to regional, international and mobile environment
1081. Alahuhta, Maija (2010) Tyypin 2 diabeteksen riskirahmään kuuluvien työikäisten henkilöiden painonhallinnan ja elintapamuutosen tunnuspiirteitä
Karolina Rasi

COLLAGEN XV AS A MATRIX ORGANIZER
ITS FUNCTION IN THE HEART AND ITS ROLE TOGETHER WITH LAMININ α4 IN PERIPHERAL NERVES